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Thèse

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par

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Caractérisation génétique de *Phytophthora alni* Brasier & S.A. Kirk, hybride interspécifique agent du dépérissement de l'aulne en Europe

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Sommaire

Lexique

Allopolyploïdie : association de plusieurs génomes distincts, et origine polyphylétique

Aneuploïdie : changement du nombre de base de chromosomes, portant sur un ou quelques chromosomes entiers, mais non sur l'ensemble du lot haploïde (*n*).

Autapomorphie : Lors d'une comparaison de caractères entre groupes-frères, cet adjectif désigne l'état dérivé (i.e. 'nouveau') d'un caractère propre à l'un de ces deux groupes. Par extension, on parle de caractère autapomorphe ou d'une autapomorphie.

Autopolyploïde : duplication du même génome et origine monophylétique

Hétéroploïde : noyau cellulaire contenant un nombre de chromosomes non diploïde.

Homéologues (ou homoéologues) : se dit des paires de chromosomes homologues qui proviennent de parents distincts suite à une hybridation (cas de l'allopolyploïdie) ou qui représentent des « copies » des paires de chromosomes homologues du taxon de ploïdie « normale » (cas de l'autopolyploïdie). Par extension, on parle d'allèles homéologues lorsqu'ils sont présents sur chacun des chromosomes homéologues.
Ex : pour un organisme diploïde qui possède deux lots haploïdes de chromosomes AA, chaque paire de chromosomes rassemble deux chromosomes homologues. Pour un autotétraploïde AA/A'A', les chromosomes similaires des génomes A et A' sont dits homéologues. Pour un allotétraploïde AA/BB, les chromosomes similaires des génomes A et B sont aussi dits homéologues.

Homoploïde : caractérise un taxon issu d'hybridation interspécifique et qui a retrouvé le même niveau de ploïdie que les espèces parentales (souvent diploïdes) dont il s'isole génétiquement par des réorganisations de son génome.

Introgression : échanges génétiques entre des hybrides et leurs espèces parentales, et par extension invasion d'un génome par du matériel génétique étranger

Orthologues : gènes d'espèces différentes dont les séquences sont homologues, dérivent d'un même gène ancestral et ont divergé à la suite d'un événement de spéciation.

Paralogues : gènes issus d'un événement de duplication au sein du génome d'un même individu.

Réticulation : hybridation interspécifique

Introduction

1. - L'aulne en Europe.

1.1. - Les différentes espèces d'aulnes en Europe.

Le genre *Alnus* appartient à la famille des bétulacées. Il comprend trente-cinq espèces réparties dans l'hémisphère nord sur les continents américain, asiatique et européen (Boullard, 1969). En Europe, quatre espèces endémiques d'aulnes sont majoritairement présentes : *A. incana* Moench (aulne blanc, « grey alder »), *A. cordata* Desf. (aulne de Corse ; « Italian alder »), *A. glutinosa* (L.) Gaetern (aulne glutineux, « black or common alder ») et *A. viridis* D.C. (aulne vert, « green alder »). Une cinquième espèce d'origine nord-américaine, *Alnus rubra* Bong., a été plantée de façon intensive dans certains pays européens.

1.1.1. - L'aulne glutineux.

Alnus glutinosa est de loin l'espèce la plus abondante en Europe. Cette espèce est très commune dans les ripisylves et les forêts humides, partout sur le continent européen (Jalas & Suominen, 1976). En revanche, dans les régions européennes plus sèches comme les zones méditerranéennes ou orientales du continent, l'aulne glutineux n'est souvent présent qu'en bordure de cours d'eau.
Bien que cette essence ait été progressivement remplacée en forêt par d'autres genres de feuillus plus rentables économiquement, sa plantation a été très encouragée sur les bords de cours d'eau, notamment en Europe de l'ouest, en raison de ses qualités écologiques (Claessens, 2003). Ces peuplements ripicoles constituent aujourd'hui la majorité de la population européenne d'aulnes glutineux, bien qu'ils subsistent encore en peuplements purs sur des grandes surfaces dans certaines régions comme la vallée du Danube ou les plaines du centre-nord de l'Europe (Turok et al., 1996).

1.1.2. - L'aulne blanc.

Alnus incana présente une répartition proche de celle de l'aulne glutineux, bien qu'il soit quasiment absent des régions méditerranéennes et rare dans les îles Britanniques (Jalas & Suominen, 1976). De plus, il semble beaucoup mieux supporter les basses températures et est beaucoup plus présent dans les régions septentrionales de la Scandinavie que l'aulne glutineux. Bien que moins tolérant à l'ennoyage que ce dernier, il est assez fréquent en bordure de plans d'eau. Etant capable de se développer sur des sols pauvres et secs, il est naturellement présent dans les zones montagneuses et est aussi utilisé en plantation pour améliorer des stations pédoclimatiques difficiles (Claessens, 2003).

1.1.3. - L'aulne vert.

Alnus viridis est un arbre qui ne dépasse pas 4 à 6 m de hauteur. C'est une espèce endémique des montagnes du centre et de l'est de l'Europe (Jalas & Suominen, 1976) qui joue un rôle important dans le prévention de l'érosion des sols et des avalanches. Le marcottage naturel est assez fréquent chez

l'aulne vert et cette espèce est très souvent pionnière dans les zones de reforestation (Claessens, 2003).

1.1.4. - L'aulne de Corse.

Alnus cordata est une essence essentiellement utilisée en plantation. Son aire de répartition géographique naturelle est limitée à la Corse et au sud-ouest de l'Italie (Jalas & Suominen, 1976). L'aulne de Corse est capable de tolérer la sécheresse et des sols assez pauvres (Claessens, 2003). Il est ainsi fréquemment utilisé dans des programmes d'amélioration du sol. Enfin, ses propriétés esthétiques (feuillage brillant) en font une espèce particulièrement utilisée en plantation ornementale, notamment en Europe de l'Ouest.

1.2. - Intérêts écologiques de l'aulne en Europe.

Les caractéristiques biologiques des aulnes en font principalement des essences pionnières. Elles associent une pollinisation anémophile et une dissémination des graines par l'eau et par le vent à une croissance juvénile très rapide. L'aulne est capable de coloniser très rapidement des sols nus ou enherbés et la plupart des espèces sont tolérantes à des niveaux élevés de nappes phréatiques, ainsi qu'à de fréquents épisodes d'inondation, grâce au développement de racines adventives. Comme pour la plupart des ligneux, des associations symbiotiques mycorrhiziennes s'établissent au niveau des radicelles entre l'aulne et certains champignons. Cette essence présente en plus la particularité d'entretenir une symbiose avec l'actinomycète *Frankia alni*, capable de fixer l'azote atmosphérique. Cette association mutualiste se traduit par le développement de nodosités racinaires qui permettent de fixer des molécules d'azote et de les rendre disponible pour l'aulne mais aussi pour son environnement (Boullard, 1990).

Ces espèces constituent donc des essences importantes pour l'établissement naturel de boisements sur des sites pédoclimatiques difficiles. La diversité de leurs caractères leur permet aussi bien de s'établir en pionnier sur des zones géographiques très contraignantes que de constituer un genre pilier dans certaines forêts climax du centre et du Nord de l'Europe (Claessens, 2003). Leur capacité à fixer l'azote atmosphérique les rend particulièrement efficaces pour l'amélioration des sols pauvres et pour favoriser l'implantation d'autres essences sur ces derniers. En Europe, l'espèce *A.glutinosa* constitue la principale essence sur de nombreuses rivières et joue un rôle vital dans l'écosystème des berges (ripisylves) qu'elle contribue aussi très fortement à stabiliser d'un point de vue mécanique, notamment lors des phénomènes inondatifs. Les alignements d'aulnes glutineux représentent une zone tampon le long de nombreux cours d'eaux et jouent un rôle important de filtration et de purification de l'eau. La zone d'ombre qu'ils apportent contribue enfin de façon notable à l'entretien de la biodiversité au bord des rivières, mais aussi au sein de leur lit (Dussart, 1999).

Enfin, les différentes espèces européennes d'aulnes apportent une contribution substantielle à l'économie locale de certaines régions, car la production de bois peut être valorisée de nombreuses

manières : bois de placage, ébénisterie, bois de chauffage, contreplaqué, panneau de particules ou encore sculpture (White & Gibbs, 2000 ; Claessens, 2003).

2. - Problèmes sanitaires décrits sur l'aulne.

2.1. - Dommages abiotiques.

L'essentiel des dommages abiotiques décrits sur l'aulne est causé par des phénomènes de sévères sécheresses estivales. Ces dernières ont été invoquées dans la mortalité de nombreux aulnes ripicoles en Europe centrale (revues par Cech & Hendry, 2003). Un assèchement indirect des ripisylves par la canalisation des rivières, la mise en place de drainages ou de captages d'eau potable ont aussi provoqué des dépérissements localisés.

Les gelées tardives ou les hivers très rigoureux ne provoquent pas de dépérissement chez cette essence, tout au plus des blessures localisées (Cech & Hendry, 2003).

Enfin, des symptômes ponctuels de dépérissement de la couronne foliaire ainsi que l'apparition de taches de couleur rouille sur le tronc ont été associés à l'application d'herbicides à proximité des aulnes, ou encore à l'étranglement mécanique des flux de sèves par des fils barbelés (Cech & Hendry, 2003).

2.2. - Problèmes liés aux maladies ou aux ravageurs.

2.2.1. - Ravageurs.

L'essentiel des affectations causées par des insectes à l'aulne sont à mettre à l'actif du charançon du saule et du peuplier (*Cryptorhynchus lapathi*), dont la larve peut provoquer des blessures sur des branches de toutes tailles, favorisant leur bris. Les blessures d'origine larvaire peuvent provoquer un dépérissement léger de la couronne, mais plus généralement ne génèrent pas plus que des nécroses dispersées et peu profondes (Cech & Hendry, 2003).

2.2.2. - Dommages d'origine bactérienne.

Une bactérie du genre *Erwinia* (*E. alni*) a été associée à des symptômes de nécroses sous-corticales limitées et de taches de couleur rouille à la surface de l'écorce (Surico et al., 1996). Cette dernière aurait été impliquée dans des dépérissements locaux d'aulnes en Toscane (Italie) durant les années 1950 (Moriondo, 1958).

Toujours en Italie, des cas de mortalité d'aulnes glutineux et d'aulnes de Corse ont été attribués à des organismes procaryotes proches des bactéries (Mycoplasm-like organisms, MLO). Ces derniers ont été isolés d'aulnes présentant des symptômes de dépérissement tels que feuilles peu nombreuses, petites et tombant prématurément, chancres corticaux et mortalité de branches (Marcone et al., 1994). Toutefois, Lederer et Seemüller (1991) ont aussi mis en évidence la présence de ces MLO sur des aulnes d'aspect

tout à fait sain, ce qui laisse supposer que les MLO n'étaient pas les seuls facteurs en cause dans les dépérissements observés.

2.2.3. - Les maladies cryptogamiques.

Un certain nombre de champignons ont été associés à des nécroses sur tronc et branches d'aulnes, mais n'apparaissent finalement qu'en tant que parasites secondaires intervenant suite à un affaiblissement de l'arbre causé par des dommages d'origine biotique (galerie ou morsure larvaire) ou abiotique (Cech & Hendry, 2003). Parmis les taxons les plus fréquemment isolés figurent *Ophiovalsa suffusa*, *Valsa oxystroma* (Cech & Hendry, 2003), *Phoma* spp., *Cylindrocarpon* spp. et *Cryptosporiopsis* sp. (Streito et al., 2002b). L'ascomycète *Hypoxylon fuscum* a été fréquemment isolé de nécroses corticales d'aulnes blancs au faciès dépérissant lors d'une prospection menée en Pologne dans une région forestière altérée par la pollution atmosphérique (Domanski & Kowalski, 1987). Il a été toutefois reconnu plus tard que l'espèce en cause n'avait qu'un rôle de parasite secondaire, profitant d'un affaiblissement général des aulnes se développant dans un contexte défavorable. Enfin, Moricca (2002) a décrit en Italie du Nord des symptômes de dépérissement sur *A. cordata* et *A. viridis* causés par *Phomopsis alnea*. La sévérité des symptômes est toutefois fortement dépendante de l'état de stress initial de l'aulne infecté.

Les basidiomycètes du genre *Armillaria* sont assez fréquents sur les aulnes (Peace, 1962) et engendrent des nécroses racinaires. Toutefois, bien que des symptômes de dépérissement de couronne n'aient jamais été associés à la présence d'armillaires, Cech & Hendry (2003) recensent quelques cas de lésions basales du tronc causées par *Armillaria* sp.

Enfin, des cas ponctuels de dépérissement d'aulnes aux Pays-Bas ont été attribués à l'action conjuguée de l'application d'herbicide à la présence d'Oomycètes du genre *Pythium* (Cech & Hendry, 2003). Certains de ces *Pythium*, appartenant au groupe « P » ont démontré leur capacité à causer des nécroses racinaires sur des jeunes semis d'aulnes.

2.3. - Dépérissements d'origine complexe.

Des dépérissements locaux assez conséquents ont été rapportés dans différents pays européens durant le XXe siècle. En Allemagne, puis en Belgique, aux Pays-Bas et au Danemark, des cas récurrents de dépérissement d'aulnes se sont manifestés sur des plantations ayant systématiquement dépassé leur 12ème année de développement. Ce type particulier de dépérissement ('Erlensterben'), initialement attribué à une mauvaise adaptation pédoclimatique des clones utilisés, n'a néanmoins jamais été totalement élucidé, bien qu'il soit acquis qu'aucune affection parasitaire ne soit mise en cause dans ce cas (Cech & Hendry, 2003).

Durant les années 1990, des cas locaux de dépérissement d'aulnes ont été observés en Ecosse, principalement le long de cours d'eau (Gregory et al., 1996). L'aspect progressif des dommages, ainsi que les symptômes nécrotiques observés sous l'écorce militaient pour une explication d'ordre

parasitaire. Néanmoins, bien que de nombreux champignons aient été isolés des nécroses (*Ophiovalsa suffusa*, *Melanconis alni*, *Cryptosporiopsis* sp., *Innonotus radiatus*, ainsi que deux autres espèces non identifiées) les tests de pathogénicité n'ont pu démontrer leur implication directe dans les dépérissements observés (Cech & Hendry, 2003). Finalement, Gregory et al. (1996) ont expliqué ces dépérissements par l'action latente de ces champignons, dont le développement aurait été favorisé lors de situations de stress ou d'affaiblissement des aulnes.

3. - Emergence récente d'une nouvelle maladie sur les aulnes en Europe.

L'aulne était donc jusqu'à présent décrit comme indemne de maladie majeure, tout comme peu sujet à des attaques sévères de ravageurs (Cech & Hendry, 2003). Toutefois, en 1993, des dépérissements massifs de populations d'aulnes en ripisylve ont été observés dans de nombreuses localités du Royaume-Uni (Gibbs et al., 1994).

3.1. - Mise en évidence de dépérissements massifs à travers l'Europe.

Les symptômes observés par Gibbs et al. (1994) étaient identiques à ceux causés par des Oomycètes du genre *Phytophthora* sur d'autres ligneux à feuilles caduques. Les feuilles étaient moins nombreuses, anormalement petites et légèrement jaunissantes (Figure 1A); une nécrose racinaire était constatée et des bandes nécrotiques de tissus sous-corticaux s'étendaient du collet en remontant vers le tronc (Figure 1B). Sur ce dernier, des taches noirâtres accompagnées d'exsudats d'aspect goudronneux étaient fréquemment visibles (Figure 1C). Les études prospectives qui suivirent mirent en évidence des dépérissements dans d'autres sites qui n'étaient pas à proximité immédiate de zones humides, et montrèrent qu'au total des des milliers d'aulnes présentaient des faciès dépérissants (Figure 1D).

A B C

D

Figure 1 : Symptômes typiques de dépérissements d'aulnes. (A) Feuilles nanisantes et chlorotiques en comparaison avec des feuilles d'aspect sain (Photo JC Streito, LNPV-UMAF). (B) Nécrose sous-corticale en forme de flamme sur sujet adulte, devenant *in fine* ceinturante (Photo C. Husson, INRA). (C) Tâches de couleur brun-rouille et exsudations brunes sur jeune sujet (Photo JC Streito, LNPV-UMAF). (D) Aspect général d'un aulne dépérissant présentant un éclaircissement caractéristique du houppier (à droite) en comparaison avec un sujet d'aspect sain (à gauche) (Photo JC Streito, LNPV-UMAF).

Suite à d'autres signalements de dépérissements massifs ailleurs en Europe, une action concertée européenne (FAIR5 CT97 3615) a été financée de 1998 à 2001. Celle-ci avait pour objectifs d'évaluer l'aire de répartition de la maladie en Europe, de produire des recommandations en matière de lutte contre la maladie et d'identifier les axes de recherche à développer en priorité vis-à-vis de cette maladie émergente. Les prospections organisées pendant la durée de cette action concertée ont montré que la maladie de dépérissement de l'aulne était présente dans de nombreux pays européens : Royaume-Uni, Pays-Bas, France, Allemagne, Suède, Autriche, Belgique, Irlande, Hongrie, Italie, Slovénie et Lituanie. En revanche, l'Europe reste actuellement le seul continent touché par la maladie. En France, la situation est relativement préoccupante, beaucoup de bordures de cours d'eau sont

contaminées et des milliers d'aulnes sont en cours de dépérissement sur la majorité du territoire (Streito et al., 2002b). Une étude récente menée sur une grande partie du bassin Rhin-Meuse a montré que cette nouvelle maladie était présente dans 80 % des sites prospectés, avec pour ces derniers une prévalence moyenne de la maladie atteignant 16 % (Thoirain et al., 2006).

3.2. - Caractéristiques de la maladie.

Les symptômes observés ailleurs en Europe où des dépérissements d'aulnes ont été mis en évidence sont identiques à ceux décrits au Royaume-Uni (Gibbs, 2003). Il a été de plus constaté qu'une abondante fructification des arbres constituait parfois une autre caractéristique de la maladie. La manifestation la plus visible et la plus immédiate de la présence du dépérissement sur l'arbre reste prioritairement le net éclaircissement du houppier, sans descente de cime. Ces arbres observés ensuite de plus près présentent très souvent des nécroses sous-corticales à la base du tronc, que l'on peut mettre en évidence en sondant l'écorce autour des tâches noirâtres à exsudats. Dans certains cas, il n'y a aucun symptôme visible à la base du tronc, et le dépérissement du houppier a été associé à des nécroses initiées à partir des racines adventives, parfois nombreuses chez *A. glutinosa* (Lonsdale, 2003). Bien que beaucoup de jeunes semis d'aulnes présentant des symptômes au collet meurent rapidement, les sujets de diamètre plus important peuvent tout à fait survivre malgré leur infection par le parasite, suffisament de tissu sous cortical restant vivant et continuant d'alimenter une partie du houppier. Cette croissance minimale peut rester tout de même limitée dans le temps et l'arbre peut finir par dépérir complètement les années qui suivent. De plus, il a été constaté que lors d'un recépage d'un aulne adulte malade, certains des rejets pouvaient contracter la maladie alors que d'autres rejets de la même cépée pouvaient rester indemnes pendant quelques années (Gibbs, 2003 ; Jung & Blaschke , 2004).

3.3. - Une nouvelle espèce de *Phytophthora* est responsable de cette maladie émergente.

L'agent causal de la maladie a été isolé pour la première fois en 1993 au Royaume-Uni (Gibbs, 2003). Un Oomycete du genre *Phytophthora* (Règne des Straménopiles) était systématiquement associé à la maladie au niveau des nécroses sous-corticales (Gibbs, 1994) et était capable de reproduire les symptômes typiques par inoculation artificielle (Brasier et al., 1995). L'étude des caractéristiques morphologiques et culturales d'une dizaine d'isolats de ce *Phytophthora* ont conclu à sa proximité avec *Phytophthora cambivora* (Petri) Buisman, agent de dépérissement de nombreuses autres espèces ligneuses (Gibbs, 1995). Toutefois, ses caractéristiques en culture *in vitro* comme la morphologie de son thalle, ses températures optimales et maximales de croissance, son type apparent de reproduction sexuelle (homothallisme) et l'absence de germination des spores sexuées (oospores), démontrèrent que cet Oomycète différait de *P. cambivora*. Les études menées par Brasier et al. (1995) suggérèrent par la

suite que le *Phytophthora* agent de dépérissement de l'aulne était probablement un hybride naturel ayant pour un des parents putatifs *P. cambivora*.

4. - Etat de l'art sur les caractéristiques de *Phytophthora alni* agent du dépérissement de l'aulne.

4.1. - Le *Phytophthora* de l'aulne est un hybride interspécifique.

4.1.1. - Le *Phytophthora* de l'aulne est un complexe de différentes entités.

Les premières études morphologiques et culturales rapportées par Brasier et al. (1995) mirent en évidence des caractéristiques singulières chez ce *Phytophthora* de l'aulne. Bien que morphologiquement proche de *P. cambivora*, avec lequel il partage la singularité de présenter des oogones ornementées (Figure 2), il s'en distingue par son homothallisme, l'avortement d'un grand nombre d'oogones en culture, son aspect cultural rasant ainsi que par ses températures optimale et maximale de croissance sensiblement inférieures (Brasier et al., 1995).

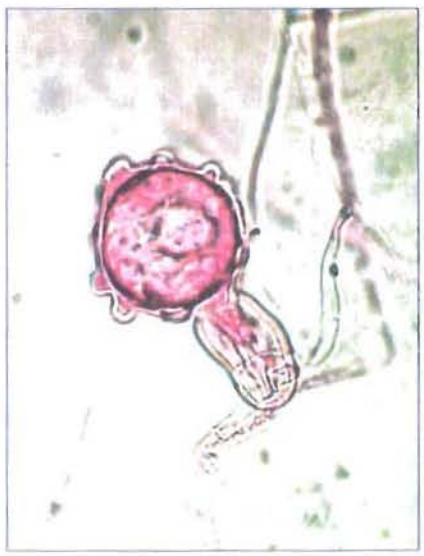

Figure 2 : Oogone ornementée de *Phytophthora alni* subsp. *alni*, et anthéridie bicellulaire amphigyne.

Le développement anormal des structures gamétangiales combiné à sa capacité à s'auto-fertiliser conduirent Brasier et al. (1995) à envisager une origine hybride pour ce nouvel agent pathogène, impliquant *P. cambivora* comme un des parents potentiels.

Une contribution majeure à la description de ce nouveau *Phytophthora* a été apportée par les travaux de Brasier et al. (1999). Ces travaux ont tout d'abord démontré que l'ensemble des souches Européennes de *Phytophthora* de l'aulne, isolées de nécroses sous-corticales ou de sol sous aulne dépérissant, n'était pas homogène, mais constituait un complexe de différentes entités hétéroploïdes. Les différents isolats ont ainsi été répartis dans plusieurs groupes, selon leurs caractéristiques

culturales, cytologiques et génétiques : un groupe « standard » et différents « variants », baptisés en fonction de leur origine géographique respective (Brasier et al., 1999).

4.1.2. - Le *Phytophthora* de l'aulne de type « standard » : *P. alni* subsp. *alni* (*Paa*).

Le type « standard » est le type majoritaire dans toute l'Europe (Brasier et al., 2004). La ploïdie classique chez les *Phytophthora* spp., y compris *P. cambivora*, est à '2n' avec un nombre de chromosome de l'ordre de 10-12. Le type « standard » présente des gamétanges (oogones et anthéridies) semblables à l'un des parents de l'hybride, *P. cambivora*. Le type « standard » est décrit comme quasi-tétraploïde (4n+2) et reste incapable de réaliser la méiose au-delà de la métaphase I (Tableau 1). L'analyse de la séquence des ITS (Internal Transcribed Spacers de l'ADNr, fréquemment utilisés en phylogénie) d'isolats du type « standard » montre qu'il existe un polymorphisme inter-isolats mais aussi intra-individu, avec jusqu'à 11 sites polymorphes (Figure 3). Cette absence d'homogénéité intra-, mais aussi inter- individu(s) du type « standard », suggère que ce type est encore en cours d'évolution. L'additivité observée à plusieurs sites au sein des ITS serait une preuve supplémentaire de la nature allopolyploïde du génome du type « standard ». Il est de plus décrit comme phénotypiquement instable (Brasier et al., 1999).

L'analyse isozymatique utilisant trois enzymes dimériques (Brasier et al., 2004) montre que tous les isolats de type « standard » étudiés présentent des profils identiques avec deux singularités : hétérozygotie à chacun des trois loci et présence d'un profil trisomique au locus *Gpi* (Tableau 1).

Cette combinaison de caractères observés chez les isolats de type « standard » a servi de base pour la description d'une nouvelle espèce : *Phytophthora alni* subsp. *alni* Brasier & SA Kirk subsp. nov. (Brasier et al., 2004)

4.1.3. - Les types « variants allemand, anglais et néerlandais » : *P. alni* subsp. *multiformis* (*Pam*).

Les « variants » isolés dans différents pays européens sont morphologiquement et génétiquement distincts du type « standard », *Paa*. Ils présentent des morphologies culturales, des types gamétangiaux ainsi que des relations température-croissance différents (Tableau 1). Ils montrent chacun par ailleurs une agressivité différente vis-à-vis de l'aulne (Brasier & Kirk, 2001 ; De Merlier et al., 2005) et sont de plus décris comme phénotypiquement instables (Brasier et al., 1999). Ces types « variants » présentent des niveaux estimés de ploïdie intermédiaires (2n+4 à 2n+7) entre le type « standard » et les parents putatifs (Tableau 1). Certains de ces types « variants » présentent des séquences ITS homogènes pour un même isolat et tendent à être très proches de l'un des deux parents putatifs proposés par Brasier et al. (1999), qui serait un taxon proche de *P. fragariae*. L'analyse isoenzymatique utilisant les mêmes enzymes dimériques que pour la caractérisation du *Paa* montre que tous les isolats étudiés de type « variant allemand, anglais et néerlandais » présentent des profils identiques, mis à part le type « variant allemand » pour lequel aucun produit correspondant à l'enzyme

*Mdh*1 n'a pu être mesuré (Brasier et al., 2004). En revanche, seules une à deux isoenzymes ont été mises en évidence à chacun des trois loci avec le locus *Gpi* clairement hétérozygote (Tableau 1).
Malgré des différences phénotypiques sensibles, les résultats d'analyses isoenzymatiques et génétiques sont identiques pour ces différents variants. Il a donc été choisi de regrouper ces trois types de variants « allemand, anglais et néerlandais » au sein d'une même sous-espèce : *Phytophthora alni* subsp. *multiformis* Brasier & SA Kirk, subsp. nov. (Brasier et al., 2004). La présence de *Pam* est aujourd'hui officiellement rapportée au Royaume-Uni, en Allemagne, aux Pays-Bas (Brasier et al, 2004), en Belgique (De Merlier et al., 2005) et en France (Ioos et al., 2005), mais *Pam* est probablement aussi présent dans d'autres pays européens (Brasier et al., 2004).

4.1.4. - Le type « variant suédois » : *P. alni* subsp. *uniformis* (*Pau*).

Le type « variant suédois » est morphologiquement et génétiquement distinct du type « standard », *Paa* et des types « variants allemand, anglais et néerlandais », *Pam*. Son agressivité sur *Alnus glutinosa* est décrite comme moindre que celle du *Paa* et du *Pam* (mis à part le type « variant anglais ») (Brasier & Kirk, 2001 ; De Merlier et al., 2005). Tout comme le *Paa* et le *Pam*, il semble phénotypiquement instable (Brasier et al., 1999). La ploïdie estimée du type « variant suédois » est en revanche proche de la diploïdie (2n+2 ; Brasier et al., 1999). Sa séquence ITS est homogène et relativement proche de celle du *P. cambivora*, qui constitue l'autre parent putatif du *Phytophthora* de l'aulne selon Brasier et al. (1999). L'analyse isozymatique montre que les isolats de type « variant suédois » sont homozygotes aux trois loci étudiés (Tableau 1).
Cette combinaison commune et singulière de caractères observés chez les isolats de type « variants suédois » a servi de base pour la description d'une nouvelle sous-espèce : *Phytophthora alni* subsp. *uniformis* Brasier & SA Kirk, subsp. nov. (Brasier et al., 2004). La présence de *Pau* est aujourd'hui officiellement rapportée en Allemagne, en Autriche, en Lituanie, (Brasier et al, 2004), en Suède (Olsson, 1999) en Italie (Santini et al., 2001) en France et en Slovénie (Ioos et al., 2005), en Belgique (De Merlier et al. 2005), en Hongrie (Nagy et al., 2003), mais *Pau* est probablement aussi présent dans d'autres pays européens (Brasier et al., 2004).

4.1.5. - Autres variants.

Brasier et al. (2004) rapportent l'existence d'autres variants phénotypiques au sein de la sous-espèce *P. alni* subsp. *multiformis* (*Pam*). Ces derniers se singularisent par des ornementations oogoniales intermédiaires entre les types « variant allemand » et « variant néerlandais », et s'en distinguent légèrement par leurs profils RAPD (Randomly Amplified Polymorphic DNA). Néanmoins, ils présentent des séquences ITS et des profils isoenzymatiques identiques aux isolats correspondant à *Pam*.
L'existence de variants mineurs au sein de la sous-espèce *Paa* est aussi décrite en Angleterre. Ces derniers seraient moins agressifs envers l'aulne et présenteraient des profils isoenzymatiques légèrement différents du *Paa* type (Brasier et al., 2004).

Enfin, Jung & Blaschke (2004) avaient rapporté l'existence en Bavière de taxons issus de rétrocroisements entre le « type standard » de *Phytophthora* de l'aulne et *P. cambivora* ainsi que de nouveaux types variants. Toutefois, la consistence de ces nouvelles entitées, déduites de séquence ITS (Jung & Blaschke, 2004) n'a pas été confirmée ni mentionnée dans la description formelle des différents taxons constituant l'espèce *P. alni sensu lato* (Brasier et al., 2004 ; C.M. Brasier, communication personnelle).

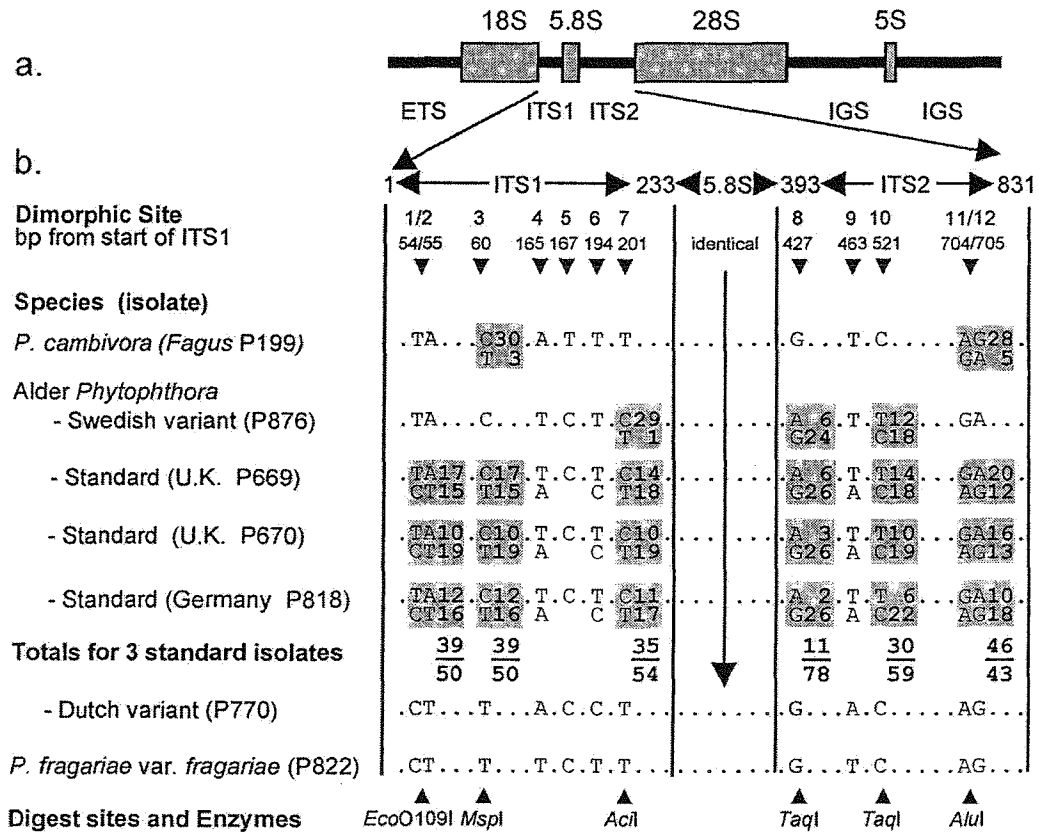

Figure 3 : Localisation des sites polymorphes au sein de l'ADN ribosomique (ADNr) de différents isolats de *Phytophthora* de l'aulne. (a) Présentation schématique de l'organisation d'une unité répétée de l'ADNr : sous-unités d'ARN ribosomique (ARNr) et espaceurs (ETS : External Transcribed Spacer ; ITS1 et ITS2 : Internal Transcribed Spacers ; IGS : InterGenic Spacers ; et sous-unités ribosomiques 18S, 5.8S, 28S, et 5S. (b) Disposition des sites polymorphes à l'intérieur de la région de 831 pb qui couvre l'ITS1, la sous unité 5.8S et l'ITS2. L'extrémité 5' de l'ITS1 a été numérotée « 1 » et les autres sites ont été numérotés à partir de cette référence. A l'endroit des sites polymorphes (régions grisées), les deux bases sont indiquées. Pour chaque site, les nombres associés aux bases correspondent au nombre de clones de produits de PCR présentant ces différentes bases à ce même site. Le nombre total de clones observés est indiqué pour l'ensemble des isolats de type « standard ». En bas de la figure sont indiquées les enzymes de restriction utilisées pour caractériser chacun des sites polymorphes. (Extrait de Brasier et al., 1999)

Tableau 1 : Principales caractéristiques culturales et cytologiques des différents entités constituant *Phytophtora alni sensu lato* et des deux espèces phylogénétiquement proches proposées comme espèces parentales par Brasier et al. (1999).

Type d'hybride *sensu* Brasier et al. (1999)	Nomenclature *sensu* Brasier et al. (2004)	Caryotype[a]	Isozymes[b]			Développement *in vitro*	Température optimale / maximale de croissance (°C)[a]	Oogone	Méiose[a]	Plantes hôtes / agressivité sur l'aulne
			Mdh-1	*Mdh*-2	*Gpi*					
Standard	*P. alni* subsp. *alni*	~4n + 2	87/100	94/100	85/93/100	Instable[a,f] Stable[f]	25/30	Ornementée[a,g] Lisse ou faiblement ornementée[e]	Incomplète	Aulne uniquement / très agressif[c], modérément à très agressif[e]
Variant allemand	*P. alni* subsp. *multiformis*	~2n + 7	abs.	94/94	85/100	Instable[a,f]	27/32	Ornementée[a] Lisse ou faiblement ornementée[e]	Incomplète	Aulne uniquement / faiblement agressif[c], modérément à très agressif[e]
Variant hollandais	*P. alni* subsp. *multiformis*	~2n + 4	100/100	94/94	85/100	Très instable[a] Stable[e,f]	27/32	Très ornementée[a] Lisse ou faiblement ornementée[e]	Complète	Aulne uniquement / relativement agressif[c], modérément à très agressif[e]
Variant anglais	*P. alni* subsp. *multiformis*	nd	100/100	94/94	85/100	nd	nd	nd	nd	Aulne uniquement / faiblement agressif[c], modérément à très agressif[e]
Variant suédois	*P. alni* subsp. *uniformis*	~2n + 2	87/87	100/100	93/93	Très instable[a] Stable à instable[c] Stable[f,g]	27/30	Lisse[a,c] Lisse ou ornementée selon T°C de culture[g]	Complète	Aulne uniquement / faiblement agressif[c], Faiblement à très agressif[e], Faiblement agressif sur *A. glutinosa* et très agressif sur *A. cordata*[d]
Parents putatifs[a]										
P. fragariae		2n	-	-	-	Stable[a,f]	25/30	Lisse[a]	Complète	Fraisier, Framboisier / non agressif[c]
P. cambivora		2n	87/87	94/100	93/93	Stable[a,f]	27/34	Ornementée[a]	Complète	Nombreux ligneux* / faiblement agressif[c]

nd : trop instable pour être déterminé ; abs. : pas de protéine résolue ; *non agressif sur *Alnus* spp.
[a]Selon Brasier et al. (1999)
[b]Selon Brasier et al. (2004) sauf pour *P. cambivora* : W. Man in't Veld, communication personnelle.
[c]Selon Brasier and Kirk (2001)
[d]Selon Santini et al. (2003)
[e]Selon De Merlier et al. (2005). *nota* : les isolats de *Pam* étudiés n'ont pas été affectés à un type particulier de variant.
[f]Selon R. Ioos, observations personnelles
[g]Selon Nagy et al., 2003

4.3. - Pathogénicité des différentes sous-espèces de *P. alni.*

4.3.1. - Le pouvoir infectieux.

Les facteurs intervenant du côté du pathogène dans les interactions hôtes/parasites sont liés au pouvoir infectieux. Ce dernier comporte deux composantes : la virulence et l'agressivité (Van der Planck, 1968). La virulence est la composante qualitative du pouvoir pathogène permettant à celui-ci d'attaquer, après reconnaissance d'un hôte donné. Elle est de type « tout ou rien » et dépend de gènes isolés et indépendants. L'agressivité est la composante quantitative du pouvoir pathogène et ne s'exprime que si le parasite est virulent. Elle varie en continu et est gouvernée par plusieurs gènes dont les effets se cumulent. Son expression est souvent influencée par les facteurs physiques du climat. D'elle dépend la vitesse de progression de l'épidémie. A l'heure actuelle, il ne semble pas exister d'espèce, ni de génotype d'aulne totalement résistant à la maladie causée par *P. alni.* L'étude du pouvoir infectieux s'est donc pour l'instant limitée à évaluer l'agressivité du parasite envers son hôte. Toutefois, certains spécimens semblent présenter des niveaux de tolérance naturelle intéressants et sont en cours d'évaluation au CRA de Gembloux, Belgique (Dr A. Chandelier, communication personnelle).

4.3.2. - Agressivité des différentes sous-espèces de *P. alni.*

Les premiers tests de pouvoir infectieux ont été réalisés par Brasier & Kirk (2001). Les tests ont consisté en l'inoculation après blessure de bûchettes vivantes d'*Alnus glutinosa* par du mycélium actif d'une série représentative d'isolats européens de *Paa, Pam, Pau, P. cambivora, P. fragariae* et d'autres espèces de *Phytophthora* communes en ripisylves ou agressives sur ligneux. Les résultats obtenus ont montré que seuls les isolats de *Paa* et le type « variant néerlandais » de *Pam* étaient hautement agressifs sur l'aulne. Les isolats de *Pau* et les autres types « variants » de *Pam* se sont avérés seulement faiblement agressifs, tout comme les isolats de *P. cambivora* (Tableau 1). En revanche, les isolats de *P. fragariae* et les autres espèces de *Phytophthora* n'ont produit que des nécroses très limitées au niveau des points d'inoculation.

De Merlier et al. (2005) ont ensuite évalué le pouvoir pathogène de différents isolats de *P. alni* collectés en Wallonie (Belgique) et comprenant des représentants de chaque sous-espèce. Ces tests ont été réalisés en inoculant de jeunes plantules d'aulnes avec des marges actives de culture de *P. alni sensu lato* au niveau de blessures artificielles. Les isolats Wallons de *Paa, Pam* et *Pau* se sont tous avérés agressifs lors de cette expérimentation. Les résultats n'ont pas permis de mettre en évidence une différence d'agressivité statistiquement significative entre les différentes sous-espèces de *P. alni.* En revanche, pris dans leur ensemble, certains isolats étaient significativement plus agressifs que d'autres mais faisaient partie indistinctement des sous-espèces *Paa, Pam* ou *Pau.*

Néanmoins, il est important de noter que les protocoles utilisés pour évaluer l'agressivité de ces *Phytophthora* ne correspondent pas tout à fait à une réalité épidémiologique car il est plus probable

que les infections naturelles se produisent par contamination par des zoospores au niveau de microblessures ou de racines adventives (cf. § 4.4.). De plus, l'aspect d'interaction hôte/parasite n'est pas correctement pris en compte puisque l'on favorise la contamination et l'infection par blessure sévère et inoculation massive. Ainsi, la faible agressivité des isolats de *Pam* (mis à part le type « variant néerlandais ») et *Pau* semble quelque peu mitigée par le fait que les isolats testés ont été isolés de nécroses sous-corticales naturelles sur aulne.

Enfin, Santini et al. (2003) se sont attachés à évaluer l'agressivité d'un isolat italien de *Pau* vis-à-vis de différentes essences forestières, y compris deux espèces d'aulne, par inoculation de mycélium sur blessure artificielle ou de mycélium dans le substrat de culture. Les auteurs ont démontré que l'agressivité de l'isolat italien de *Pau* était significativement plus élevée sur les deux espèces d'aulnes (*A. glutinosa* et *A. cordata*) que sur chêne rouvre (*Quercus robur*), noyer (*Juglans regia*) et châtaignier (*Castanea sativa*). Dans la même étude, l'isolat italien de *Pau* s'est montré significativement plus agressif sur *A. cordata* (essence à partir duquel il avait été initialement isolé) que sur *A. glutinosa*. Les auteurs rapportent à ce titre la présence de *Pau* sur *A. glutinosa* sans symptôme apparent en pépinière.

4.4. - Eléments d'épidémiologie.

Le dépérissement massif de peuplements d'aulnes est un phénomène relativement récent à l'échelle de la pathologie végétale en tant que science. Ainsi, les premiers travaux sur la maladie ne datent que du milieu des années 1990, et se poursuivent actuellement. Toutefois, les différentes études menées depuis la première description de l'agent pathogène responsable ont permis d'apporter quelques éléments sur l'épidémiologie de cette nouvelle maladie de l'aulne.

4.4.1. - Sources d'inoculum.

Les tissus nécrotiques sous-corticaux du tronc ainsi que les tissus racinaires lignifiés infectés semblent évidemment constituer une source importante d'inoculum. Le potentiel infectieux de ces tissus a été confirmé expérimentalement par le biais de pièges biologiques utilisant ces tissus (Lonsdale, 2003). Jung & Blaschke (2004) sont parvenus à provoquer en quelques mois l'infection de plants d'aulnes en plaçant des fragments de tissus infectés par *P. alni* sur le substrat de culture. D'abondantes formations de sporanges ont été observées à la surface de tels tissus immergés quelques heures dans de l'eau (T. Jung, données non publiées). De plus, Streito (2003) rapporte qu'il est possible de ré-isoler *P. alni* à partir de fragments de nécroses sous corticales enfouies sous terre pendant au moins trois mois. Néanmoins, les tissus nécrotiques ne semblent pas systématiquement héberger *P. alni* sous une forme viable puisque ce dernier n'est pas toujours fructueusement isolé de nécroses, d'apparence pourtant actives (Streito et al., 2002a). A ce titre, les résultats de Brasier & Kirk (2001) montrent qu'il existe un effet saisonnier significatif sur l'agressivité de *P. alni* (faible de

novembre à mars et nulle en avril), ce qui pourrait aussi expliquer qu'il peut être plus difficile d'isoler l'agent pathogène à certaines périodes.

Les fines racines pourraient constituer une source d'inoculum non négligeable, comme c'est le cas pour de nombreuses espèces de *Phytophthora*. La capacité à produire des sporanges à partir de fines racines inoculées artificiellement par *P. alni* a été vérifiée expérimentalement (Lonsdale, 2003) et la production de sporanges et de zoospores pourrait être favorisée en milieu naturel lors des inondations périodiques.

Enfin, il peut être envisagé que des fragments de *P. alni* sous différentes formes se trouvent dans le sol ou l'eau. Les piégeages biologiques effectués à partir du sol prélevé sous des aulnes infectés ont mis en évidence la présence de *P. alni* à plusieurs reprises (Lonsdale, 2003 ; Jung & Blaschke, 2004 ; C. Husson & R. Ioos, données non publiées). Le type de structures de l'oomycète présentes dans le sol n'est pas connu, mais il peut parfaitement être imaginé que l'inoculum en question est simplement constitué de fragments de racines infectées. En revanche, Streito et al. (2002a) sont parvenus à isoler *P. alni* par piégeage biologique à partir de l'eau d'une rivière présentant des aulnes atteints de dépérissement. *Phytophthora alni* est en effet capable de se disséminer par voie végétative grâce à ses spores motiles dans l'eau (zoospores).

4.4.2. - Résistance et survie.

Il n'existe pas de données publiées quant à la définition exacte des organes de conservation utilisés par *P. alni* pour survivre dans les substrats desquels il a été isolé (sol, nécroses). Néanmoins, l'œuf issu de la reproduction sexuée (oospore) est une structure de résistance bien décrite chez les espèces du genre *Phytophthora*. *Phytophthora alni* produit par ailleurs des quantités considérables d'oospores en culture (Delcán & Brasier, 2001). Néanmoins, Delcán & Brasier (2001) n'ont jamais observé de germination d'oospores de *P. alni* dans leurs conditions expérimentales. Ils ont de plus montré qu'une grande majorité de ces derniers n'étaient pas viables. Lonsdale (2003) rapporte l'absence de formation d'oospores dans des tissus sous corticaux d'aulne naturellement contaminés par *P. alni,* bien qu'en ayant observé un très faible nombre dans des conditions d'inoculation artificielle. Il semble donc peu probable que les oospores jouent un rôle notable dans la conservation de *P. alni.*

La formation de chlamydospores, structures de conservation produites par voie végétative dans le mycélium par certaines espèces de *Phytophthora* n'a jamais été décrite chez aucune sous-espèce de *P. alni.* En revanche, ce dernier produit parfois en culture des cellules à paroi épaissies qui pourraient mimer le rôle de chlamydospores (Lonsdale, 2003).

Finalement, bien qu'il soit acquis que *P. alni* soit capable de survivre plusieurs mois sous forme de mycélium dans les tissus qu'il infecte, on ne sait pas encore précisément comment il est capable de subsister plusieurs saisons successives. Jung & Blaschke (2004) rapportent toutefois que la survie dans le sol de *P. alni sensu lato* est limitée dans le temps, eu égard à sa faible compétitivité dans ce substrat avec d'autres micro-organismes, notamment d'autres *Phytophthora* spp. A titre d'exemple, quelques

pépinières initialement infectées par *P. alni sensu lato* et dans lesquelles des plant d'aulnes n'ont volontairement plus été produits pendant trois années sont apparamment apparues naturellement « désinfectées » suite à cette période de privation de la plante hôte (Jung & Blaschke, 2004). Ces auteurs recommandent ainsi une rotation minimale de trois ans dans les pépinières contaminées par ce parasite pour assurer son extinction.

4.4.3. - Structures de dissémination.

Chez beaucoup d'espèces du genre *Phytophthora*, les zoospores sont des agents majeurs de dissémination et d'infection. Dans le cas de *P. alni*, il apparaît comme très vraisemblable que ces derniers contribuent de façon substantielle à la dissémination de l'agent pathogène, spécialement en ce qui concerne les peuplements d'aulnes ripicoles. Les zoospores de *P. alni* sont capables d'initier des infections chez l'aulne à partir des racines (Lonsdale, 2003 ; Jung & Blaschke, 2004), des microblessures et des racines advendives (Jung & Blaschke, 2004). La dissémination des zoospores de *P. alni* est aisée le long des cours d'eau et les périodes d'inondation permettent à la fois de stimuler la production de sporanges et l'émission de zoospores infectieuses (Jung & Blaschke, 2004). La majorité des symptômes initiaux d'infection par *P. alni* se situe au collet des arbres, facilement inondés et donc constituant des cibles idéales pour les zoospores.

Le transport de terre contenant des formes viables de *P. alni* (engins de terrassement ou plantation plants infectés) a aussi été invoqué pour expliquer la dissémination de l'agent pathogène de régions contaminées vers des régions initialement saines (Jung & Blaschke, 2004).

4.4.4. - Ebauche de cycle épidémiologique.

Il apparaît que la conservation et la dissémination des différentes sous-espèces de *P. alni* ne fait appel qu'à la reproduction asexuée. Bien que de nombreux paramètres restent encore inconnus au sujet de la biologie de ce parasite émergent, il est possible d'imaginer un cycle de développement relativement simple. Le mycélium de *P. alni* infectant différents tissus de l'aulne produit des sporanges dans de l'eau libre ou un sol très humide. Ces derniers produisent à leur tour par différentiation cellulaire des zoospores bi-flagellées motiles dans l'eau libre qui vont par chimiotactisme s'accumuler à la surface de tissus sensibles de l'hôte, comme les microblessures, les racines adventives ou les lenticelles (Lonsdale, 2003). Les zoospores germent et le mycélium de *P. alni* va alors progressivement coloniser les tissus sains de l'aulne. Le mycélium présent dans les lésions de surface pourra alors produire à son tour des sporanges, si les conditions le permettent. La contamination et l'infection des parties distales de l'appareil racinaire ne semblent pas causer de dépérissement sévère de l'aulne. En revanche, l'infection de la base du tronc ou des racines superficielles lignifiées se traduisent souvent *in fine* par une nécrose ceinturante qui va réduire puis bloquer les flux de sèves, entraînant la mort de l'aulne (Lonsdale, 2003).

4.4.5. - Facteurs de risques concernant la maladie.

Deux études épidémiologiques ont été menées à l'échelle régionale dans le sud de la Grande-Bretagne (Gibbs et al., 1999) et dans l'Est de la France (Thoirain et al., 2006), deux régions particulièrement affectées par des dépérissements massifs d'aulnes. Ces études ont démontré que la probabilité d'infection d'aulnes situés à moins d'un mètre du bord d'un rivière était sept fois supérieure à celle d'aulnes se développant plus loin (Gibbs et al., 1999) et que la prévalence de la maladie était négativement corrélée à la vitesse du cours d'eau (Thoirain et al., 2006). La proximité de l'aulne au cours d'eau et un faible courant augmentent vraisemblablement la probabilité pour les zoospores infectieuses de contaminer leur plante hôte. Une texture fine du sol, en particulier les sols argilo-limoneux, a un effet statistique favorable sur la prévalence de la maladie, comme déjà démontré pour plusieurs espèces de *Phytophthora* en milieu forestier (Thoirain et al., 2006). De plus, l'augmentation de la température des cours d'eau est liée de façon positive à la prévalence de la maladie, ce qui correspond bien au comportement *in vitro* de l'agent pathogène (Thoirain et al., 2006). Enfin, bien que Gibbs et al., (1999) aient montré une bonne corrélation entre la teneur en oxydes d'azote et prévalence de la maladie dans le sud de l'Angleterre, Thoirain et al. (2006) n'ont pu confirmer une telle corrélation en France. Dans les deux études, il a été supposé que la présence de nitrate dans les rivières n'était pas le facteur explicatif, mais plutôt la présence d'une activité humaine plus importante, présentant donc un risque d'introduction de la maladie plus élevé.

Néanmoins, les facteurs de risques cités ne vont s'exprimer qu'une fois la maladie introduite, et *in fine* il est clair que le facteur de risque le plus important reste l'introduction du parasite dans une zone préalablement saine par plantation d'aulnes infectés, ou l'apport d'eau ou de sol contaminés.

Une fois infecté, la vitesse et la probabilité de mort de l'arbre vont dépendre de nombreux facteurs : localisation de l'infection, vitesse de croissance du parasite dans les tissus qu'il va infecter, nature et âge des tissus (diamètre du tronc), fréquence d'exposition à l'inoculum, conditions environnementales, etc. Des études de modélisation épidémiologique sont en cours au sein de l'équipe de Pathologie Forestière, UMR IAM de l'INRA de Champenoux (F. Elegbede, thèse en cours), en s'appuyant sur les données recueillies dans le cadre d'un suivi pluriannuel d'un transect contaminé de la rivière Sarre. L'analyse des premières données obtenues montre que de 2002 à 2005, 35% des arbres de ce transect présentant des symptômes l'année *n* étaient morts l'année *n+3*. Ce chiffre global masque en fait le poids considérable de la mortalité des jeunes plants (diamètre <5 cm) pour lesquels 50% des sujets présentant des symptômes en 2002 étaient mort en 2005. En revanche, pour des sujets moyens (5 cm <diamètre< 30 cm) le pourcentage de mortalité en trois ans s'établit à 13% alors que pour les les sujets « adultes » (dimamètre > 30 cm) la mortalité tombe à 3% (B. Marçais & F. Elegbede, données non publiées).

4.5. - Relations entre *Paa*, *Pam*, *Pau*, et les espèces phylogénétiquement proches.

4.5.1. - Relations entre *Phytophthora alni sensu lato* et les espèces phylogénétiquement proches.

Les travaux de Brasier et al. (1999) ont montré qu'il existait une forte affinité entre *P. alni sensu lato* et deux autres espèces de *Phytophthora* : *P. cambivora* et *P. fragariae*. En effet, les trois espèces présentent des caractéristiques similaires en termes de morphologies gamétangiales et de caractéristiques culturales. De plus, la séquence des ITS de *P. alni*, bien que présentant des sites polymorphes, est très proche de celle de ces deux espèces (Figure 3 ; Brasier et al., 2004). Une étude phylogénétique des *Phytophthora* produite par Cooke et al. (2000) et basée sur ces mêmes régions d'ADN ribosomique a placé sans ambiguïté *P. cambivora* et les deux variétés de *P. fragariae* (var. *fragariae* et var. *rubi*) dans le même clade (« 7a »). Une étude phylogénétique du genre *Phytophthora* portant sur plusieurs loci nucléaires et mitochondriaux a été récemment conduite par Kroon et al. (2004). Cette dernière n'a pas pris en compte toutes les sous-espèces de *P. alni* mais a montré la forte proximité phylogénétique entre *P. fragariae sensu lato* et *Pam.*

Enfin, l'analyse des empreintes génétiques générées par AFLP (Amplified Fragment Length Polymorphism) sur l'ADN total de ces trois espèces (Brasier et al., 1999) (Figure 4), le partage d'allèles communs lors d'analyses isoenzymatiques (Nagy et al., 2003, W. Man in't Veld, communication personnelle, cf. Tableau 1) et le partage de fragments de restriction communs résolus par digestion de l'ADN mitochondrial (Nagy et al., 2003) confortent la proximité génétique entre *P. cambivora*, *P. fragariae* et *P. alni*.

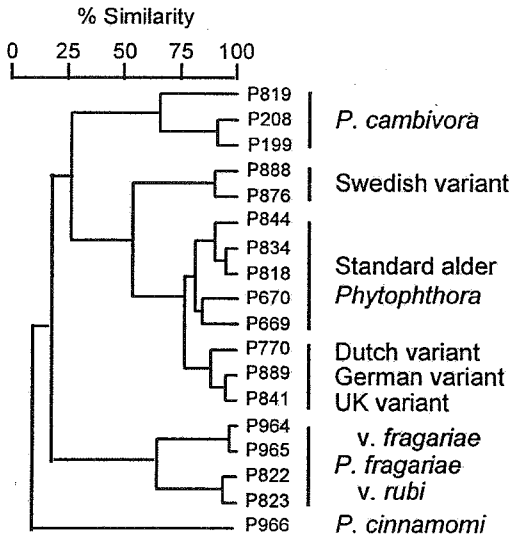

Figure 4 : Dendrogramme construit à l'aide de l'algorithme UPGMA (Unweigth Pair Group Method with Arithmetic mean) à partir d'empreintes AFLP générées à partir de l'ADN total d'isolats de *Paa* (ex « standard type»), *Pam* (ex « Dutch, German, and UK variant »), *Pau* (ex « Swedish variant »), *P. cambivora* et *P. fragariae sensu lato*. *P. cinnamomi* a été utilisé comme groupe externe. Extrait de Brasier et al. (1999).

En se basant sur les ploïdies estimées et par la mise en évidence de fréquents mésappariements des chromosomes lors des méioses, Brasier et al. (1999) ont démontré que le *Phytophthora* de l'aulne était

une entité hybride. La présence de bandes hétérodimériques chez *Paa* lors d'analyses isoenzymatiques utilisant des enzymes dimériques confirme bien le caractère hybride (Olsson, 1999 ; Nagy et al., 2003 ; Brasier et al., 2004). Brasier et al. (1999) ont émis l'hypothèse que les espèces parentales de cet hybride étaient *P. cambivora*, espèce hétérothallique, et un taxon proche de *P. fragariae*, espèce homothallique. Si cette hypothèse était confirmée, il s'agirait du premier cas décrit d'hybride naturel d'agents pathogènes présentant une spécificité parasitaire radicalement différente des parents, en d'autres termes un « super-pathogène » (Brasier, 1995).

4.5.2. - Relations entre les différentes sous-espèces *Paa*, *Pam* et *Pau*.

Les différents entités constituant *P. alni* sensu lato ont été longtemps définies comme des « types », « standard » (*Paa*) ou « variants » (*Pam* et *Pau*) avant de se voir attribuer formellement le rang de sous-espèces (Brasier et al., 2004). Les trois sous-espèces partagent toutes les caractéristiques d'être agressives sur l'aulne (mais à des niveaux variables, cf. 4.3.), d'être homothalliques et de présenter des oogones ornementées (dans une moindre mesure pour *Pau* ; Nagy et al., 2003). De plus, les analyses AFLP effectuées par Brasier et al. (1999) semblent indiquer une origine commune pour les trois sous-espèces, et montrent une plus forte proximité entre *Pam* et *Paa* qu'entre *Pau* et *Paa* (Figure 4).

Les isolats de *P. alni* subsp. *uniformis* présentent une relative uniformité de caractères morphologiques et génétiques. D'autre part, les différents types de variants constituant l'entité *P. alni* subsp. *multiformis* présentent une gamme remarquable de différentes caractéristiques morphologiques, et présentent une ploïdie apparente variant de 2n+4 à 2n+7. En revanche, les différents types « variants » de *Pam* partagent des caractéristiques moléculaires similaires, différentes de *Pau* et *Paa*, ce qui justifie leur regroupement au sein de la même sous-espèce (Brasier et al., 2004). Enfin, *Paa*, qui constitue de loin la sous-espèce la plus fréquente (Brasier et al. 2004) se distingue de *Pam* et *Pau* par sa ploïdie (4n+2), ses empreintes génétiques et son profil alloenzymatique (trois allèles pour le locus *Gpi* ; W. Man in't Veld, communication personnelle). Brasier et al. (1999) ont par ailleurs montré que les profils AFLP de *Paa* étaient presque entièrement constitués de ceux reproduits chez *Pau* et *Pam*.

Brasier et al. (1999) ont émis trois hypothèses concernant les relations entre les trois sous-espèces : *Pam* et *Pau* sont *i)* des descendants de *Paa* produits par ségrégation génétique ou *ii)* des taxons issus de rétro-croisements de *Paa* avec les espèces parentales, ou encore *iii)* des taxons issus d'autres évènements de réticulation (hybridation interspécifique).

La première hypothèse a été évaluée expérimentalement par Delcán & Brasier (2001). Ces derniers ont étudié la descendance de différents isolats de *Paa* et de *Pam* dérivant d'isolement mono-zoospores (200 zoospores par isolat) ou issu de repiquages mono-hyphaux (200 repiquage par isolat) et en ont conclu que la descendance observée se conformait strictement au phénotype parental. Bien que cette première hypothèse ne puisse encore être complètement écartée, l'origine de *Pam* et *Pau* reste donc incertaine.

5. - Objectifs scientifiques de la thèse.

5.1. - Mise au point d'outils moléculaires de détection.

La maladie du dépérissement de l'aulne causée par *P. alni* semble d'origine relativement récente. Bien que l'action concertée européenne 1998-2000 ait permis d'avancer significativement dans la connaissance de cette maladie émergente, beaucoup d'inconnues subsistent, notamment en matière épidémiologique. *P. alni* peut présenter un cycle biologique relativement simple (cf.§ 4.4.4.) mais certains paramètres de ce dernier sont encore mal connus. En particulier, la (ou les) forme(s) de conservation hivernale de *P. alni* est (sont) encore inconnue(s). Sa capacité à se conserver dans le sol, dans les limons des plans ou cours d'eau, ou dans les nécroses sous corticales ou racinaires est encore mal cernée. De même, bien qu'une agressivité variable au cours de l'année puisse être mise en évidence expérimentalement (Brasier & Kirk, 2001), on connaît encore mal comment évolue son activité au cours des saisons. De plus, il existe encore peu de données permettant de décrire dans le temps et dans l'espace ses phases de pathogénicité, de dissémination, de conservation et éventuellement sa phase saprophytique.

Le principal frein aux études épidémiologiques reste la difficulté à mettre en évidence la présence de *P. alni* dans les différents substrats. Il est relativement difficile à isoler sur milieu synthétique à partir de nécroses sous-corticales (Streito et al., 2002a) et semble peu compétiteur vis à vis d'autres espèces de la famille des Pythiacées lorsque des piégeages biologiques tentent de mettre en évidence sa présence dans du sol (C. Husson, communication personnelle). Des outils spécifiques, fiables et sensibles sont donc requis pour poursuivre les indispensables études épidémiologiques concernant ce parasite émergent.

Le premier objectif de la thèse a donc consisté à produire des outils de détection de *P. alni sensu lato*. Ces outils de détection devaient être spécifiques, et permettre notamment de distinguer les trois taxons qui le constituent. Ces outils devraient en outre présenter une robustesse et une sensibilité compatible avec une utilisation sur de nombreux prélèvements de différents substrats, dans le cadre des études épidémiologiques entreprises par l'équipe de pathologie forestière de l'UMR «Interaction Arbres-Microorganismes ».

5.2. - Caractérisation génétique des différents taxons *Paa*, *Pam* et *Pau* et origine du taxon hybride *Paa*.

Les travaux de Brasier et al. (1999) ont permis de mettre en évidence la nature hybride de *P. alni*. Si son allopolyploïdie semble bien supportée par la mise en évidence de sites polymorphes dans la région des ITS (Brasier et al., 1999 ; Brasier et al. 2004), la nature exacte des espèces parentales reste encore uniquement au stade d'hypothèse. *P. cambivora* et un taxon proche de *P. fragariae* ont été

suggérés comme parents potentiels de l'hybride, en se basant sur leur proximité morphologique et la forte homologie de leur séquence ITS avec les différentes sous-espèces de *P. alni* (Brasier et al. 1999). Néanmoins, bien que cette hypothèse soit retenue dans la littérature scientifique (Brasier, 2000 ; Brasier, 2001 ; Brasier & Kirk, 2001 ; Jung & Blaschke, 2004), l'identité des espèces parentale reste encore à démontrer en se basant sur des données plus robustes que les seules similitudes morphologiques et les séquences d'ITS. De même, les relations entre les différentes sous-espèces de *P. alni* restent encore aussi à éclaircir (cf. § 4.5.2.).

Le second objectif de la thèse a donc consisté à tester d'une part l'hypothèse de Brasier et al. (1999) concernant l'origine du taxon *P. alni* subsp. *alni*, et d'autre part à tenter de mieux comprendre la relation entre les différents taxons *Paa*, *Pam* et *Pau*.

5.3. - Développement de marqueurs microsatellites pour *Phytophthora alni sensu lato*.

L'apparition relativement récente de la maladie du dépérissement de l'aulne laisse supposer plusieurs hypothèses (liste non exhaustive) :

i) Un ou plusieurs des agents pathogènes responsables sont d'origine exotique à l'Europe et y ont été récemment introduits,

ii) la ou les hybridations ayant conduit à la création des différentes sous-espèces se sont produites récemment à partir d'espèces déjà bien établies en Europe,

iii) Les agents responsables de la maladie étaient déjà tous présents depuis assez longtemps et un ou plusieurs changement(s) écologique(s) a permis à la maladie de s'exprimer avec beaucoup plus de sévérité.

Les différents taxons constituant *P. alni sensu lato* ne semblent se multiplier et se disséminer qu'en ayant recours à la voie végétative (Delcán et Brasier, 2001 ; Brasier et al., 2004). Brasier et al. (1995) et Nagy et al. (2003) supposent que la dissémination de *P. alni*, et en particulier de *Paa* à travers l'Europe, s'est effectuée sous la forme d'un simple clone. Afin de mieux comprendre la structure spatiale et temporelle de la population des différents taxons constituant *P. alni sensu lato*, l'étude de leur variabilité génétique devrait être entreprise. Les marqueurs microsatellites représentent actuellement un des outils les plus puissants en matière d'étude de génétique des populations. Ces marqueurs co-dominants (permettant de distinguer les états alléliques homozygotes et hétérozygotes) sont de plus particulièrement utiles pour répondre aux questions évolutives chez les membres du règne des Champignons (Weising et al., 1995). De plus, ces outils présenteraient l'avantage de donner des indications intéressantes concernant la ploïdie des organismes étudiés, en se basant sur le nombre d'allèles (Bruno et Brinegar, 2004 ; Pandey et al, 2004 ; Truong et al., 2005) et les ratios d'amplification de ces derniers aux différents loci microsatellites étudiés (Truong et al., 2005 ; Christiansen, 2005). Enfin, le caractère spécifique des marqueurs microsatellites en font des outils

intéressants pour aider à confirmer ou infirmer les différentes hypothèses concernant l'identité des espèces parentales dans le cas d'un hybride, comme démontré dans le cas d'hybrides interspécifiques de champignons du genre *Verticillium* (Barbara et al., 2005).

5.4. – L'hybridation interspécifique dans le genre *Phytophthora.*

L'hybridation interspecifique est un phénomène relativement commun dans le règne végétal (Arnold, 2004) et son importance commence à être reconnue dans le règne animal (Mallet, 2005). Jusqu'à récemment, peu de cas d'hybridations naturelles ont été décrits dans le règne des Champignons (Fungi) (Burnett, 2003). Ces dernières années, il est toutefois apparu un nombre croissant de descriptions d'hybrides naturels impliquant des espèces fongiques ou des Oomycètes, impliquant principalement des agents phytopathogènes (Olson & Stenlid, 2002). En particulier, au sein du genre *Phytophthora*, plusieurs nouveaux taxons hybrides naturels ont été décrits durant cette dernière décade, dont *P. alni*. A côté de ces hybrides naturels, de nombreux travaux ont été consacrés à la caractérisation d'hybrides artificiels entre différentes espèces de *Phytophthora*. Il est donc apparu intéressant d'appréhender l'ensemble du phénomène d'hybridation dans ce genre d'Oomycète, particulièrement important puisque la plupart des espèces qui le composent constituent de sévères agents pathogènes (Brasier, 2002). Les données obtenues suite à des croisements interspécifiques artificiels et les résultats de caractérisation des quelques hybrides naturels décrits à ce jour, y compris les résultats obtenus dans le cadre de cette étude de *P. alni sensu lato* ont été synthétisées dans un article de synthèse qui est en cours de préparation.

Chapitre I

Mise au point d'outils moléculaires de détection des différents taxons constituant *Phytophthora alni sensu lato.*

I.1. – Présentation de la stratégie scientifique.

L'isolement sur milieu synthétique a été pendant longtemps la technique privilégiée pour la détection des champignons phytopathogènes. L'avènement des techniques basées sur la biologie moléculaire, en particulier celles utilisant le principe de la PCR (Polymerase Chain Reaction ; Mullis & Faloona, 1987), a quelque peu révolutionné la pathologie végétale. En général, les techniques basées sur la PCR sont plus rapides, plus spécifiques, plus sensibles et peuvent être mises en œuvre par du personnel n'ayant pas de compétences spécifiques en taxonomie. De plus, elles permettent de mettre en évidence la présence d'organismes non ou difficilement cultivables. Cette dernière caractéristique représente un atout considérable dans le cas de *P. alni* puisque ce taxon a été décrit comme relativement difficile à isoler sur milieu synthétique (Streito et al., 2002a ; Streito 2003). De plus, *P. alni* subsp. *alni* étant un hybride interspécifique (Brasier et al., 2004), les outils classiques de mycologie (isolement et caractérisation morphologique) peuvent présenter des défauts de non-spécificité puisque l'hybride pourrait présenter par nature les caractéristiques phénotypiques parentales (Brasier, 2001).

Nous avons donc entrepris de rechercher dans le génome des différentes sous-espèces de *P. alni* des régions spécifiques de ce dernier, afin de les utiliser comme séquences cibles d'amorces de tests PCR.

La région des ITS est très fréquemment utilisée pour le développement d'amorces « espèce-spécifiques ». Malheureusement, la région des ITS présente des sites polymorphes intra-individuel chez *Paa* et *Pau* et semble encore en cours d'évolution (Brasier et al., 1999), elle ne peut donc convenir au développement d'amorces spécifiques de *P. alni*. Une alternative à l'utilisation dc régions bien caractérisées comme les ITS réside dans la recherche de régions spécifiques à partir d'empreintes génétiques générées en utilisant la technique de RAPD (Randomly Amplified Polymorphic DNA). Nous avons donc généré des marqueurs SCARs (Sequence Characterized Amplified DNA) à partir d'ADN extrait d'isolats de *Paa* et cherché à les utiliser pour définir une série d'amorces de PCR spécifiques de *P. alni*.

Les résultats obtenus sont présentés dans la publication suivante :

Publication 1 :

Ioos R., Husson C., Andrieux A., and Frey P. (2005) SCAR–based PCR primers to detect the hybrid pathogen *Phytophthora alni* and its subspecies causing alder disease in Europe. *European Journal of Plant Pathology* 112, 323-335.

Le transfert et l'application de ces tests PCR aux études épidémiologiques a fait l'objet d'une communication orale lors du 3ème congrès international de l'IUFRO Forest *Phytophthora* Research Workshop, à Freising, Allemagne (2004). Ce travail de transfert ainsi que les premiers résultats

obtenus dans le cadre d'études épidémiologiques ont fait l'objet d'un article qui sera publié dans les annales de ce congrès, dans un numéro spécial du périodique « Forest Research Bulletin ».

Publication 2:

Ioos R., Husson C., Marçais B., and Frey P. (2006) Development of PCR tests to detect the hybrid Phytophthora causing alder disease in Europe in natural samples. In: Forest Research Bulletin *Proceedings of 3rd IUFRO Forest Phytophthora Research Workshop*, Freising, Germany, 11-17 Sept. 2004. Sous presse.

Publication 1

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SCAR–based PCR primers to detect the hybrid pathogen *Phytophthora alni* and its subspecies causing alder disease in Europe

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Abstract

Since the 1990s, a new *Phytophthora* species hybrid has been jeopardizing the natural population of alders throughout Europe. This new *Phytophthora, P. alni*, has been suggested as a natural hybrid between two closely related species of *Phytophthora*. Little is known about the epidemiology of this pathogen, because its direct isolation is not always satisfactory. In this study we developed three pairs of Polymerase Chain Reaction (PCR) primers derived from Sequence Characterized Amplified Regions (SCAR) that allow discrimination among the three subspecies of *P. alni*: *P. alni* subsp. *alni*, *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*. These molecular tools were successfully used to detect *P. alni* directly in different substrates such as infested river water and soil, and necrotic alder bark, without the need for any prior baiting or isolation stages. An Internal Amplification Control (IAC) was included to help discriminate against false negative samples due to the potential presence of inhibitory compounds in DNA extracts. These molecular tools should be useful for epidemiological studies on this emerging disease.

Introduction

At the beginning of the 1990s, a new destructive and lethal disease of Alder (*Alnus* spp.) was described in Great Britain in riparian populations as well as horticultural shelterbelts (Gibbs et al., 1994). The disease exhibited characteristic symptoms: thinning of the crown, sometimes with abnormally small, sparse and yellowish leaves and tarry or rusty exudations on the stems. These external symptoms were consequences of the destruction of strips of inner bark and/or roots necrosis (Gibbs et al., 1994; Gibbs, 1995). The disease has since been described throughout Europe and has had a particularly destructive impact in Great Britain, but is also found in France, Belgium and Germany where it represents an increasing threat to natural riparian alder populations (Brasier et al., 1995; Gibbs, 1995; Streito et al., 2002a; Gibbs et al., 2003; Jung and Blaschke, 2004). The disease was initially shown to be caused by a previously unknown *Phytophthora* sp. resembling *P. cambivora* (Gibbs et al., 1994; Brasier et al., 1995). Further investigations led Brasier et al. (1999) to hypothesize that the *Phytophthora* involved was a natural hybrid between *P. cambivora* and another unknown taxon of *Phytophthora* close to *P. fragariae*. According to cultural features, cytological evidence, ITS sequences and genomic DNA fingerprinting, Brasier et al. (1999) also showed that the alder *Phytophthora* consisted of a range of heteroploid species hybrids. These can be divided into a 'standard' type and

several variants, all pathogenic to the different species of European alder. The standard type is nearly tetraploid (4n + 2, n = 18–22 chromosomes) and exhibits an unusual ITS polymorphism, i.e. dimorphic sites within ITS sequences for a single isolate. On the other hand, the respective ploidy for the different variants ranges from 2n + 2 for the Swedish variant to 2n + 7 for the German variant. In contrast to the standard type, the variants show a nearly homogenous ITS sequence. The ITS sequence for the Dutch, German and UK variants only differs from *P. fragariae* by a few bases whereas the ITS sequence for the Swedish variant is very close to the *P. cambivora* sequence (Brasier et al., 1999). The respective aggressiveness of the different types of alder *Phytophthora* are slightly different (Brasier and Kirk, 2001; Santini et al., 2003) but these hybrids are the only known *Phytophthora* species to be pathogenic to alder. In contrast, these hybrids are not pathogenic to other woody hosts such as *Quercus*, *Acer* or *Fagus* (Brasier and Kirk, 2001). Recently, Brasier et al. (2004) formally named these different types of alder *Phytophthora* as *P. alni*. Moreover, according to extensive morphological, cytological and genetic data, Brasier et al. (2004) have split *P. alni* into three subspecies: *P. alni* subsp. *alni* corresponding to the standard type, *P. alni* subsp. *uniformis* corresponding to the Swedish variant type and *P. alni* subsp. *multiformis* including the UK, German and Dutch variant types.

Recent studies have demonstrated that the pathogens were able to disseminate along rivers by producing large quantities of waterborne zoospores (Streito et al., 2002a, b) and could also be brought from infected areas to an initially healthy area by planting infected alder plants (Jung and Blaschke, 2004). However, the epidemiology as well as the aetiology of this new disease are still unclear, perhaps because direct isolation requires a certain level of technical skill and experience in correctly recognizing the symptoms and the hyphae produced by the pathogen. In addition, Streito et al. (2002b) and Streito (2003) reported that the efficiency of classical detection techniques such as direct isolation or baiting with this Oomycete could be poor. Moreover, hybrid fungi and hybrid Oomycetes are unlikely to be identified or detected by conventional methods which are mainly based on morphology, as their features are close or identical to those of the parental species (Brasier, 2001). During the last decade, molecular markers have proven to be useful for species-specific detection of plant pathogens. Generally, based on a Polymerase Chain Reaction (PCR), reliable and accurate diagnostic tests are now widely used, especially for economically important plant pathogens such as quarantine listed fungi (Bonants et al., 1997, 2003; Ioos and Frey, 2000). Nevertheless, Internal Transcribed Spacers (ITS)-based PCR techniques, despite being used very frequently for species discrimination, are not appropriate in the case of *P. alni*. Indeed, the ITS homology between the putative parental species and the variant types, on the one hand, and the running ITS sequence rearrangement for the standard type, on the other, make these regions inappropriate for diagnostic purposes.

Alternative strategies were developed for the design of species or strain-specific markers using randomly selected sequences (Wiglesworth et al., 1994; Boehm et al., 2001). Sequence Characterized Amplified Regions (SCAR) can be selected from RAPD (Random Amplified Polymorphic DNA) fingerprints and used to mark specific alleles (Paran and Michelmore, 1993) or generate species-specific PCR markers (Schilling et al., 1996; Schubert et al., 1999).

The aims of our study were first to generate SCARs from RAPDs carried out on a panel of different subspecies of *P. alni* and closely related species, i.e. *P. cambivora* and *P. fragariae*, in order to find markers that could be specific to the hybrids. Secondly, we designed sets of PCR primers that exhibit a complementary range of specificity, including a universal primer pair that enables the specific detection of the different subspecies of *P. alni* in various substrates. The distinction of the different subspecies might be useful for epidemiological purposes as, in contrast to *P. alni* subsp. *alni*, *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis* were shown to be able to complete meiosis, despite the fact that no germination of the resulting oospores has ever been observed during *in vitro* studies (Delcan and Brasier, 2001).

Materials and methods

Cultures of Oomycetes

French isolates of *P. alni* or *Phytophthora* spp. were obtained by isolation from naturally infected

tissues on PARPHY medium (Robin et al., 1998). Foreign isolates of *P. alni* and *Phytophthora* spp. were obtained from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) or from collaborative researchers (Table 1). Assignation of the isolates to one of the three subspecies of *P. alni* was achieved by combining the examination of the morphological features of each isolate in pure culture according to Brasier et al. (1995) and restriction patterns of the ITS region using a series of enzymes, according to Brasier et al. (1999) and Cooke et al. (2000) (data not shown). All the cultures were kept at 10 °C in the dark on V8 agar slants (Miller, 1955) and as small V8 agar blocks flooded with sterile distilled water (SDW). Oomycete DNA was extracted from 5-day-old cultures grown in shaken liquid V8-juice medium (Miller, 1955) at 20 °C.

Zoospore production

The zoospores used in this study were produced by an isolate of *P. alni* subsp. *alni* (1429-6b) isolated from a bark necrosis on alder in France. Sporangia were produced by incubating 20 plugs of active margin culture of *P. alni* subsp. *alni* isolate 1429-6b for 48 h in the dark in 25 ml of pond water previously filtered through a 47 mm dia 5 µm pore Durapore® membrane (Millipore, Molsheim, France). Then, agar plugs bearing numerous sporangia were carefully rinsed with SDW and transferred into a sterile Petri dish. To release zoospores, 30 ml of pre-chilled pond water previously filtered through a 0.2 µm pore cellulose acetate filter were added and the plugs were incubated for 2 h under fluorescent light at 20 °C. The zoospore suspension was filtered through a 45 µm sieve to remove mycelium and agar plugs. One millilitre of the initial suspension was thoroughly vortexed for 2 min in a microcentrifuge tube to encyst zoospores in order to facilitate counting. The initial concentration of zoospores was determined by using a haemocytometer under a microscope at 250× magnification. First, 50 ml of river water were filtered through a 100 µm mesh sieve to eliminate large debris. The filtrate was subsequently filtered through a 47 mm dia Durapore® membrane with 5 µm pores. An initial suspension of 50×10^3 zoospores ml^{-1} was diluted by aliquots of 50 ml of filtered river water in order to obtain different quantities of zoospores. We tested six series of 50 ml of artificially contaminated river water containing from 1.5×10^6 to 30 zoospores.

DNA extraction from Oomycete and fungal cultures

DNA was extracted using a plant DNA extraction kit (DNeasy plant mini kit®, Qiagen, Courtaboeuf, France) following the manufacturer's instructions with slight modifications. For pure Oomycete culture, 200 mg of fresh mycelium was harvested and mixed in a 2 ml tube with 400 µl of lysis buffer and 4 µl of the RNase provided. The mixture was ground for 2 min with two 3 mm tungsten carbide beads at a frequency of 30 Hz, using a mixermill grinder (Tissuelyser® Qiagen, Courtaboeuf, France). The ground solution was subsequently centrifuged for 5 min at 14,000 rpm to compact the debris and the supernatant was treated following the manufacturer's instructions. DNA concentrations were estimated using a spectrophotometer (BioPhotometer®, Eppendorf, Le Pecq, France)

DNA extraction from lignified woody tissues, soil and water

For DNA extraction from woody tissues, thin wood shavings were taken from symptomatic tissues (bark necrosis) using a sterile scalpel blade. The shavings were transferred to a sterile 2 ml microcentrifuge tube with 500 µl of DNeasy® lysis buffer, 500 µl of powdered skimmed milk (0.2 g/25 ml distilled water) and 4 µl of the RNase provided by the manufacturer. The sample was ground with two 3 mm tungsten carbide beads and DNA was extracted as described above. For soil DNA extraction, about 1 g of sampled soil was transferred to a 2 ml centrifuge tube and DNA was extracted as described above for woody tissues. Water DNA was extracted following a protocol derived from Kong et al. (2003) with slight modifications. Fifty millilitres of river water artificially inoculated with *P. alni* subsp. *alni* zoospores was filtered through a 47 mm dia Durapore® membrane with 5 µm pores. The membrane was removed carefully from the filtering unit and cut into pieces of approximately 0.25 cm^2 using sterile forceps and scissors. All the pieces were transferred to a sterile 2 ml microcentrifuge

Table 1. Polymerase chain reaction amplification of DNA from isolates of different subspecies of *Phytophthora alni* recovered from different geographic locations, isolates of different species from the genus *Phytophthora* and *Pythium* and isolates of fungi commonly recovered from alder bark necrosis, using the three *P. alni* primer pairs designed in this study

Species	Code	Host	Geographical origin	Year	Isolator /supplier	PA-F/R	PAM-F/R	PAU-F/R	ITS1–ITS4 or ITS6–ITS4*
P. alni subsp.*alni*	2N0685	*Alnus glutinosa*	France	2002	J.C. Streito	+	+	+	+
	71T1	*Alnus glutinosa*	France	1997	J.C. Streito	+	+	+	+
	77T4	*Alnus glutinosa*	France	1997	J.C. Streito	+	+	+	+
	82T1A	*Alnus glutinosa*	France	1997	J.C. Streito	+	+	+	+
	84T2	*Alnus glutinosa*	France	1997	J.C. Streito	+	+	+	+
	9900715.6	*Alnus glutinosa*	Belgium	1999	J.C. Streito	+	+	+	+
	98-7-5	*Alnus glutinosa*	France	1998	J.C. Streito	+	+	+	+
	98-7-6	*Alnus glutinosa*	France	1998	J.C. Streito	+	+	+	+
	2N0529	*Alnus glutinosa*	France	2002	J.C. Streito	+	+	+	+
	DSFO98172	*Alnus glutinosa*	France	1998	J.C. Streito	+	+	+	+
	AUL026/1	*Alnus glutinosa*	France	1999	J.C. Streito	+	+	+	+
	9900783.4	*Alnus glutinosa*	France	1999	J.C. Streito	+	+	+	+
	1R0152	*Alnus glutinosa*	France	2001	J.C. Streito	+	+	+	+
	1N0201	*Alnus glutinosa*	France	2001	J.C. Streito	+	+	+	+
	9500802	*Alnus glutinosa*	France	1995	J.C. Streito	+	+	+	+
	PD2010953	*Alnus* sp.	The Netherlands	ND	W. Man in't Veld	+	+	+	+
	P1275	*Alnus glutinosa*	Scotland	2000	G. Mackaskill	+	+	+	+
	P1272	*Alnus viridis*	Scotland	2000	J. Gibbs	+	+	+	+
	P1271	*Alnus glutinosa*	Scotland	2000	J. Gibbs	+	+	+	+
	P1270	*Alnus glutinosa*	Scotland	2000	J. Delcan	+	+	+	+
	P1960	*Alnus glutinosa*	England	1997	J. Delcan	+	+	+	+
	P957[a]	*Alnus glutinosa*	England	1997	J. Delcan	+	+	+	+
	P950[a]	*Alnus glutinosa*	England	1997	J. Delcan	+	+	+	+
	P937	*Alnus glutinosa*	England	1997	J. Delcan	+	+	+	+
	P850	*Alnus glutinosa*	England	1996	S. Gregory	+	+	+	+
	P834[e]	*Alnus glutinosa*	England	ND	C. Brasier	+	+	+	+
	2198[c]	*Alnus glutinosa*	Belgium	1999	D. De Merlier	+	+	+	+
	2295[c]	*Alnus glutinosa*	Belgium	2001	D. De Merlier	+	+	+	+
	6[d]	*Alnus glutinosa*	Hungary	2001	Z. Nagy	+	+	+	+
	8[d]	*A. glutinosa* soil	Hungary	2001	Z. Nagy	+	+	+	+
	9[d]	*A. glutinosa* soil	Hungary	2001	Z. Nagy	+	+	+	+
	1a[d]	*A. glutinosa* soil	Hungary	2001	Z. Nagy	+	+	+	+
	4-2[d]	*Alnus glutinosa*	Hungary	2001	Z. Nagy	+	+	+	+
	P1bisa	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	P3a	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	Priva	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	Privb	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	P6-2	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	P6-1	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	Ainvelle Sol	*A. glutinosa* soil	France	2003	C. Husson	+	+	+	+
	2ALD03	*Alnus glutinosa*	France	2003	C. Husson	+	+	+	+
	102-1	*Alnus glutinosa*	France	2003	C. Husson	+	+	+	+
	Moselle	*Alnus glutinosa*	France	2002	C. Husson	+	+	+	+
	370-2	*Alnus glutinosa*	France	2002	C. Husson	+	+	+	+
	3N10094-5a	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	3N10094-5c	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	3N10048-3a	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	3N10048-3b	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	3N10048-3f	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	Ainvelle4-4	*Alnus glutinosa*	France	2003	C. Husson	+	+	+	+
	Ainvelle1-2	*Alnus glutinosa*	France	2003	C. Husson	+	+	+	+
	Ainvelle1-1	*Alnus glutinosa*	France	2003	C. Husson	+	+	+	+
	703	*Alnus glutinosa*	France	2003	G. Capron	+	+	+	+
	1429-6b	*Alnus glutinosa*	France	2003	R. Ioos	+	+	+	+
	Sol A15	*A. glutinosa* soil	France	2003	C. Husson	+	+	+	+
	Sol A1	*A. glutinosa* soil	France	2003	C. Husson	+	+	+	+
	Sol A7	*A. glutinosa* soil	France	2003	C. Husson	+	+	+	+

Table 1. (Continued).

Species	Code	Host	Geographical origin	Year	Isolator /supplier	PA-F/R	PAM-F/R	PAU-F/R	ITS1–ITS4 or ITS6–ITS4*
	BBA 23/00	*Alnus glutinosa*	Germany	2000	K. Kaminski	+	+	+	+
	PO 192	*Alnus glutinosa*	Poland	ND	G. Skuta	+	+	+	+
	PO 193	*Alnus glutinosa*	Poland	ND	G. Skuta	+	+	+	+
	PO 203	*Alnus glutinosa*	Poland	ND	G. Skuta	+	+	+	+
	PO 205	*Alnus glutinosa*	Poland	ND	G. Skuta	+	+	+	+
	Pucking B10	*Alnus glutinosa*	Austria	ND	T. Cech	+	+	+	+
P. alni subsp. *uniformis*	AUL028	*Alnus glutinosa*	France	1999	J.C. Streito	+	–	+	+
	155-a[d]	*Alnus glutinosa*	Hungary	1999	Z. Nagy	+	–	+	+
	155-b[d]	*A. glutinosa* soil	Hungary	1999	Z. Nagy	+	–	+	+
	155-c[d]	*A. glutinosa* soil	Hungary	1999	Z. Nagy	+	–	+	+
	CBS109280[e]	*Alnus cordata*	Italy	ND	P. Capretti	+	–	+	+
	P875[a,b,c,f]	*Alnus glutinosa*	Sweden	ND	C. Olsson	+	–	+	+
	2271[c]	*Alnus glutinosa*	Belgium	2001	D. De Merlier	+	–	+	+
	Phy-A-Slo	*Alnus glutinosa*	Slovenia	2003	A. Munda	+	–	+	+
P. alni subsp. *multiformis*	W1139	*Alnus* sp.	The Netherlands	ND	W. Man in't Veld	+	+	–	+
	P972[a,c,f]	*Alnus* sp.	The Netherlands	ND	W. Man in't Veld	+	+	–	+
	P841[a,c,f]	*Alnus glutinosa*	UK	1996	S. Gregory	+	+	–	+
	DSFO/0125	*Alnus glutinosa*	France	2000	J.C. Streito	+	+	–	+
P. cambivora	463	*Castanea sativa*	France	ND	INRA Bordeaux	–	–	–	+
P. cambivora	643	*C. sativa* soil	France	ND	INRA Bordeaux	–	–	–	+
P. cambivora	JC17	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. cambivora	GA1	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. cambivora	99428	*Castanea sativa*	France	ND	R. Ioos	–	–	–	+
P. cambivora	ST3R1	*Quercus petraea*	France	ND	C. Delatour	–	–	–	+
P. cambivora	627	ND	France	ND	INRA Bordeaux	–	–	–	+
P. cambivora	1A21	*Quercus* sp. soil	France	ND	INRA Bordeaux	–	–	–	+
P. fragariae var. *fragariae*	1	*Fragaria x ananassa*	ND	ND	K. Hughes	–	–	–	+
P. fragariae var. *fragariae*	209.46	*Fragaria x ananassa*	ND	ND	CBS	–	–	–	+
P. fragariae var. *fragariae*	309.62	*Fragaria x ananassa*	ND	ND	CBS	–	–	–	+
P. fragariae var. *rubi*	FVR 59	*Rubus* sp.	UK	ND	D. Cooke	–	–	–	+
P. fragariae var. *rubi*	163-2	*Rubus* sp.	France	ND	A. Baudry	–	–	–	+
P. fragariae var. *rubi*	2	*Rubus* sp.	UK	ND	K. Hughes	–	–	–	+
P. fragariae var. *rubi*	967.95	*Rubus* sp.	UK	ND	CBS	–	–	–	+
P. fragariae var. *rubi*	109.892	*Rubus* sp.	UK	ND	CBS	–	–	–	+
P. cactorum	CAC4810/TJ	ND	France	ND	C. Delatour	–	–	–	+
P. cinnamomi	DSFO2N0964	*Castanea sativa*	France	ND	J.C. Streito	–	–	–	+
P. cinnamomi	DSFA970060	*Quercus suber*	France	ND	J.C. Streito	–	–	–	+
P. cinnamomi	DSFO990050	*C. sativa* soil	France	ND	J.C. Streito	–	–	–	+
P. cinnamomi	P382	*Nothofagus procera* soil	UK	ND	C. Brasier	–	–	–	+
P. citricola	2N0750-171	ND	France	ND	J.C. Streito	–	–	–	+
P. citricola	AUL 045 AP7	*Alnus glutinosa*	France	ND	J.C. Streito	–	–	–	+
P. citricola	2AE5	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. citricola	3N1345-17	*Alnus glutinosa*	France	ND	R. Ioos	–	–	–	+
P. citrophthora	2N1021	*Rosa* sp.	France	ND	J.C. Streito	–	–	–	+
P. cryptogea	990675	*Actinidia chinensis*	France	ND	J.C. Streito	–	–	–	+
P. erythroseptica	960713	*Polygonum oberti*	France	ND	J.C. Streito	–	–	–	+
P. europaea	AL5	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. europaea	2AU2	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. gonapodyides	Gonap 4	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. gonapodyides	AB4	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. humicola	3N1245-j	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
P. ilicis	3N1245-l	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
P. inundata	9500802	*A. glutinosa* soil	France	ND	J.C. Streito	–	–	–	+
P. lateralis	98093.1-SPV	*Chamaecyparis* sp.	France	ND	J.C. Streito	–	–	–	+
P. megasperma	3N1245-m	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
P. megasperma	BK1	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. megasperma	03-12	water under *Quercus* sp.	France	ND	C. Delatour	–	–	–	+
P. megasperma	mega 1	ND	Germany	ND	T. Jung	–	–	–	+

Table 1. (Continued).

Species	Code	Host	Geographical origin	Year	Isolator /supplier	PA-F/R	PAM-F/R	PAU-F/R	ITS1–ITS4 or ITS6–ITS4*
P. megasperma	8RPOC3	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. nicotianae	960579	*Nicotiana tabacum*	France	ND	J.C. Streito	–	–	–	+
P. taxon forestsoil	8CARPPOC1	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. palmivora	970423	*Hedera* sp.	France	ND	J.C. Streito	–	–	–	+
P. parasitica	970029	*Lycopersicon esculentum*	France	ND	J.C. Streito	–	–	–	+
P. taxonPgchlamydo	Haye,3,1	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. pseudosyringae	EW5	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. psychrophila	FF20	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. quercina	FNA	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. quercina	Mers2	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
P. ramorum	2N0983	*Rhododendron* sp.	France	ND	C. Saurat	–	–	–	+
P. ramorum	3N0003	*Viburnum* sp.	France	ND	C. Saurat	–	–	–	+
P. sojae	443	*Glycine max*	No	ND	F. Panabières	–	–	–	+
P. syringae	2JZ2	*Quercus* sp. soil	France	ND	C. Delatour	–	–	–	+
Pythium aphanidermatum	Ctsa	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
Pythium sylvaticum	0675/a	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
Pythium intermedium	02/84/1	ND	France	ND	S. Verger	–	–	–	+
Pythium irregulare	02/57/1	ND	France	ND	S. Verger	–	–	–	+
Pythium ultimum	433/3	ND	France	ND	S. Verger	–	–	–	+
Pythium sp.	3N1345-11	*A. glutinosa* soil	France	ND	R. Ioos	–	–	–	+
Botryosphaeria obtusa	467a	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Trichoderma harzanium	1790a	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Fusarium avenaceum	1790b	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Microsphaerosis olivaceae	467b	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Phoma sp.	1790c	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Epicoccum nigrum	1790	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Fusarium sporotrichioides	P1bis1	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Aspergillus sp.	Priv1	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Alternaria sp.	A6b	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+
Graphium sp.	P3-3	*Alnus glutinosa*	France	2003	R. Ioos	–	–	–	+

ND, not determined.
* ITS6 and ITS4 primers were used for *Phytophthora* and *Pythium* spp. whereas ITS1 and ITS4 primers were used for other fungi (White et al., 1990).
[a]Also studied by Delcan and Brasier (2001).
[b]Also studied by Brasier et al. (1999).
[c]Also studied by De Merlier et al. (2005).
[d]Also studied by Nagy et al. (2003).
[e]Also studied by Santini et al. (2003).
[f]Also studied by Brasier and Kirk (2001).

tube and DNA was extracted following the same protocol described above for woody tissues.

RAPD and PCR amplification conditions

The amplification reactions were carried out on a Genamp 9700 thermocycler (Applied Biosystems, Foster City, California). The cycling profile for RAPD included an initial denaturation step at 95 °C for 3 min followed by 40 cycles of denaturation, annealing and elongation for respectively 30 s at 94 °C, 30 s at 36 °C and 1 min at 72 °C, and a final extension step at 72 °C for 7 min. RAPDs were carried out in a 20 μl mixture containing 1× *Taq* DNA polymerase buffer (Sigma-Aldrich, Lyon, France), 2 mM $MgCl_2$, 0.5 μM of 10-mer RAPD primer (kit OPE, OPF, OPG and OPH, Operon Technologies, Alameda, California), 150 μM dNTPs, 0.8 $\mu g\ \mu l^{-1}$ Bovine Serum Albumin (BSA), 1 unit of *Taq* DNA Polymerase (Sigma-Aldrich), 2 μl of template DNA and molecular biology grade water was added to 20 μl. The cycling profile for PCR was the same as that described above except that the annealing tem-

perature was raised to 58 °C and only 35 amplification cycles were necessary to obtain a significantly positive signal. PCRs were carried out in a 20 µl mixture containing 1× polymerase buffer (Sigma-Aldrich), 1.8 M $MgCl_2$, 0.45 µM of each primer, 180 µM dNTPs, 0.7 µg μl^{-1} BSA, 0.6 unit of *Taq* DNA Polymerase (Sigma-Aldrich), 2 µl of template DNA and molecular biology grade water was added to 20 µl. RAPD and PCR fragments were separated, together with a 100 bp DNA ladder (Invitrogen, Cergy Pontoise, France), by a 4 h and a 1 h electrophoresis, respectively, on a 1% agarose gel at 4 V cm^{-1}. Gels were stained with ethidium bromide and images were recorded with a CCD camera and a GELDOC 2000® system (Biorad, Marne-La-Coquette, France).

Cloning and sequencing of RAPD fragments

All the RAPD fragments were cloned from products generated with DNA from *P. alni* subsp. *alni* isolate 703 with the pCR® 4-TOPO® – TA cloning kit (Invitrogen, Cergy Pontoise, France). Ten microlitres of the bulk RAPD products containing the band(s) of interest were subjected to a 30 min elongation step at 72 °C with 0.5 µl of 4 × 25 mM dNTPs mix and 0.3 U *Taq* DNA Polymerase in order to ensure the addition of an adenosyl base at each 3′ end of the amplicons, as recommended by the manufacturer. Five microlitres were then transferred to a sterile 1.5 ml microcentrifuge tube and the amplicons were ligated to a TOPO® vector (Invitrogen) as recommended by the manufacturer in the presence of 1 µl of the salt solution provided. Ligated plasmids were used to transform TOP 10® competent cells (Invitrogen) according to the manufacturer's instructions. Positive clones were selected by PCR amplifications of inserts with M13 sequencing primers. The PCRs were carried out directly with a suspension of transformed bacteria in ultrapure water. Clones containing the RAPD band of interest were selected according to the expected PCR product size. PCR products were then purified using Millipore purification microplates (Millipore, Molsheim, France) on a vacuum manifold (Millipore). Double strand DNA sequencing was performed by the di-deoxy-chain termination method using a T3–T7 sequencing kit on a CEQ 2000 XL DNA sequencer (Beckman, Fullerton, California).

Construction of an Internal Amplification Control (IAC)

A heterologous DNA template with 5′ and 3′ ending sequences identical to the primer pair PA-F and PA-R was constructed according to the protocol described by Langrell (2002), with slight modifications. Briefly, DNA extracted from leaves of *Populus trichocarpa* × *P. deltoides* 'Beaupré' was subjected to RAPD following the protocol described above, except that 0.45 mM of 10-mer primer was replaced by 0.45 mM of each of the 20-mer primers PA-F and PA-R. A typical RAPD pattern was revealed by electrophoresis on a 1% agarose gel and an 850-bp fragment was chosen as IAC for PA-F/R specific PCR. The entire RAPD product was cloned with the pCR® 4-TOPO® – TA cloning kit (Invitrogen), using the protocol described above. The clone containing the selected fragment was screened by PCR with M13 primers. Clones containing the selected fragment were screened according to the expected PCR product size. These clones were also tested in three different PCRs: one with the primer PA-F, one with the primer PA-R and the last one with both primers to ensure that the selected clones exhibit both recognition sites in 5′ and in 3′. Ready-to-use IAC templates were stored as a suspension of transformed bacteria in ultra pure water at −20 °C until used for PCR. A series of concentrations of IAC copies were mixed with a 200 ng to 0.5 pg range of Oomycete DNA and tested by PCR with PA-F/R primers. Six hundred copies of IAC in each PCR tube proved to be adequate to allow the amplification of both targets in the presence of a wide range of *P. alni* DNA concentrations and this method was therefore chosen to be used in routine analysis.

Primer design

Forward and reverse sequences were edited with Sequencher software (Gene Codes, Ann Arbor, Michigan). The presence of the RAPD primer was checked at both ends of the sequences and generally two sets of primers were designed with the help of Primer 3 software (Rozen and Skaletsky, 2000). Commonly, the first primer pair consisted mainly of the 10 bases of the RAPD primer completed by the following 3′ base sequence to design a 20–23 mer primer. In addition, a set of internal primers was designed within the SCAR. These

internal primers were chosen to have GC contents between 50 and 60% with a GC clamp at the 3′ end. Primers were custom synthesized by Invitrogen (Cergy Pontoise, France).

Results

Sixty-eight RAPD primers were tested with a panel representing two isolates of *P. alni* subsp. *alni* (703 and 1429-6b), one isolate of *P. alni* subsp. *multiformis* (DSFO/0125), one isolate of *P. alni* subsp. *uniformis* (AUL028), and isolates of two closely related species: *Phytophthora cambivora* (PC463, PC643) and *P. fragariae* var. *rubi* (FVR 59, 163-2) (Table 1). For each primer, RAPDs were carried out twice to confirm reproducibility of the patterns, with low stringency conditions. Sixty-eight primers were tested and 41 bands were selected on the different patterns. Those bands seemed to be specific for either, *P. alni* subsp. *alni* and one of the other subspecies of *P. alni*, or specific for *P. alni* subsp. *alni* and the two other subspecies of *P. alni*. Thirty-nine out of these 41 bands could be cloned and sequenced. Firstly, the sequences obtained were investigated using the blastn and blastx programme (http://www.ncbi.nlm.nih.gov/BLAST/) to check for any similarity with known sequences in the Genbank database. Except for one SCAR showing 18% identity with a retrotransposon sequence in *P. infestans* and another in which translation showed partial identity with an ABC transporter protein (data not shown), no other significant similarity was found. Secondly, the SCAR sequences were compared to sequences retrieved from the *Phytophthora sojae* genome sequencing project (http://genome.jgi-psf.org/physo/). *Phytophthora sojae* occurs in a different ecological niche from *P. alni*, *P. cambivora* and *P. fragariae* but lies in the same phylogenetic clade (Cooke et al., 2000). Eighteen out of 41 SCARs showed partial or complete identity to *P. sojae* sequences. These regions were subsequently not used to design PCR primers within them.

Finally, 122 primers were designed from the SCAR sequences. Sixty-seven primer pairs were tested by PCR with a panel of 18 representative isolates of *P. alni* from different geographical origins and different subspecies, two isolates of *P. cambivora*, one isolate of *P. fragariae* var. *fragariae*, one isolate of *P. fragariae* var. *rubi*, and three species of *Phytophthora* frequently isolated from riparian ecosystems: *P. inundata*, *P. megasperma* and *P. gonapodyides* (Brasier et al., 2003a, b). Primer pairs producing a unique PCR product with the alder *Phytophthora* isolates but yielding no amplification with the other *Phytophthora* species described above were selected to be tested by a more exhaustive PCR assay, including all the isolates of *P. alni*, *Phytophthora* spp. and the other Oomycetes and fungi listed in Table 1.

Finally, 34 out of 67 primer pairs showed cross-reactions with at least one of *Phytophthora cambivora*, *P. fragariae* var. *fragariae*, *P. fragariae* var. *rubi*, *P. inundata*, and were not used in subsequent experiments for detection purposes. Interestingly, several PCR primer pairs showed different specificity patterns with *Phytophthora alni*.

One primer pair designed from RAPD with OPF4 primer produced a unique PCR amplicon of approximately 450 bp with all the isolates of different subspecies of *P. alni* (Figure 1A.) but yielded no amplicon with the other species of *Phytophthora* or with the other Oomycete or fungal species (Table 1). Nevertheless, very faint bands were visible when DNA extracts from *P. cambivora* isolates were tested, but their weakness and their much larger size (> 700 bp), allowed easy distinction from the *P. alni* isolates. Moreover, increasing the annealing temperature up to 62 °C overcame this problem. These forward and reverse primers were designated 'PA-F' and 'PA-R' respectively (Table 2).

Eight primer pairs designed from RAPD with OPG3, OPG8, OPG10 and OPH19 primers were shown to be specific to both *P. alni* subsp. *alni* and *P. alni* subsp. *multiformis* but did not cross-react with *P. alni* subsp. *uniformis*, or with the other species tested. One primer pair producing an amplicon of approximately 590 bp (Figure 1B.) was selected and designated as 'PAM-F/R' (Table 2). In addition, one primer pair designed from RAPD with OPF2 primer, was shown to be specific to both *P. alni* subsp. *alni* and to *P. alni* subsp. *uniformis* and did not cross-react with *P. alni* subsp. *multiformis*, or with the other species tested. This primer pair produced a unique PCR amplicon of approximately 750 bp (Figure 1C.) and was designated as 'PAU-F/R' (Table 2). Nevertheless, PAU-F/R amplification produced a

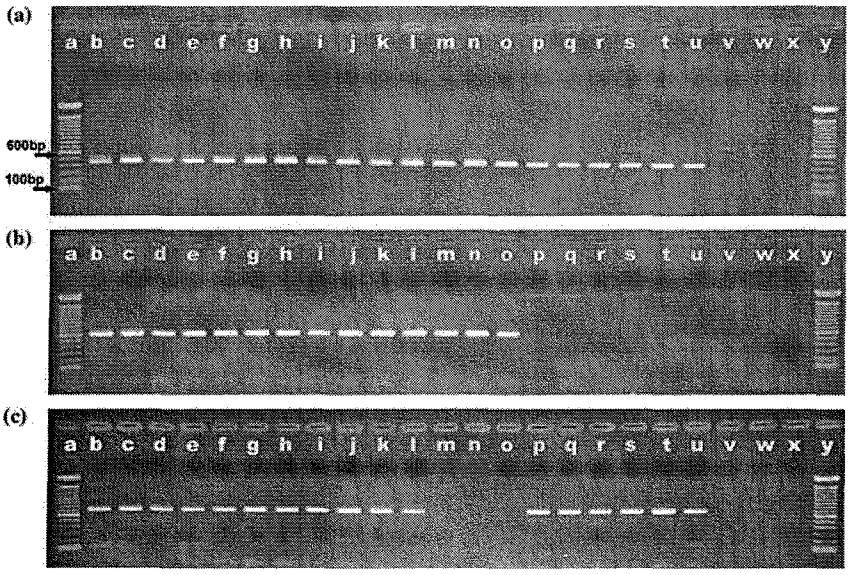

Figure 1. PCR products obtained with the primer pair PA-F/R (a), the primer pair PAM-F/R (b) and the primer pair PAU-F/R (c). Lanes b to l: *Phytophthora alni* subsp. *alni* 2N0685, 71T1, DSFO98172, PD2010953, P1272, P950, 2198, 155-a, BBA23/00, Pucking B10, PO192, Lanes m to o: *P. alni* subsp. *multiformis* isolates DSFO/0125, W1139, P841. Lanes p to u: *P. alni* subsp. *uniformis* isolates P875, CBS109280, 6, AUL028, Phy-A-SLO, 2271. Lane v: *P. cambivora* isolate PC643. Lane w: *P. fragariae* var. *rubi* isolate 163-2. Lane x: negative control with sterile ultra pure water. Lanes a and y: 100-bp DNA ladder.

very faint band of >1500 bp with *P. alni* subsp. *multiformis* DNA extracts. Although the size of this amplicon did not affect the interpretation of the results, this problem was overcome by increasing the annealing temperature to 62 °C.

The three selected primer pairs were tested with dilution series of purified DNA from several isolates of different subspecies of *P. alni* (Table 2). Primer pairs PA-F/R and PAM-F/R yielded the expected PCR product down to 0.5 pg of target DNA, whereas PAU-F/R could detect *P. alni* subsp. *alni* down to 5 pg and *P. alni* subsp. *uniformis* down to 50 pg (Table 2).

PCR tests using primer pairs PA-F/R were successfully carried out directly on total DNA extracted from inoculated or naturally infested plant samples and soil. In artificially contaminated river water, PCR with the PA-F/R primers yielded positive results for all five zoospore quantities from 1.5×10^6 down to 190 zoospores (Figure 2).

To help discriminate against false negatives due to the presence of inhibitory compounds in DNA extracts from naturally infected samples, an 850 bp heterologous fragment with identical primer recognition sites at both ends was constructed from *Populus* DNA using both primers PA-F/R under low stringency PCR conditions. The amplicon size chosen was larger than the *P. alni* target in order not to outcompete the efficient amplification of the Oomycete target DNA in routine PCR analysis. Two PCR products of the expected sizes were obtained when the IAC was

Table 2. Sequence, selectivity and sensitivity of the three *Phytophthora alni* primer pairs developed in this study

Primer pair	Sequence (5′–3′)	Amplicon size	Specificity	Sensitivity
PA-F	GGT GAT CAG GGG AAT ATG TG	450 bp	*P. alni* subsp. *alni*	<0.5 pg
PA-R	ATG TCC GAG TGT TTC CCA AG		*P. alni* subsp. *multiformis*	<0.5 pg
			P. alni subsp. *uniformis*	<0.5 pg
PAM-F	CTG ACC AGC CCC TTA TTG GC	590 bp	*P. alni* subsp. *alni*	<0.5 pg
PAM-R	CTG ACC AGC CAT CCC ACA TG		*P. alni* subsp. *multiformis*	<0.5 pg
PAU-F	GAG GAT CCC TAA CAC TGA ATG G	750 bp	*P. alni* subsp. *alni*	<5 pg
PAU-R	GAT CCC TGG TTG AAG CTG AG		*P. alni* subsp. *uniformis*	<50 pg

added to the DNA extracted from naturally infected alder tissues, from naturally infected alder soil or from *P. alni* zoospores trapped on the Durapore® membrane (Figure 3). In contrast, no amplification of the IAC was obtained when a large amount of target *P. alni* DNA was added as a template in the PCR tube, e.g. genomic DNA extracted from pure culture of the Oomycete (Figure 3, lane 7). However, since the target DNA could be amplified, the absence of the IAC amplification product only meant that the large amount of target DNA prevented the IAC amplification by outcompeting and that no inhibitory compound was present. In the case where only the IAC band was produced while no *P. alni* target was amplified, we concluded that the DNA extract did not contain a detectable amount of *P. alni* DNA. This was the case with DNA extracted from a hornbeam forest soil (Figure 3, lane 5).

Discussion

No generalized dieback of alders was reported in France before 1990. *Phytophthora alni* was isolated for the first time in France in 1996, but was supposed to have caused damage since the beginning of the 1990s (Streito et al., 2002a). Streito et al. (2002a) demonstrated that the disease is now widespread in France with particularly high damage in north-eastern and western France. The spread and severity of the disease in France are comparable with those recently observed in Bavaria, Germany (Jung and Blaschke, 2004). The disease has also been reported with a lower impact in most of the European countries (Streito, 2003). However, several European countries, as well as countries on other continents, are so far officially free of this destructive pathogen. Recently, the Canadian Food Inspection Agency added *P. alni* to its quarantine list (Anonymous, 2003). Once introduced into a river system, no efficient means of control exists to prevent downstream contamination of alder trees. Therefore, plants entering disease-free countries should be strictly checked with a reliable detection tool to limit the pathogen spread.

Up to the present time, direct isolation of *P. alni* using a non selective medium (Streito, 2003), a selective medium (Streito et al., 2002a; De Merlier et al., 2005) or a biological baiting (Jung and Blaschke, 2004) were the unique means of detecting the Oomycete in alder necrosis, infected soil or contaminated water. Unfortunately, the efficiency of these methods remains often poor and requires an active form of the Oomycete for successful isolation. Streito (2003) also reported that the onset of activity by the pathogen could also vary from season to season and from year to year. This

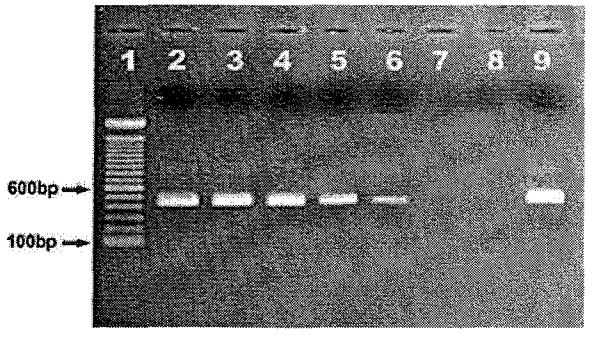

Figure 2. PCR products obtained with the PA-F/R primer pair and DNA extracted from different quantities of *Phytophthora alni* subsp. *alni* zoospores introduced into 50 ml of zoospore-free river water. Lane 1: 100-bp DNA ladder. Lanes 2 to 7: 1.5×10^6, 4.1×10^5, 7×10^4, 1.1×10^3, 190 and 30 zoospores, respectively, in 50 ml of river water. Lane 8: river water without introduced *P. alni* subsp. *alni* zoospore. Lane 9: positive control (genomic DNA from *P. alni* subsp. *alni* isolate 1429-6b).

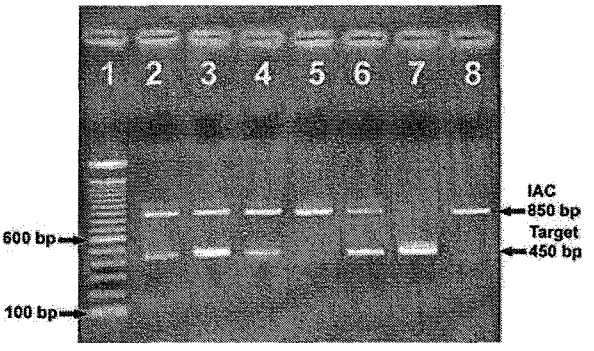

Figure 3. PCR products obtained with the primer pair PA-F/R and different DNA extracts. The internal amplification control was added directly in the PCR mixture to a final number of 600 IAC copies per PCR tube. Lane 1: 100-bp DNA ladder. Lanes 2 and 3: DNA extracted from inner bark of alder naturally infected by *Phytophthora alni* subsp. *alni*. Lane 4: DNA extracted from 50 ml of river water artificially contaminated with 1200 zoospores of *P. alni* subsp. *alni*. Lane 5: DNA extracted from an aliquot of *Carpinus betulus* forest soil. Lane 6: DNA extracted from an aliquot of river bank soil sampled in the vicinity of a *P. alni* subsp. *alni* infected alder. Lane 7: DNA extracted from pure culture of *P. alni* subsp. *alni* isolate 1429-6b. Lane 8: negative control (ultra pure water).

may therefore have a direct influence on the likelihood of successful isolation of *P. alni*. Moreover, competition between several microorganisms may occur during the isolation stage. Recently, De Merlier et al. (2005) successfully developed a SCAR-based PCR primer pair that is specific to *P. alni* subsp. *alni* and *P. alni* subsp. *uniformis*. However, *P. alni* subsp. *multiformis* isolates remained undetected with this primer pair. Moreover, this PCR test does not allow discrimination among *P. alni* subsp. *alni* and *P. alni* subsp. *uniformis* isolates and was only developed to detect these two subspecies in bark necrosis. The PCR primer pairs we developed enabled the specific detection of any subspecies of *P. alni* in different substrates like soil, water or woody tissues.

PCR tests with the PA-F/R primers were able to detect down to 0.5 pg of *P. alni* DNA. Theoretically, this amount represents less than the DNA content of one nucleus of the diploid *P. infestans* (Tooley and Therrien, 1987). Nevertheless, we could not achieve positive detection of *P. alni* subsp. *alni* with less than 190 zoospores trapped on a filtration membrane. There are probably several reasons why we could not get a positive signal below this threshold, such as DNA shearing during the DNA extraction process or suboptimal PCR conditions. However, we successfully detected *P. alni* in naturally infected soil as well as in fresh or old bark necrosis on alder trees from which we failed to isolate the Oomycete (data not shown). This probably means that there is often enough DNA of the pathogen to be detected by the PCR test we developed, even in cases where active forms of the Oomycete were absent. These molecular tools could therefore be useful for diagnosis purposes to confirm the presence of *P. alni* when symptoms of alder disease are observed, and when isolation proves to be difficult. These tools are currently under assessment in a large-scale epidemiological study of alder disease, requiring the routine analysis of hundreds of soil, water, and plant samples (Husson et al., 2004). This PCR test could also be of great interest to check plant stocks, in nurseries for example, as a means of preventing the spread of the pathogen into disease-free areas. With this in mind, the Internal Amplification Control we have developed in association to the PA-F/R primers will help to eliminate the 'false-negative' samples. Indeed, the presence of inhibiting compounds in DNA extracts from soil or woody tissues might be frequent and might lead to false negative PCR results if such an IAC is not used (Langrell, 2002).

Brasier et al. (1999) stressed that many hybrids or introgressants are unlikely to be detected by the conventional diagnosis methods used in international quarantine surveys. Even diagnoses using ITS-based PCR would not be appropriate if homogenization of the rDNA had occurred, thus returning to the parental species sequences. In the case of *P. alni* subsp. *alni*, which is the most frequent subspecies in Europe, ITS arrays are not yet stabilized and are still in the process of fixation (Brasier et al. 1999). We successfully found *P. alni*-specific markers in other regions of the genome through a large screening of RAPD profiles. The SCAR-based primers we designed did not cross-react with the closely related species *P. cambivora* and *P. fragariae*, even with low stringency PCR conditions. An initial explanation for this was to consider that the target sequences belonged to another unknown parental *Phytophthora* species. None of the *Phytophthora* species tested in this study appeared to be this unknown parent. Consequently, the SCAR primers we described here might also be used to identify the *Phytophthora* species involved in this particular case of natural hybrid. Another hypothesis is that these hybrid-specific sequences were generated during or after the hybridization process and did not exist in the genome of the parental species. This recombination phenomenon has already been described within the ITS of *P. alni* subsp. *alni* by Brasier et al. (1999).

In this study, we developed three primer pairs with distinct sub-specificity. The first primer pair PA-F/R allowed the specific detection of all the subspecies of *P. alni* we had in collection. However, the primer pair PAM-F/R gave a positive PCR signal with all the *P. alni* subsp. *alni* and all the *P. alni* subsp. *multiformis* isolates, whereas the primer pair PAU-F/R gave a positive PCR signal with all the *P. alni* subsp. *alni* and all the *P. alni* subsp. *uniformis* isolates. As we selected SCARs from RAPD profiles obtained with a *P. alni* subsp. *alni* isolate, we could not expect to find sequences exclusive to *P. alni* subsp. *multiformis* or subsp. *uniformis*. Nevertheless, these results confirm the close relationship between *P. alni* subsp. *alni* (i.e. the 'Standard' type) and the two other subspecies (i.e. the 'Variant' types) already demonstrated by Brasier et al. (1999) from AFLP data.

Interestingly, despite their different geographical origins, all the isolates belonging to a subspecies gave a positive signal with their respective subspecific primer pairs, as well as with other pairs of subspecific primers derived from the SCARs we had screened (Ioos, unpublished data). This might indicate that the different European isolates of *P. alni* have conserved markers characteristic of each subspecies within their genome. However, the origin of these subspecies of *P. alni* remains unclear and further investigations should be made to understand the hybridization process as well as the occurrence of the three subspecies throughout Europe.

Finally, although the primer sets we described in this paper were successfully checked with a large collection of *P. alni*, we can not totally exclude the possibility that their reliability might be challenged in the future, as this pathogen still seems to be evolving, especially for *P. alni* subsp. *alni* (Brasier et al., 1999). In addition, we could only investigate a few representative isolates of *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*. As hypothesized by Brasier et al. (2004), we cannot anticipate the existence of other types or the outbreak of new types of alder *Phytophthora*. Therefore, the specificity of our primers should be checked continuously in the future, even though they were successfully used with all of the recently collected European *P. alni* isolates that we have tested so far. Even though there is so far no 'non-pathogenic' isolates of *P. alni* described in the literature, we cannot exclude their occurrence. Therefore, any positive PCR detection of *P. alni*, directly in soil or in water, for example, would not discriminate between a pathogenic and a non-pathogenic isolate and should be interpreted carefully. Continuous checking of the pathogenicity of the different isolates of *P. alni* is necessary and, in this view, isolation of the pathogen by classical means remains therefore very useful.

Acknowledgements

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References

Anonymous (2003) Import requirements of non-manufactured wood and other non-propagative wood products, except solid wood packaging material, from all areas other than the continental United States. Directive D-02-012, September 15, 2003.

Boehm EWA, Ma Z and Michailides TJ (2001) Species-specific detection of *Monilinia fructicola* from California stone fruits and flowers. Phytopathology 91: 428–439.

Bonants P, Hagenaar-de Weerdt M, VanGent-Pelzer M , Lacourt I, Cooke D and Duncan J (1997) Detection and identification of *Phytophthora fragariae* Hickman by the polymerase chain reaction. European Journal of Plant Pathology 103: 345–355.

Bonants PJM, Carroll GC, de Weerdt M , vanBrouwershaven IR and Baayen RP (2003) Development and validation of a fast PCR-based detection method for pathogenic isolates of the citrus black spot fungus *Guignardia citricarpa*. European Journal of Plant Pathology 109: 503–513.

Brasier CM (2001) Rapid evolution of introduced plant pathogen via interspecific hybridization. Bioscience 51: 123–133.

Brasier CM and Kirk S (2001) Comparative aggressiveness of standard and variant hybrid alder *Phytophthora, Phytophthora cambivora* and other *Phytophthora* species on bark of *Alnus, Quercus* and other woody hosts. Plant Pathology 50: 218–229.

Brasier CM, Rose J and Gibbs JN (1995) An unusual *Phytophthora* associated with widespread alder mortality in Great Britain. Plant Pathology 44: 999–1007.

Brasier CM, Cooke DEL and Duncan JM (1999) Origin of a new *Phytophthora* pathogen through interspecific hybridization. Proceedings of the National Academy of Sciences of the USA 96: 5878–5883.

Brasier CM, Cooke DEL, Duncan JM and Hansen EM (2003a) Multiple taxa from trees and riparian ecosystems in *Phytophthora gonapodyides-P. megasperma* ITS clade 6 which tend to be high-temperature tolerant and either inbreeding or sterile. Mycological Research 107: 277–290.

Brasier CM, Sanchez-Hernandez E and Kirk S (2003b) *Phytophthora inundata* sp. nov. a part heterothallic pathogen of trees and shrubs in wet or flooded soils. Mycological Research 107: 477–484.

Brasier CM, Kirk SA, Delcan J, Cooke DEL, Jung T and Man in't Veld WA (2004) *Phytophthora alni* sp. nov. and its variants: designation of emerging allopolyploid hybrid pathogens spreading on *Alnus* trees. Mycological Research 108: 1172–1184.

Cooke DEL, Duncan JM, Williams NA, Hagenaar-de-Weerdt M and Bonants PJM (2000) Identification of *Phytophthora* species on the basis of restriction enzyme fragment analysis of the internal transcribed spacer regions of ribosomal RNA EPPO Bulletin 30: 519–523.

De Merlier D, Chandelier A, Debruxelles N, Noldus M, Laurent F, Dufays E, Classens H and Cavelier M (2005) Characterization of alder *Phytophthora* isolates from Wallonia and development of SCAR primers for their specific detection. Journal of Phytopathology 153: 99–107.

Delcan J and Brasier CM (2001) Oospore viability and variation in zoospore and hyphal tip derivatives of the hybrid alder Phytophthoras. Forest Pathology 31: 65–83.

Gibbs JN (1995) *Phytophthora* root disease of alder in Britain. EPPO Bulletin 25: 661–664.

Gibbs J, Strouts R, Rose J and Brasier C (1994) An unusual *Phytophthora* associated with disease of common alder. Report on Forest Research, pp. 27–28 HMSO, London.

Gibbs JN, van Dijk C and Webber JF, (eds.) (2003) Phytophthora disease of alder in Europe. Forestry Commission Bulletin 126, 82 pp. HMSO, London.

Husson C, Thoirain B, Caël O, Ioos R and Marçais B (2004) Epidemiology of the *Phytophthora*-induced alder decline in northeastern France. 3rd Workshop of IUFRO Working Party 7.02.09 'Phytophthora in Forests and Natural Ecosystems' 11th–17th Sept. 2004, Freising, Germany.

Ioos R and Frey P (2000) Genomic variation within *Monilinia laxa, M. fructigena* and *M. fructicola*, and application to species identification by PCR. European Journal of Plant Pathology 106: 373–378.

Jung T and Blaschke M (2004) *Phytophthora* root and collar rot of alders in Bavaria: distribution modes of spread and possible management strategies. Plant Pathology 53: 197–208.

Kong P, Hong C, Jeffers SN and Richardson P (2003) A species-specific polymerase chain reaction assay for rapid detection of *Phytophthora nicotianae* in irrigation water. Phytopathology 93: 822–831.

Langrell SRH (2002) Molecular detection of *Neonectria galligena* (Syn. *Nectria galligena*). Mycological Research 106: 280–292.

Miller PM (1955) V-8 juice agar as a general purpose medium for fungi and bacteria. Phytopathology 45: 461–462.

Nagy ZA, Bakonyi J and Ersek T (2003) Standard and Swedish variant types of the hybrid alder *Phytophthora* attacking alder in Hungary. Pest Management Science 59: 484–492.

Paran I and Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theoretical and Applied Genetics 85: 985–993.

Robin C, Desprez-Loustau ML, Capron G and Delatour C (1998) First record in France and pathogenicity of *Phytophthora cinnamomi* on cork and holm oak. Annales des Sciences Forestières 55: 869–883.

Rozen S and Skaletsky HJ (2000). Primer3 on the WWW for general users and for biologist programmers pp. 365–386 Krawetz & Misener *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, Humana Press, Totowa, NJ.

Santini A, Barzanti GP and Capretti P (2003) Susceptibility of some mesophilic hardwoods to alder *Phytophthora*. Journal of Phytopathology 151: 406–410.

Schilling AG, Möller EM and Geiger HH (1996) Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum, F. graminearum* and *F. avenaceum*. Phytopathology 86: 515–522.

Schubert R, Bahnweg G, Nechwatal J, Jung T, Cooke DEL, Duncan JM, Moller-Starck G, Langebartels C, Sandermann H and Osswald W (1999) Detection and quantification for *Phytophthora* species which are associated with root-rot diseases in European deciduous forests by species-specific polymerase chain reaction. European Journal of Forest Pathology 29: 169–188.

Streito J-C (2003) *Phytophthora* disease of alder: identification and distribution. In Gibbs JN, Van Dijk C, Webber JF, (eds) *Phytophthora* disease of alder in Europe. Forestry commission Bulletin No. 126: 25–38. HMSO, London.

Streito J-C, Legrand P, Tabary F and Jarnouen de Villartay G (2002a) *Phytophthora* disease of alder (*Alnus glutinosa*) in France: investigations between 1995 and 1999. Forest Pathology 32: 179–191.

Streito J-C, Jarnouen de Villartay G and Tabary F (2002b) Methods for isolating the alder *Phytophthora*. Forest Pathology 32: 193–196.

Tooley PW and Therrien CD (1987) Cytophotometric determination of the nuclear DNA content of 23 Mexican and 18 non-Mexican isolates of *Phytophthora infestans*. Experimental Mycology 11: 19–26.

Wiglesworth MD, Nesmith WC, Schardl CL, Li DX and Siegel MR (1994) Specific repetitive sequences in *Peronospora tabacina* for the early detection of the tobacco blue mold pathogen. Phytopathology 84: 425–430.

White TJ, Bruns T, Lee S and Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322 In: Innis Gelfand. DH, Sninsky JJ and White TJ (eds) *PCR protocols: a guide to method and applications*, Academic Press, NewYork.

Publication 2

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Development of PCR tests to detect the hybrid *Phytophthora* causing alder disease in Europe in natural samples.

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Introduction

At the beginning of the 1990s, a new destructive and lethal disease of Alder (*Alnus* spp.) was described in Great Britain in riparian populations as well as horticultural shelterbelts (Gibbs *et al.*, 1994). The disease exhibited characteristic symptoms: thinning of the crown, sometimes with abnormally small, sparse and yellowish leaves and tarry or rusty exudations on the stems. These external symptoms were consequences of the destruction of strips of inner bark and/or roots necrosis (Gibbs *et al.*, 1994).

The disease has since been described throughout Europe and has had a particularly destructive impact in Great Britain, but is also found in France, Belgium and Germany where it represents an increasing threat to natural riparian alder populations (Streito *et al.*, 2002; Gibbs *et al.*, 2003; Jung and Blaschke, 2004).

Combining data obtained from cultural features, cytological studies, ITS sequences and genomic DNA fingerprinting, Brasier *et al.* (1999) also showed that the alder *Phytophthora* consisted of a range of heteroploid species hybrids. Recently, Brasier *et al.* (2004) formally named these different types of alder *Phytophthora* as *Phytophthora alni*. Moreover, according to extensive morphological, cytological and genetic data, Brasier *et al.* (2004) has split *P. alni* into three subspecies: *P. alni* subsp. *alni* (*Paa*), *P. alni* subsp. *uniformis* (*Pau*) and *P. alni* subsp. *multiformis* (*Pam*). The respective aggressiveness of the different subspecies of *P. alni* are slightly different (Brasier and Kirk, 2001; Santini *et al.*, 2003) but these hybrids are the only known *Phytophthora* species to be pathogenic to alder.

Recent studies have demonstrated that *P. alni* was able to disseminate along rivers by producing large quantities of waterborne zoospores (Streito *et al.*, 2002) and could also be brought from infected areas to an initially healthy area by planting infected alder plants (Jung and Blaschke, 2004). However, the epidemiology as well as the aetiology of this new disease is still unclear, essentially because this pathogen remains difficult to isolate on artificial medium (Streito, 2003). Moreover, hybrid fungi and hybrid Oomycetes are unlikely to be identified or detected by conventional methods which are mainly based on morphology, as their features are close or identical to those of the parental species. Nevertheless, reliable and accurate DNA-based diagnosis tests are now widely used, especially for economically important plant pathogens such as quarantine listed fungi. Therefore, the aims of our study were first to generate SCARs (Sequence Characterize Amplified Regions) from RAPDs (Random Amplified Polymorphic DNA) carried out on a panel of different subspecies of *Phytophthora*

alni and closely related species, i.e. *P. cambivora* and *P. fragariae*, in order to find markers that could be specific to the hybrids. Secondly, we designed sets of PCR primers that exhibit a complementary range of specificity, including a universal primer pair that enables the specific detection of the different subspecies of *P. alni* in various substrates.

Results and Discussion

Design of *P. alni*-subspecific PCR primers

We successfully developed three primer pairs showing complementary specificity (Ioos *et al.*, 2005). The specificity of the primer pairs was checked with a wide collection of *P. alni* originating from different European countries and of *Phytophthora* spp. The first primer pair PA-F/R allowed the specific detection of all the isolates and all the subspecies of *P. alni* we had in collection. The primer pair PAM-F/R gave a positive PCR signal with all the *Paa* and all the *Pam* isolates, whereas the primer pair PAU-F/R gave a positive PCR signal with all the *Paa* and all the *Pau* isolates (Figure 1). Primers' sequences are given in Table 1. PCR tests with the PA-F/R primers were able to detect down to 0.5 pg of pure *P. alni* DNA.

In addition, an internal amplification control (IAC) was built and stabilized as a bacterial plasmid to help discriminate between false negative samples due to the potential presence of inhibitory compounds in DNA extracts. This IAC is made of a fragment of *Populus* DNA flanked by the respective PA–F/R targets (Ioos *et al.*, 2005). The IAC is directly introduced in the PCR master mix and is systematically amplified when no PCR inhibitory compounds are present in the DNA extract to be tested (Figure 2).

Detection of *P. alni* from bark lesions

These molecular tools were successfully used to detect directly *P. alni* in alder bark necrosis, without the need for any prior baiting or isolation step. The PCR tests were carried out following a DNA extraction using a commercial plant DNA extraction kit, with the addition of powdered skimmed milk (Ioos *et al.*, 2005). We successfully detected *P. alni* in fresh or old bark necrosis on alder trees from which we failed to isolate the Oomycete. This probably means that there is often enough DNA of the pathogen to be detected by the PCR test we developed, even in cases where active forms of the Oomycete were absent. These molecular tools could therefore be useful for diagnosis purposes to confirm the presence of *P. alni* when symptoms of alder disease are observed, and when isolation proves to be difficult.

Detection of *P. alni* in river water

The protocol of detection of *P. alni* in water as described in Ioos *et al.*, (2005) was further improved and adapted for an 'on-site' application. Briefly, river water is sampled using a suction pump and directly filtered through a succession of 500 µm and 80 µm filters and finally runs through a 11 µm pore Durapore® (Millipore, Molsheim, France) membrane (Figure 3). From one up to nine litres of river water can be filtered, depending of the turbidity of the water. The 11 µm membrane is stored in a sterile Petri dish and brought to the lab to be analyzed. Each membrane is cut into pieces of

approximately 4 mm^2 using sterile forceps and scissors. Total DNA is extracted using a commercial plant DNA extraction kit as described in Ioos *et al.* (2005) but slightly improved by the addition of 3-4 mg of polyvinylpolypyrolidone (PVPP) in the lysis buffer. The detection threshold was estimated to be less than 30 zoospores / litre. This protocol was used to detect the occurrence of *P. alni* at numerous locations along a 4 km section of the Sarre River (France), which is a severely infected area. The first results we obtained showed that *P. alni* zoospores could be detected in water sampled beneath diseased alders as well as beneath healthy looking alders (Husson C., unpublished results).

Detection of *P. alni* in soil

The detection of *P. alni* was efficiently achieved in 500 mg soil samples by testing the PA F/R primers with a DNA extract obtained using a Fast DNA Spin kit for soil (QBiogen, Illkirch, France), supplemented by the addition of 3-4 mg of PVPP. The detection threshold varied according to the soil texture: 10 nuclei of *P. alni* in a loamy sand soil compared to 30 nuclei in a clay loam soil.

During a survey of the Sarre River, about 60 soil samples were collected beneath alder saplings, either healthy-looking or showing typical symptoms of the disease. The soil samples were all first tested by biological baiting using rhododendron leaves. The *P. alni*-specific primers were tested by PCR with DNA extracts obtained directly from soil samples as described above, but also with the DNA extracted from lesions excised from the rhododendron leaves. As a result, *P. alni* was detected more frequently by PCR carried out on DNA extracted from soil than on DNA extracted from biological baitings (Husson C., unpublished results). Nevertheless, and despite apparently less sensitive, PCR following biological baits may be a useful tool for epidemiological studies, thanks to the fact this method only detects active forms of *P. alni*.

Conclusions

These tools are currently under assessment in a large-scale epidemiological study of alder disease, requiring the routine analysis of hundreds of soil, water, and plant samples (Husson *et al.*, 2004). This PCR test, combined with an appropriate DNA extraction protocol could also be of great interest to check plant stocks, in nurseries for example, as a means of preventing the spread of the pathogen into disease-free areas. With this in mind, the Internal Amplification Control we have developed in association to the PA-F/R primers will help to eliminate the 'false-negative' samples.

References

Brasier CM, Cooke DEL and Duncan JM (1999) Origin of a new *Phytophthora* pathogen through interspecific hybridization. Proceedings of the National Academy of Sciences of the USA 96: 5878-5883.

Brasier CM and Kirk S (2001) Comparative aggressiveness of standard and variant hybrid alder *Phytophthora*, *Phytophthora cambivora* and other *Phytophthora* species on bark of *Alnus*, *Quercus* and other woody hosts. Plant Pathology 50: 218-229.

Brasier CM, Kirk SA, Delcan J, Cooke DEL, Jung T and Man in't Veld WA (2004) *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. Mycological Research 108: 1172-1184

Gibbs J, Strouts R, Rose J and Brasier C (1994) An unusual *Phytophthora* associated with disease of common alder. Report on Forest research. HMSO, London, 27-28.

Gibbs JN, van Dijk C and Webber JF (eds.) (2003) *Phytophthora* disease of alder in Europe. Forestry commission Bulletin 126, 82 pp. HMSO, London.

Husson C., Thoirain B., Caël O., and Marçais B. (2004). Epidemiology of the *Phytophthora*-induced alder decline in northeastern France. Third International Meeting on *Phytophthora* in Forests and Natural Ecosystems, Freising (Germany), 11-17 september 2004.

Ioos, R., Husson, C., Andrieux, A., and Frey, P. (2005) SCAR–based PCR primers to detect the hybrid pathogen *Phytophthora alni* and its subspecies causing alder disease in Europe. Eur. J. Plant Pathol. 112, 323-335.

Jung T and Blaschke M (2004) *Phytophthora* root and collar rot of alders in Bavaria: distribution, modes of spread and possible management strategies. Plant Pathology 53: 197-208.

Santini A, Barzanti GP and Capretti P (2003) Susceptibility of some mesophilic hardwoods to alder *Phytophthora*. Journal of Phytopathology 151: 406-410.

Streito J-C (2003) *Phytophthora* disease of alder: identification and distribution. In Gibbs JN, Van Dijk C and Webber JF (eds.) *Phytophthora* disease of alder in Europe. Forestry commission Bulletin N°126: 25-38. HMSO, London.

Streito J-C, Legrand P, Tabary F and Jarnouen de Villartay G (2002) *Phytophthora* disease of alder (*Alnus glutinosa*) in France: investigations between 1995 and 1999. Forest Pathology 32: 179-191.

Table 1: Sequence of the different subspecific *P. alni*-PCR primers and amplicon sizes

Primer pair	Sequence (5'- 3')	Amplicon size
PA-F	GGT GAT CAG GGG AAT ATG TG	450 bp
PA-R	ATG TCC GAG TGT TTC CCA AG	
PAM-F	CTG ACC AGC CCC TTA TTG GC	590 bp
PAM-R	CTG ACC AGC CAT CCC ACA TG	
PAU-F	GAG GAT CCC TAA CAC TGA ATG G	750 bp
PAU-R	GAT CCC TGG TTG AAG CTG AG	

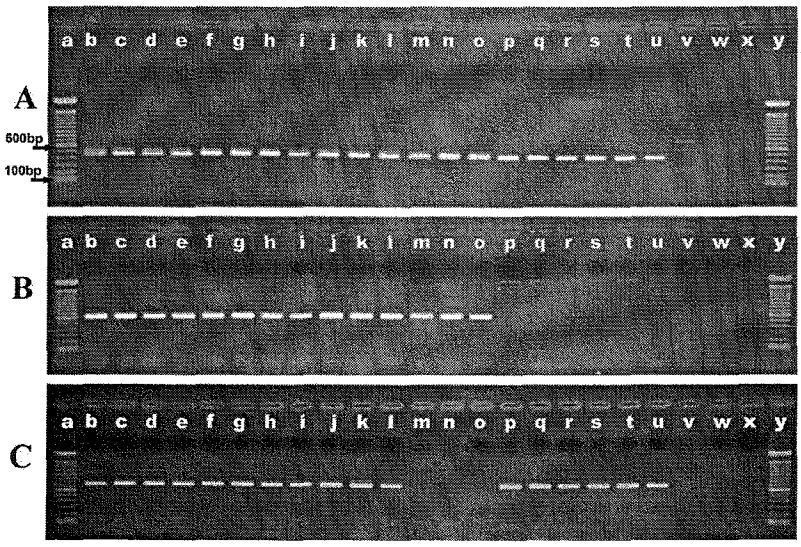

Figure 1: PCR products obtained with the primer pair PA-F/R (A), the primer pair PAM-F/R (B) and the primer pair PAU-F/R (C). Lanes b to l: *P. alni* subsp. *alni* 2N0685, 71T1, DSFO98172, PD2010953, P1272, P950, 2198, 155-a, BBA23/00, Pucking B10, PO192, Lanes m to o: *P. alni* subsp. *multiformis* isolates DSFO/0125, W1139, P841. Lanes p to u: *P. alni* subsp. *uniformis* isolates P875, CBS109280, 6, AUL028, Phy-A-SLO, 2271. Lane v: *P. cambivora* isolate PC643. Lane w: *P. fragariae* var. *rubi* isolate 163-2. Lane x: negative control with sterile ultra pure water. Lanes a and y: 100-bp DNA ladder. With kind permission of Springer Science and Business Media

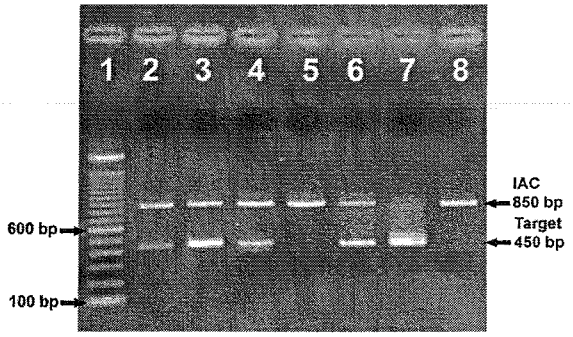

Figure 2: PCR products obtained with the primer pair PA-F/R and different DNA extracts. The internal amplification control was added directly in the PCR mixture to a final number of 600 IAC copies per PCR tube. Lane 1: 100-bp DNA ladder. Lanes 2 and 3: DNA extracted from inner bark of alder naturally infected by *P. alni* subsp. *alni*. Lane 4: DNA extracted from 50 ml of river water artificially contaminated with 1200 zoospores of *P. alni* subsp. *alni*. Lane 5: DNA extracted from an aliquot of *Carpinus betulus* forest soil. Lane 6: DNA extracted from an aliquot of river bank soil sampled in the vicinity of a *P. alni* subsp. *alni* infected alder. Lane 7: DNA extracted from pure culture of *P. alni* subsp. *alni* isolate 1429-6b. Lane 8: negative control (ultra pure water).
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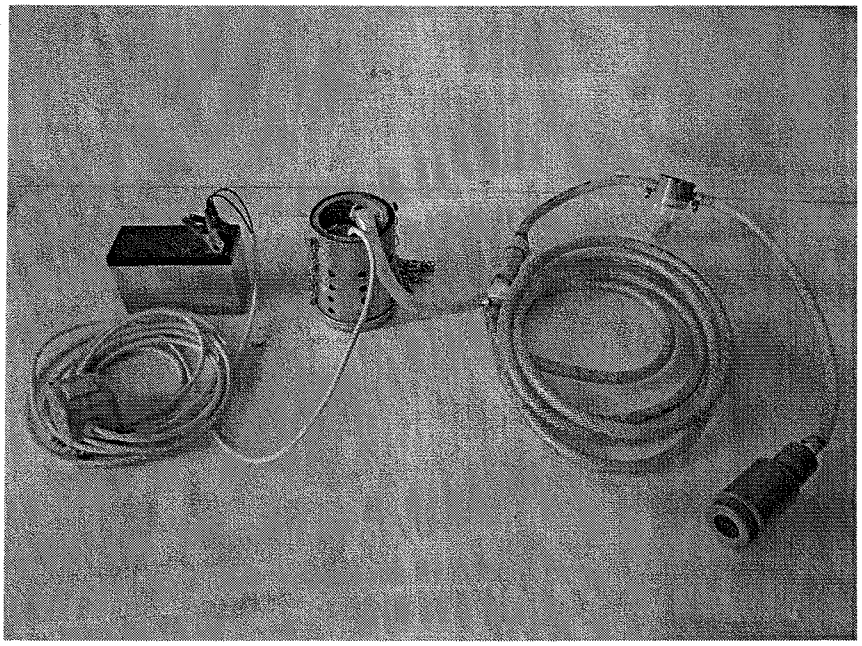

Figure 3: 'On-site' materials used to take samples from river water. From the left to the right: remote switch, battery, suction pump, tubing equipped with a 500 µm filter and an 80 µm filter, successively. The 11 µm pore filter is connected on the outlet pipe.

I.2. - Discussion des résultats obtenus.

La mise en évidence de régions spécifiques de *P. alni sensu lato*, à l'exclusion des espèces les plus proches d'un point de vue phylogénétique (*P. cambivora* et *P. fragariae*) a permis de développer trois couples d'amorces de PCR. Chacun des trois couples présente un niveau de spécificité particulier et leur utilisation combinée permet de détecter et d'identifier chacun des différents taxons : *Paa*, *Pam* et *Pau*. Des procotoles ont été développés pour la détection des trois taxons dans les nécroses sous-corticales, le sol et dans l'eau de rivière. Ces protocoles sont actuellement utilisés par l'équipe de pathologie forestière pour réaliser des études épidémiologiques sur la maladie de dépérissement de l'aulne (Husson et al., 2004 ; Thoirain et al., 2006).

Outre l'aspect pratique apporté par le développement de ces trois couples d'amorces, cette étude a permis de mettre en évidence la présence de régions communes à *Paa* et *Pau* d'une part et à *Paa* et *Pam* d'autre part. En effet, aucun des couples d'amorces évalués dans le cadre de cette étude n'a été en mesure de détecter spécifiquement et uniquement *Paa*, et non *Pam* ou *Pau*. En revanche, le couple d'amorces PA-F/R, capable de détecter indifféremment *Paa*, *Pam* ou *Pau* semble donc être capable de cibler une région commune aux trois taxons.

Le fait que les trois couples d'amorces ne produisent aucun amplifiat avec l'ADN total de *P. cambivora* et de *P. fragariae* pouvait sembler contradictoire avec leur possible implication en tant qu'espèces parentales de *P. alni* (Brasier et al., 1999). Il pouvait alors être imaginé que les trois couples d'amorces ciblaient *i)* des régions spécifiques d'une ou de deux autres espèces parentales, encore inconnues ou *ii)* des régions devenues spécifiques aux différents taxons générées par réarrangements génomiques suite à l'hybridation.

Dans la continuité de cette étude, il a été remarqué que les amplifiats générés par PCR utilisant le couple PA-F/R présentaient, après électrophorèse, des faciès suggérant la présence d'hétéroduplex avec des extraits d'ADN de *Paa* et de *Pam*. Le produit de PCR PA-F/R a donc été cloné et séquencé et il a pu être montré que le couple d'amorce PA-F/R amplifiait en fait respectivement trois et deux allèles de séquences légèrement différentes chez *Paa* et *Pam*. En revanche, une seule séquence était amplifiée chez *Pau*. Ces résultats inattendus se sont ensuite avérés en parfait accord avec les caractéristiques des différents taxons constituant *P. alni sensu lato* mises en évidence dans le chapitre II.

Chapitre II

Caractérisation génétique des différents taxons constituant l'entité *Phytophthora alni sensu lato* et investigations sur l'origine du taxon hybride *Phytophthora alni* subsp. *alni*.

II.1. - Présentation de la stratégie scientifique.

Brasier et al. (1999, 2004) suggèrent que les différents taxons constituant *P. alni sensu lato* ont été générés suite à différents événements dont l'événement initial et fondateur fut une hybridation interspécifique entre *P. cambivora* et un taxon proche de *P. fragariae*. Afin de tester cette hypothèse et de vérifier les relations phylogénétiques qui lient les différents taxons constituant *P. alni sensu lato* entre eux mais aussi avec *P. cambivora* et *P. fragariae sensu lato*, nous avons entrepris de caractériser les trois espèces selon deux stratégies complémentaires.

II.1.1. – Etude de gènes nucléaires simple copie à introns et de l'ADN mitochondrial

La première approche visait à combiner l'étude de gènes nucléaires simple copie à celle de l'ADN mitochondrial. La comparaison des séquences de ces gènes, notamment dans des régions potentiellement polymorphes telles que les introns, devait permettre d'estimer la proximité génétique entre les différentes espèces et sous-espèces *sensu* Brasier et al. (2004). Le caractère « simple copie » des gènes nucléaires choisis était un prérequis aux études de distribution dans les différents génomes, puisque pour pouvoir comparer directement les séquences de ces différents gènes chez les trois espèces, c'est-à-dire les orthologues, il fallait s'assurer *a priori* de l'absence de paralogues générés par duplication, et ayant pu évoluer différemment. Le choix des gènes étudiés (*ASF*-like, *GPA*1, *RAS-Ypt* et *TRP*1) s'est basé sur plusieurs publications caractérisant des gènes présents en simple copie dans le génome de différentes espèces de *Phytophthora*. Nous avons pu confirmer que ces différents gènes étaient aussi présents en simple copie et récupérer la séquence orthologue chez *P. sojae* et *P. ramorum*, grâce à l'accès au génome complet de ces deux espèces via le portail du Joint Genome Institute (http://genome.jgi-psf.org/, université de Californie). En complément, l'étude de l'ADN mitochondrial, décrit comme transmis uniparentallement lors de la reproduction sexuée chez *Phytophthora* (Whittaker et al., 1994), y compris dans les cas d'hybridations interspécifiques (Man in't Veld et al., 1998), devait pouvoir apporter des données intéressantes en matière d'orientation de l' (des) événement(s) d'hybridation. L'ADN mitochondrial a été étudié de façon globale par RFLP (Restriction Fragment Length Polymorphism) et par séquençage de deux gènes (*cox*1, *nadh*1).

Les résultats obtenus par cette première approche sont résumés dans l'article suivant, publié dans la revue « Fungal Genetics and Biology » :

Publication 3 :

Ioos R., Andrieux A., Marçais B., and Frey P. (2006) Genetic characterization of the natural hybrid species *Phytophthora alni* as inferred from nuclear and mitochondrial DNA analyses. *Fungal Genetics and Biology* 43, 511-529.

Publication 3

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Genetic characterization of the natural hybrid species *Phytophthora alni* as inferred from nuclear and mitochondrial DNA analyses

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Abstract

The different subspecies of *Phytophthora alni*, *P. alni* subsp. *alni* (*Paa*), *P. alni* subsp. *uniformis* (*Pau*), and *P. alni* subsp. *multiformis* (*Pam*), are recent and widespread pathogens of alder in Europe. They are believed to be a group of emergent heteroploid hybrids between two phylogenetically close *Phytophthora* species. Nuclear and mitochondrial DNA analyses were performed, using a broad collection of *P. alni* and two closely related species, *P. cambivora* and *P. fragariae*. *Paa* possesses three different alleles for each of the nuclear genes we studied, two of which are present in *Pam* as well, whereas the third matches the single allele present in *Pau*. Moreover, *Paa* displays common mtDNA patterns with both *Pam* and *Pau*. A combination of the data suggests that *Paa* may have been generated on several occasions by hybridization between *Pam* and *Pau*, or their respective ancestors. *Pau* might have *P. cambivora* as a species ancestor, whereas *Pam* seems to have either been generated itself by an ancient reticulation or by autopolyploidization.

Keywords: *RAS-Ypt*; *ASF*-like; *TRP*1; *GPA*1; mtDNA-RFLP; *cox*1; *nadh*1; Interspecific hybridization

1. Introduction

The role of natural hybridization in the evolutionary history of many animal and plant species is well recognized today. Numerous examples illustrate that it has contributed also to the evolution of many pests and pathogens (Arnold, 2004). Until recently, only a few natural species hybrids were reported in the Fungi or *Stramenipila* kingdoms (Burnett, 1983). However, since the 1990s, there have been several reports of hybridization events among true fungi or oomycetes, especially involving plant pathogens (Olson and Stenlid, 2002). Man in't Veld et al. (1998) demonstrated that a new unknown *Phytophthora* pathogen active on *Primula* and *Spathiphyllum* in hydroponic culture systems was actually a hybrid between *P. cactorum* and *P. nicotianae*. Another recently described *Phytophthora* hybrid, highly aggressive on alder trees (*Alnus* spp.), is currently spreading in natural ecosystems across Europe (Gibbs et al., 2003). Gibbs (1995) previously described this alder *Phytophthora* as a taxon similar to *P. cambivora*, which is a common pathogen on hardwood trees. Brasier et al. (1999) then demonstrated that this pathogen was actually an interspecific hybrid. Based on morphological, serological, and genotypic traits, Brasier et al. (2004) formally described this new pathogen as *Phytophthora alni*. Because this new *Phytophthora* hybrid does not consist of a single entity but comprises a range of phenotypically diverse allopolyploid genotypes, *P. alni* was split into three subspecies: *P. alni* subsp. *alni* (*Paa*), *P. alni* subsp. *uniformis* (*Pau*), and *P. alni* subsp. *multiformis* (*Pam*) (Brasier et al., 2004).

In contrast with typical *Phytophthora* species, which are diploid organisms, *Paa* was shown to be near-tetraploid, consistent with possessing an allopolyploid genome (Brasier et al., 1999). *Paa* also displays an unusual internal transcribed spacer (ITS) polymorphism, i.e., numerous

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dimorphic sites within ITS sequences for a single isolate. On the other hand, the ploidy levels for the two other subspecies range from $2n+2$ to $2n+7$ for *Pau* and *Pam*, respectively. In contrast with *Paa*, nearly homogeneous ITS sequences were observed in both *Pam* and *Pau*: the ITS sequence for *Pam* differs from *P. fragariae* var. *fragariae* (*Pff*) or *P. fragariae* var. *rubi* (*Pfr*) by only a few bases, whereas the ITS sequence for *Pau* is very close to the *P. cambivora* (*Pc*) sequence (Brasier et al., 1999). According to chromosome karyotyping, ITS sequences, and amplified fragment length polymorphism (AFLP) fingerprinting, Brasier et al. (1999) hypothesized that *P. cambivora* was a parent of *P. alni*. On the other hand, recent isozyme analysis precludes the possibility that *P. fragariae* is involved *sensu stricto* in the hybridization process (Brasier, 2003; Nagy et al., 2003). Although *P. alni* was demonstrated to be a hybrid species, its origin remains unclear and additional data are required to trace back the ancestry to the parental species.

To address this problem, the use of molecular markers is particularly useful, and both nuclear and mitochondrial data may contribute to the understanding of the hybridization process. First, nuclear single-copy genes should be of particular interest for phylogeny purposes. In addition, the biparental inheritance and the absence of intergenomic concerted evolution in comparison to low copy or high copy nuclear genes make them particularly useful for studying the origin of hybrids and polyploidy lineages. Moreover, the use of genes containing introns, which are generally highly polymorphic regions, could potentially be of great interest for the discrimination of closely related *Phytophthora* species, such as the putative parents of *P. alni* (Brasier et al., 2004). Although the number of genes containing introns is much lower in *Phytophthora* sp. than in true Fungi (B. Tyler, personal communication), a few nuclear genes with introns have already been described for two *Phytophthora* species: *P. parasitica* (*TRP*1, Karlowsky and Prell, 1991) and *P. infestans* (*RAS-Ypt*; Chen and Roxby, 1996; *GPA*1, Laxalt et al., 2002). Besides, mitochondrial DNA analysis provides valuable information in the case of hybridization between two taxa; in the case of sexual mating for the *Phytophthora* genus, mitochondrial DNA is exclusively inherited from one of the parental lines (Whittaker et al., 1994).

In the present work, we studied the allelic distribution for orthologs of *TRP*1, *RAS-Ypt*, and *GPA*1 genes, along with another recently described single-copy gene containing one intron (*ASF*-like, Munakata et al., 2000). Mitochondrial DNA was studied through restriction pattern analysis and by the sequencing of two mitochondrial genes, cytochrome *c* oxidase subunit 1 (*cox*1) and NADH dehydrogenase subunit 1 (*nadh*1) (Kroon et al., 2004). This research, combining nuclear and mitochondrial data obtained from a large Europe-wide collection of *Paa*, *Pam*, *Pau*, *Pc*, *Pff*, and *Pfr* isolates, provides new insights into the hybrid status of *P. alni*.

2. Materials and methods

2.1. Source and culture of Oomycete isolates for DNA extraction

The Oomycete isolates used in this study are listed in Table 1. French isolates of *P. alni* or *Phytophthora* spp. were obtained by isolation from diseased plant material or biological baits carried out on soil sampled beneath the host, using the *Phytophthora*-selective PARBHY medium (Robin et al., 1998). Foreign isolates of *P. alni* and *Phytophthora* spp. were obtained from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) or from several European colleagues. Assignment of the isolates to one of the three subspecies of *P. alni* was achieved by combining the examination of the morphological features of each isolate in pure culture according to Brasier et al. (2004), and analyzing restriction patterns of the ITS region using a series of enzymes, according to Brasier et al. (1999) and Cooke et al. (2000). The assignment was further confirmed using subspecific SCAR-based PCR primers (Ioos et al., 2005).

A panel of 15 isolates was selected from among *Paa*, *Pau*, *Pam*, *P. cambivora*, and *P. fragariae* and used for cloning and sequencing: five isolates of *P. alni* subsp. *alni* (PAA129, PAA130, PAA143, PAA151, and PAA162), three isolates of *P. alni* subsp. *uniformis* (PAU60, PAU84, and PAU89), three isolates of *P. alni* subsp. *multiformis* (PAM54, PAM71, and PAM73), two isolates of *P. cambivora* (PC643 and PCJC17), one isolate of *P. fragariae* var. *fragariae* (PFF309), and one isolate of *P. fragariae* var. *rubi* (PFR109), chosen from different geographical locations.

All the isolates were grown shaking in 6 ml of liquid V8 juice medium (Miller, 1955). After incubation at 22 °C for 4–7 days, the mycelium was harvested by filtration on a sterile Whatman N°1 paper (Maidstone, England) and stored in this condition at −20 °C until DNA extraction. DNA was extracted using commercial plant DNA extraction kits (DNeasy plant mini kit®, Qiagen, Courtaboeuf, France) and as previously described (Ioos et al., 2005).

2.2. RFLP of mitochondrial DNA

Total DNA was extracted using commercial plant DNA extraction kits (DNeasy plant mini kit®) as previously described (Ioos et al., 2005), except that the quantity of dried mycelium was increased from 200 to 600 mg, and 10 µl of proteinase K (20 mg/ml) was added to the lysis buffer. Incubation time with the lysis buffer was also increased to 20 min. Four to 8 µg of total DNA was typically recovered. Mitochondrial DNA is present in multiple copies and can be separated from genomic DNA by digestion with restriction enzymes, which cut regions rich in G+C (Spitzer et al., 1989). Accordingly, 20 µl of total DNA were digested twice for 12 h with each time five units of *Hae*III or *Hpa*II and mtDNA patterns were resolved after a 15 h electrophoresis (0.6 V/cm) on a 0.8% agarose gel in TBE 0.5× buffer. Gels were stained

Table 1
List of the isolates of *Phytophthora* spp. and *Pythium* spp. used in this study

Species	Isolate	Isolator/supplier, reference	Host	Origin	Year isolated	mtDNA pattern
P. alni* subsp. *alni	PAA2	J.C. Streito (2N0685)	*Alnus glutinosa*	France (Yrieu lake, Landes)	2002	M
	PAA20	J.C. Streito (71T1)	*Alnus glutinosa*	France (Oise river, Aisne)	1997	*nd*
	PAA23	J.C. Streito (82T1A)	*Alnus glutinosa*	France (Oise river, Aisne)	1997	M
	PAA24	J.C. Streito (84T2)	*Alnus glutinosa*	France (Nied river, Bas-Rhin)	1997	*nd*
	PAA34	J.C. Streito (98-7-5)	*Alnus glutinosa*	France (La Combauté river, Haute-Saône)	1998	M
	PAA35	J.C. Streito (98-7-6)	*Alnus glutinosa*	France (La Combauté river, Haute-Saône)	1998	*nd*
	PAA38	J.C. Streito (2N0529)	*Alnus glutinosa*	France (Aa river, Pas-de-Calais)	2002	M
	PAA44	J.C. Streito (DSFO98172)	*Alnus glutinosa*	France (Thouet river, Maine-et-Loire)	1998	*nd*
	PAA47	J.C. Streito (AUL026/1)	*Alnus glutinosa*	France (Moselle river, Vosges)	1999	U
	PAA52	J.C. Streito (9900783.4)	*Alnus glutinosa*	France (Meurthe river, Meurthe-et-Moselle)	1999	*nd*
	PAA53	J.C. Streito (1R0152)	*Alnus glutinosa*	France (Nied Française river, Moselle)	2001	M
	PAA58	J.C. Streito (1N0201)	*Alnus glutinosa*	France (Rhône river, Savoie)	2001	M
	PAA100	R. Ioos (P1bisa)	*Alnus glutinosa*	France (Semouse river, Haute-Saône)	2003	M
	PAA102	R. Ioos (P3d)	*Alnus glutinosa*	France (Semouse river, Haute-Saône)	2003	M
	PAA107	R. Ioos (Priva)	*Alnus glutinosa*	France (Semouse river, Haute-Saône)	2003	M
	PAA111	C. Husson (Ainvelle Sol)	*Alnus glutinosa* soil	France (Ainvelle forest, Haute-Saône)	2003	M
	PAA112	C. Husson (2ALD03)	*Alnus glutinosa*	France (Moine river, Maine-et-Loire)	2003	M
	PAA113	C. Husson (102-1)	*Alnus glutinosa*	France (Sarre river, Moselle)	2003	M
	PAA114	C. Husson (Moselle)	*Alnus glutinosa*	France (Moselle river, Vosges)	2002	M
	PAA115	C. Husson (370-2)	*Alnus glutinosa*	France (Sarre river, Moselle)	2002	M
	PAA116	R. Ioos (3N10094-5a)	*Alnus glutinosa*	France (Ognon river, Haute-Saône)	2003	M
	PAA120	R. Ioos (3N10048-3a)	*Alnus glutinosa*	France (Ognon river, Haute Saône)	2003	*nd*
	PAA126	C. Husson (Ainvelle4-4)	*Alnus glutinosa*	France (Semouse river, Haute-Saône)	2003	M
	PAA129*	G. Capron (703)	*Alnus glutinosa*	France (Luy river, Landes)	2003	U
	PAA130*	R. Ioos (1429-6b)	*Alnus glutinosa*	France (Saône river, Haute-Saône)	2003	M
	PAA131	C. Husson (Sol A15)	*Alnus glutinosa* soil	France (Ainvelle forest, Haute Saône)	2003	M
	PAA133	C. Husson (Sol A7)	*Alnus glutinosa* soil	France (Ainvelle forest, Haute Saône)	2003	*nd*
	PAA147	B. Thoirain (478-4)	*Alnus glutinosa*	France (Sarre river, Moselle)	2004	M
	PAA149	B. Thoirain (19BT)	*Alnus glutinosa*	France (Vezouze river, Meurthe et Moselle)	2004	M
	PAA150	B. Thoirain (10BT)	*Alnus glutinosa*	France (Vezouze river, Meurthe et Moselle)	2004	M
	PAA151*	B. Thoirain (2051000-D12)	*Alnus glutinosa*	France (Moselle river, Vosges)	2004	U
	PAA153	B. Thoirain (C2.1-2070250)	*Alnus glutinosa*	France (Meurthe river, Meurthe et Moselle)	2004	M
	PAA160	B. Thoirain (C9)	*Alnus glutinosa*	France (Mortagne river, Meurthe et Moselle)	2004	M
	PAA161	B. Thoirain (C18)	*Alnus glutinosa*	France (Chiers river, Meurthe et Moselle)	2004	M
	PAA185	R. Ioos (4N1605)	*Alnus glutinosa*	France (Ill river, Bas-Rhin)	2004	M
	PAA195	R. Ioos (Jouy2)	*Alnus glutinosa*	France (Eure river, Eure)	2005	M
	PAA29	J.C. Streito (9900715.6)	*Alnus incana*	Belgium (Train river, Brabant)	1999	*nd*
	PAA86	D. De Merlier (2198[c])	*Alnus glutinosa*	Belgium (Salm river, Luxembourg)	1999	M
	PAA88	D. De Merlier (2295[c])	*Alnus glutinosa*	Belgium (Lesse river, Luxembourg)	2001	M
	PPA70	W. Man in't Veld (PD 20010933)	*Alnus* sp.	The Netherlands (Horst, Southern-Limburg)	Unknown	M
	PAA74	G. Mackaskill (P1275)	*Alnus glutinosa*	Great Britain (Scotland)	2000	U
	PAA75	J. Gibbs (P1272)	*Alnus viridis*	Great Britain (Scotland)	2000	M
	PAA76	J. Gibbs (P1271)	*Alnus glutinosa*	Great Britain (Scotland)	2000	M
	PAA77	J. Delcan (P1270)	*Alnus glutinosa*	Great Britain (Scotland)	2000	M
	PAA78	J. Delcan (P1960)	*Alnus glutinosa*	Great Britain (England)	1997	*nd*
	PAA79	J. Delcan (P957[a])	*Alnus glutinosa*	Great Britain (England)	1997	*nd*
	PAA80	J. Delcan (P950[a])	*Alnus glutinosa*	Great Britain (England)	1997	*nd*
	PAA81	J. Delcan (P937)	*Alnus glutinosa*	Great Britain (England)	1997	U
	PAA82	S. Gregory (P850)	*Alnus glutinosa*	Great Britain (England)	1996	M
	PAA85	C. Brasier (P834[e])	*Alnus glutinosa*	Great Britain (England)	Unknown	M
	PAA91	Z. Nagy (6[d])	*Alnus glutinosa*	Hungary (Hévíz)	2001	M′
	PAA92	Z. Nagy (8[d])	*Alnus glutinosa* soil	Hungary (Hévíz)	2001	M′
	PAA93	Z. Nagy (9[d])	*Alnus glutinosa* soil	Hungary (Hévíz)	2001	M′
	PAA94	Z. Nagy (1a[d])	*Alnus glutinosa* soil	Hungary (Hévíz)	2001	M′
	PAA95	Z. Nagy (4-2[d])	*Alnus glutinosa*	Hungary (Hévíz)	2001	M′
	PAA134	K. Kaminski (BBA 23/00)	*Alnus glutinosa*	Germany (Hessen)	2000	M
	PAA162*	R. Ioos (9a)	*Alnus glutinosa*	Germany (Bavaria)	2004	U
	PAA176	R. Ioos (1c)	*Alnus glutinosa*	Germany (Bavaria)	2004	U
	PAA141	T. Cech (Pucking B10)	*Alnus glutinosa*	Austria (Donau Valley)	Unknown	U′

(*continued on next page*)

Table 1 (*continued*)

Species	Isolate	Isolator/supplier, reference	Host	Origin	Year isolated	mtDNA pattern
	PAA143*	L. Orlikowski (PO 192)	*Alnus glutinosa*	Poland (Pilicia river, Bialobrzegi)	2002	M″
	PAA144	L. Orlikowski (PO 193)	*Alnus glutinosa*	Poland (Vistula river, Koszyce)	2003	M″
	PAA145	L. Orlikowski (PO 203)	*Alnus glutinosa*	Poland (Pisia river, Radziejowice)	2004	M″
	PAA146	L. Orlikowski (PO 205)	*Alnus glutinosa*	Poland (Vistula river, Pulawy)	2002	M″
	PAA178	L. Orlikowski (PO318)	*Alnus glutinosa*	Poland (Notec river, Naklo)	2003	M″
	PAA180	L. Orlikowski (PO355)	*Alnus glutinosa*	Poland (Rudawka river, Morawica)	2004	M″
	PAA181	L. Orlikowski (PO379)	*Alnus glutinosa*	Poland (Bug river, Wlodawa)	2004	M″
	PAA182	L. Orlikowski (PO385)	*Alnus glutinosa* soil	Poland (Bug river, Wlodawa)	2004	M″
	PAA183	L. Orlikowski (PO399)	*Alnus glutinosa* soil	Poland (nursery, Siedliska)	2004	M″
	PAA184	L. Orlikowski (PO400)	*Alnus glutinosa*	Poland (nursery, Kornie)	2004	M″
	PAA189	L. Orlikowski (*P. alni* soil)	*Alnus glutinosa* soil	Poland (nursery, Siedliska)	2004	M″
	PAA191	L. Orlikowski (121 AL/KO/03)	*Alnus glutinosa*	Poland (Vistula river, Koszyce)	2004	M″
	PAA192	L. Orlikowski (106 AL/PV)	*Alnus glutinosa*	Poland (Bug river, Wlodawa)	2004	M″
	PAA193	L. Orlikowski (107/AL/B/03)	*Alnus glutinosa*	Poland (Bug river, Wlodawa)	2004	M″
P. alni* subsp. *uniformis	PAU60*	J.C. Streito (AUL028)	*Alnus glutinosa*	France (Moselle river, Vosges)	1999	U″
	PAU84*	C. Olsson (P875[a,b,c,f])	*Alnus glutinosa*	Sweden (Gothenburg)	1997	U′
	PAU87	D. De Merlier (2271[e])	*Alnus glutinosa*	Belgium (Amblève river, Liège)	2001	U
	PAU187	D. De Merlier (2276[e])	*Alnus glutinosa*	Belgium (Canal du Centre, Hainaut)	2001	U
	PAU188	D. De Merlier (2277[e])	*Alnus incana*	Belgium (Rulles river, luxembourg)	2001	U
	PAU89*	P. Capretti (CBS109280[e])	*Alnus cordata*	Italy (Northern Tuscany)	2000	U
	PAU96	Z. Nagy (155-a[d])	*Alnus glutinosa*	Hungary (Hanság)	1999	U
	PAU97	Z. Nagy (155-b[d])	*Alnus glutinosa* soil	Hungary (Hanság)	1999	U
	PAU98	Z. Nagy (155-c[d])	*Alnus glutinosa* soil	Hungary (Hanság)	1999	U
	PAU142	A. Munda (Phy-A-Slo)	*Alnus glutinosa*	Slovenia (Lubljana)	2003	U
P. alni* subsp. *multiformis	PAM54*	J.C. Streito (DSFO/0125)	*Alnus glutinosa*	France (Aff river, Ille et Vilaine)	2000	M
	PAM71*	W. Man in't Veld (W1139)	*Alnus glutinosa* soil	The Netherlands (De Wieden, Overijssel)	Unknown	M
	PAM90	W. Man in't Veld (P972[a,c,f])	*Alnus glutinosa* soil	The Netherlands (De Wieden, Overijssel)	Unknown	M
	PAM73*	S. Gregory (P841[a,c,f])	*Alnus glutinosa*	Great Britain (England)	1996	M
	PAM186	D. De Merlier (2274[e])	*Alnus glutinosa*	Belgium (Sambre river, Namur)	2001	M′
P. cambivora	PC463	INRA Bordeaux	*Castanea sativa*	France (Lot et Garonne)	1994	C2
P. cambivora	PC643*	INRA Bordeaux	*Castanea sativa* soil	France (Aude)	2000	C1
P. cambivora	PCJC17*	C. Delatour	*Quercus* sp. soil	France (Haute Saône)	1999	C2
P. cambivora	PCGA1	C. Delatour	*Quercus* sp. soil	France (Haute Saône)	1999	C2
P. cambivora	PC99428	R. Ioos	*Castanea sativa*	France	1999	C2
P. cambivora	PCST3R1	C. Delatour	*Quercus petraea*	France (Gironde)	1999	C2
P. cambivora	PC627 (oecdc)	INRA Bordeaux	*Castanea sativa*	Italy	2000	C2
P. cambivora	PC1A21	INRA Bordeaux	*Quercus* sp. soil	France (Vienne)	1999	C2
P. cambivora	PC4N1425	LNPV-UMAF	*Castanea sativa*	France (Yvelines)	2004	C2
P. cambivora	PC4N444	LNPV-UMAF	*Castanea sativa*	France (Eure)	2004	C2
P. fragariae var. *fragariae*	PFF1	K. Hughes	*Fragaria x ananassa*	Unknown	Unknown	*nd*
P. fragariae var. *fragariae*	PFF209.46	CBS (CBS209.46)	*Fragaria x ananassa*	Great Britain (England)	1946	FF
P. fragariae var. *fragariae*	PFF309*	CBS (CBS 309.62)	*Fragaria x ananassa*	Great Britain (Scotland)	1962	FF
P. fragariae var. *rubi*	PFRVR 59	D. Cooke (FVR 59)	*Rubus* sp.	Great Britain	Unknown	FR
P. fragariae var. *rubi*	PFR163-2	A. Baudry (163-2)	*Rubus* sp.	France	Unknown	FR
P. fragariae var. *rubi*	PFR2	K. Hughes	*Rubus* sp.	Great Britain	Unknown	FR
P. fragariae var. *rubi*	PFR967.95	CBS (CBS967.95)	*Rubus* sp.	Great Britain (Scotland)	1985	FR
P. fragariae var. *rubi*	PFR109*	CBS (CBS109.892)	*Rubus* sp.	Great Britain (Scotland)	1991	FR
P. cactorum	CAC4810/TJ	C. Delatour	Unknown	France	Unknown	

Table 1 (*continued*)

Species	Isolate	Isolator/supplier, reference	Host	Origin	Year isolated	mtDNA pattern
P. cinnamomi	DSFO2N0964	J.C. Streito	*Castanea sativa*	France	2002	
P. cinnamomi	DSFA970060	J.C. Streito	*Quercus suber*	France	1997	
P. cinnamomi	DSFO990050	J.C. Streito	*Castanea sativa* soil	France	1999	
P. cinnamomi	P382	C. Brasier	*Nothofagus procera* soil	Great Britain	1980	
P. citricola	2N0750-171	J.C. Streito	Unknown	France	2002	
P. citricola	AUL 045 AP7	J.C. Streito	*Alnus glutinosa*	France	1999	
P. citricola	2AE5	C. Delatour	*Quercus* sp. soil	France	1998	
P. citricola	3N1345-17	R. Ioos	*Alnus glutinosa*	France	2003	
P. citrophthora	2N1021	J.C. Streito	*Rosa* sp.	France	2002	
P. cryptogea	990675	J.C. Streito	*Actinidia sinensis*	France	1999	
P. erythroseptica	960713	J.C. Streito	*Polygonum oberti*	France	1999	
P. europaea	AL5	C. Delatour	*Quercus* sp. soil	France	1998	
P. europaea	2AU2	C. Delatour	*Quercus* sp. soil	France	1999	
P. gonapodyides	Gonap 4	C. Delatour	*Quercus* sp. soil	France	1998	
P. gonapodyides	AB4	C. Delatour	*Quercus* sp. soil	France	1998	
P. humicola	3N1245-j	R. Ioos	*Alnus glutinosa* soil	France	2003	
P. ilicis	3N1245-l	R. Ioos	*Alnus glutinosa* soil	France	2003	
P. inundata	9500802	J.C. Streito	*Alnus glutinosa* soil	France	1998	
P. lateralis	98093.1-SPV	J.C. Streito	*Chamaecyparis* sp.	France	1998	
P. megasperma	3N1245-m	R. Ioos	*Alnus glutinosa* soil	France	2003	
P. megasperma	BK1	C. Delatour	*Quercus* sp. soil	France	1998	
P. megasperma	03-12	C. Delatour	water under *Quercus* sp.	France	1998	
P. megasperma	mega 1	T. Jung	Unknown	Germany	1998	
P. megasperma	8RPOC3	C. Delatour	*Quercus* sp. soil	France	1998	
P. nicotianae	960579	J.C. Streito	*Nicotiana tabacum*	France	1996	
P. taxon forestsoil	8CARPPOC1	C. Delatour	*Quercus* sp. soil	France	1998	
P. palmivora	970423	J.C. Streito	*Hedera* sp.	France	1997	
P. parasitica	970029	J.C. Streito	*Lycopersicon esculentum*	France	1997	
P. taxon Pgchlamydo	Haye,3,1	C. Delatour	*Quercus* sp. soil	France	1998	
P. pseudosyringae	EW5	C. Delatour	*Quercus* sp. soil	France	1998	
P. psychrophila	FF20	C. Delatour	*Quercus* sp. soil	France	1998	
P. quercina	FNA	C. Delatour	*Quercus* sp. soil	France	1999	
P. quercina	Mers2	C. Delatour	*Quercus* sp. soil	France	1999	
P. ramorum	2N0983	C. Saurat	*Rhododendron* sp.	France	2002	
P. ramorum	3N0003	C. Saurat	*Viburnum* sp.	France	2002	
P. sojae	443	F. Panabières	*Glycine max*	Unknown	Unknown	
P. syringae	2JZ2	C. Delatour	*Quercus* sp. soil	France	1999	
Pythium aphanidermatum	Ctsa	R. Ioos	Unknown	France	2003	
Pythium sylvaticum	0675/a	R. Ioos	Unknown	France	2003	
Pythium intermedium	02/84/1	S. Verger	Unknown	France	Unknown	
Pythium irregulare	02/57/1	S. Verger	Unknown	France	Unknown	
Pythium ultimum	433/3	S. Verger	Unknown	France	Unknown	
Pythium sp.	3N1345-11	R. Ioos	*Alnus glutinosa* soil	France	2003	

The different mitochondrial DNA patterns resolved in this study are indicated.
nd, not determined.
[a] Also studied by Delcan and Brasier (2001).
[b] Also studied by Brasier et al. (1999).
[c] Also studied by De Merlier et al. (2005).
[d] Also studied by Nagy et al. (2003).
[e] Also studied by Santini et al. (2003).
[f] Also studied by Brasier and Kirk (2001).
* Isolate used for sequencing in this study.

with ethidium bromide and images were recorded with a CCD camera and a GELDOC 2000® gel documentation system (Biorad, Marne-La-Coquette, France).

2.3. Design of nuclear gene-specific degenerate primers

For each of the four nuclear genes studied, namely *ASF*-like, *GPA*1, *RAS-Ypt*, and *TRP*1, the original sequence deposited in GenBank was retrieved and used as a basis for similarity research in other *Phytophthora* DNA resources (see accession numbers in Table 2). Nucleotide similarities were searched for in the GenBank database, and the *P. ramorum* and the *P. sojae* assembled sequences recently released in the public domain (http://genome.jgi-psf.org/), using the BLASTn algorithm and the *Phytophthora* Functional Genomics Database, which gathers sequences from *P. infestans* and *P. sojae*, and using the PFGD search filter (http://www.pfgd.org/pfgd/filter.html).

For each gene, all the available orthologous sequences from different *Phytophthora* species were then aligned using ClustalW (Thompson et al., 1994) and a series of degenerate primer pairs were manually designed in highly conserved regions located in exons. The location of the primers was chosen in order to enable PCR amplification of the largest part of the gene, including as many intronic regions as possible. The primer pairs that best amplified the target region in all species of the 15-isolate panel were retained and used for cloning. Table 2 lists the respective forward and reverse PCR primers chosen for each of the four nuclear genes.

2.4. Amplification and cloning of the nuclear and the mitochondrial genes

Each of the four nuclear genes was amplified by PCR for each of the 15 isolates of the panel. Amplification of the four nuclear genes was carried out in a 20-µl mixture containing 1× *Taq* polymerase buffer (Sigma–Aldrich, L'Isle d'Abeau, France), 1.8 mM $MgCl_2$ (1.5 mM for *TRP*1), 0.7 µg/µl bovine serum albumin (Sigma), 0.45 µM (0.2 µM for *TRP*1) of each forward and reverse gene-specific primer, 180 mM dNTPs, 0.6 U of *Taq* DNA Polymerase (Sigma–Aldrich), 2 µl of template DNA (30–80 ng), and molecular biology grade water was added to 20 µl. The cycling profile for PCR included an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 62 °C (58 °C for *RAS-Ypt*), and elongation for 1 min at 72 °C, and a final extension step at 72 °C for 7 min.

Amplification of the two mitochondrial genes, *nadh*1 and *cox*1, was performed for a limited panel of six isolates: *Paa* (PAA129 and PAA130), *Pam* (PAM54), *Pau* (PAU60), and *Pc* (PCJC17 and PC643). PCRs were carried out with the mitochondrial gene-specific primers designed by Kroon et al. (2004) using the same parameters as described above for the nuclear genes with slight modifications. The annealing temperatures were lowered to 58 and 53 °C for *nadh*1 and *cox*1 amplifications, respectively, and the concentration of $MgCl_2$ was raised to 3.5 mM for *cox*1, as suggested by Kroon et al. (2004).

Table 2
List of the degenerate primers designed in this study

Gene	Original reference (GenBank Accession No.)	Additional sequences[a]	Primer (forward/reverse)	Sequence (5′–3′)	Size[b]	Intron(s)[c]
ASF-like	*Homo sapiens* (AB028628)	*P. sojae* (Scaffold_6 1068853-1069936)	ASF-E1-1F	ACCAACATCACCGTGCTGGAC	388–402	1
		P. ramorum (Scaffold_43 348071–349019)	ASF-E2-2R	CGTTGTTGACGTAGTAGCCCAC		
*GPA*1	*P. infestans* (AY050536), *P. palmivora* (AY050537)	*P. sojae* (Scaffold_34 298473–301143)	GPA-E1-1F	GGACTCTGTGCGTCCCAGATG	286–312	1
		P. ramorum (Scaffold_59 316212–318351)	GPA-E2-1R	ATAATTGGTGTGCAGTGCCGC		
RAS-Ypt	*P. infestans* (U30474)	*P. cinnamomi* (AF454368) *P. cryptogea* (AF454367) *P. citricola* (AF454369) *P. sojae* (Scaffold_30 367461–369077) *P. ramorum* (Scaffold_16 280386–282150)	RAS-E1-1F RAS-E5-1R	ATGAACCCCGAATAGTRCGTGC TGTTSACGTTCTCRCAGGCG	666–698	4
*TRP*1	*P. parasitica* (M64473)	*P. sojae* (Scaffold_25 46183–57099)	TRP-E1-1F	GAGGAGATCGCGGCGCAGCG	661–765	2
		P. ramorum (Scaffold_52 330699–333530)	TRP-E3-1R	GCGCACATRCCGAGVTTGTG		

[a] Refer to GenBank accession number for other species or localization of the respective similar sequence in the *P. sojae* and the *P. ramorum* JGI sequencing projects.
[b] Range of amplicon length yielded in silico using the designed primer pair with all available sequences.
[c] Number of introns that are located within the amplified region using the designed primers.

Each of the gene-specific PCR products was cloned for each isolate of the panel, using a TOPO®-TA cloning kit (Invitrogen, Cergy Pontoise, France) and following the manufacturer's instructions.

2.5. Selection of the clones and sequencing

For each isolate, hundreds of positive clones were typically recovered for each of the cloned genes. For each isolate x gene combination, 10 positive clones were randomly selected and tested by heteroduplex analysis in order to detect sequence polymorphisms among the 10 inserts, following a protocol adapted from Pinar et al. (1997). Heteroduplex analysis is a conformational technique that makes it possible to detect mutations in PCR-amplified products. The migration of heteroduplex DNA in agarose gel electrophoresis is different from that of homoduplex DNA because of an altered tri-dimensional structure. Briefly, for each group of 10 clones derived from the same gene, the insert was amplified by PCR using M13 F/R primers, following the PCR conditions recommended by the cloning kit's manufacturer. The ten PCR products were tested against each other by mixing directly 4 µl of each PCR products in a PCR tube, corresponding to a total of 45 pairwise combinations. An extra 8-µl sample of a unique PCR product was also prepared to be used as a control for homoduplex patterns. All the mixtures were then placed in a Genamp PCR system 9700 (Applied Biosystem, Foster City, California). DNA strands were denatured at 96 °C for 3 min and slowly cooled to 20 °C at a rate of 0.25 °C/s. The entire 8-µl mixture was then loaded onto a 1% agarose gel and separated by electrophoresis for 90 min at 3.6 V/cm. Heteroduplex banding patterns were distinguished from among all of the 45 combinations and, eventually, the 10 clones could be discriminated and clustered based on the occurrence of detectable sequence polymorphisms. For both nuclear and mitochondrial genes and for each isolate, all the polymorphic inserts were selected and double-strand DNA sequencing was performed by the di-deoxy-chain termination method using a T3-T7 sequencing kit on a CEQ 2000 XL DNA sequencer (Beckman, Fullerton, California). Forward and reverse sequences were assembled in Sequencher 4.2 (Genecodes, Ann Arbor, MI) and aligned using an on-line version of ClustalW software (http://www.genebee.msu.su/clustal/basic. html). The sequence obtained for the four nuclear and the two mitochondrial genes was deposited in the GenBank database (Table 3).

2.6. Phylogenetic analyses

Phylogenetic analyses were performed with PAUP version 4.0 (Swofford, 2002), using the maximum parsimony method. Individual phylogenetic analyses were conducted for each mitochondrial and nuclear gene. Heuristic searches were performed using "tree-bisection–reconnection" (TBR) branch swapping algorithm, zero-length branches were collapsed and all characters equally weighted. Subsequent parsimony bootstrap analyses used 1000 replicates with TBR branch swapping.

2.7. Design and testing of allele-specific PCR primers within the nuclear genes

All the sequences obtained for each nuclear gene and for all of the 15 isolates were aligned using ClustalW. Based on different clusters of sequences, a series of allele-specific primers were manually designed from polymorphic regions or around insertion/deletion (indel) loci mainly located within introns. Some of the sequences could not be subjected to primer design due to an insufficient polymorphism level or scattered substitutions. To test the absence or the presence of the different alleles within the genome of the different *Phytophthora* isolates, all the primer pairs were tested by PCR, first with the 15-isolate panel to check their reliability and specificity, and then with all the isolates of the *P. alni*/*Phytophthora* spp. collection listed in Table 1. Allele-specific PCRs were carried out as described above for gene-specific amplification except that the $MgCl_2$ concentration was increased to 2.2 mM and that no BSA was used. The allele-specific annealing temperatures used are indicated in Table 4.

3. Results

3.1. Mitochondrial DNA patterns

Digestion of total DNA with the two endonucleases generated patterns with several discrete bands. Both *Hae*III and *Hpa*II revealed polymorphism in the mtDNA pattern of the isolates we tested. Four and five distinct patterns were revealed with *Hae*III and *Hpa*II restrictions, respectively, among the studied isolates from the three subspecies of *P. alni*, respectively (Fig. 1). Combining the patterns of the two enzymes, isolates of the three *P. alni* subspecies could be placed in six groups: M, M′, and M″ groups encompassing only *Paa* and *Pam* isolates, and U, U′, and U″ groups, which included only *Paa* and *Pau* isolates. Most of the *Pam* isolates displayed the M pattern, whereas most of the *Pau* isolates displayed the U pattern. The pattern M′ was only observed for a single *Pam* isolate originating from Belgium (PAM186). Likewise, the two patterns U′ and U″ were both displayed by a single Pau isolate. Pattern U′ was encountered in PAU84 from Sweden, whereas pattern U″ was encountered in PAU60 from France.

Five out of the six patterns, M, M′, M″, U, and U′, were observed among the different *Paa* isolates we examined. No strong relationship could be established between the pattern observed and isolate origin or sampling date (Table 1). M was the most frequent pattern encountered in the *Paa* isolates. By contrast, the M′ pattern was characteristic of

Table 3
GenBank accession numbers of the sequences obtained for the six genes cloned in this study for the 15-isolate panel

Species	Isolate	Nuclear genes				Mitochondrial genes	
		ASF-like	*GPA*1	*RAS-Ypt*	*TRP*1	*cox*1	*nadh*1
P. alni subsp. *alni*	PAA 129	DQ092818	DQ179047	DQ093974	DQ093997	DQ202499	DQ202488
		DQ092819	DQ179048	DQ179054	DQ093998		DQ202489
		DQ092820	DQ179049				
	PAA 130	DQ092821	DQ179050	DQ093975	DQ202482	DQ202500	DQ202490
		DQ092822	DQ179051	DQ093976	DQ202483		DQ202491
		DQ092823		DQ093977	DQ212060		
	PAA 143	DQ092824	DQ092851	DQ093978	DQ093999		
		DQ092825	DQ179052	DQ093979	DQ094000		
		DQ092831		DQ093980			
	PAA 151	DQ092826	DQ092852	DQ093981	DQ094001		
		DQ092827	DQ092853	DQ093982	DQ094002		
			DQ092854				
	PAA 162	DQ092828	DQ092855	DQ093983	DQ094003		
		DQ092829	DQ092856	DQ093984	DQ094004		
		DQ092830					
P. alni subsp. *uniformis*	PAU 60	DQ092811	DQ092857	DQ093971	DQ202480	DQ202498	DQ202486
		DQ092812					DQ202487
		DQ092813					
	PAU 84	DQ092814	DQ092849	DQ093972	DQ093996		
		DQ092815					
		DQ092816					
	PAU 89	DQ092817	DQ092850	DQ093973	DQ202481		
P. alni subsp. *multiformis*	PAM 54	DQ092806	DQ092843	DQ093966	DQ093991	DQ202496	DQ202484
		DQ092807	DQ092844	DQ179053	DQ093992	DQ202497	DQ202485
	PAM 71	DQ092808	DQ092845	DQ093967	DQ093993		
		DQ179044	DQ179046	DQ093968	DQ202479		
		DQ179045					
	PAM 73	DQ092809	DQ092847	DQ093969	DQ093994		
		DQ092810	DQ092848	DQ093970	DQ093995		
P. cambivora	PC643	DQ092834	DQ092861	DQ093987	DQ094007	DQ202502	DQ202494
		DQ092835	DQ092862	DQ093988		DQ202503	DQ202495
		DQ092836					
		DQ092837					
		DQ092838					
	PCJC17	DQ092839	DQ092860	DQ093989	DQ094008,	DQ202501	DQ202492
		DQ092840		DQ093990	DQ094009		DQ202493
		DQ092841					
		DQ092842					
P. fragariae var. *fragariae*	PFF309	DQ092832	DQ092858	DQ093985	DQ094005		
P. fragariae var. *rubi*	PFR109	DQ092833	DQ092859	DQ093986	DQ094006		

Paa isolates from Hungary, whereas M″ was only found in Polish isolates. Despite being less frequent than M, the U pattern was also identified in *Paa* from locations throughout Europe, whereas the closely related U′ pattern was only detected for one isolate originating in Austria (PAA141).

Additional patterns were resolved for *P. cambivora* isolates (C1 and C2) and for *P. fragariae* for which var. *fragariae* and var. *rubi* could be separated into FF and FR patterns, respectively (Fig. 1).

3.2. Cloning and sequencing of the four nuclear and the two mitochondrial genes

Each of the four nuclear genes (*ASF*-like, *GPA*1, *RAS-Ypt*, and *TRP*1) could be efficiently amplified using the designed exon-based primer pairs. Table 2 lists the series of primer pairs that best enabled the amplification of each gene. The two mitochondrial genes, *cox*1 and *nadh*1, were also successfully amplified using the gene-specific primers designed by Kroon et al. (2004).

Thanks to the heteroduplex analysis carried out for each individual isolate of the panel, several clones containing polymorphic inserts for each of the six genes could be selected.

The sequencing of the selected clones potentially containing different insert sequences showed that each isolate possessed one to four different alleles, depending on the nuclear gene, and either one or two slightly different sequences for the mitochondrial genes. For each of the four nuclear genes, all the coding regions of the sequences obtained were first

Table 4
List of the allele-specific primers designed in this study

Gene	Allele-specific primer (F = forward, R = reverse)	Sequence (5′–3′)	Annealing temperature (°C)
ASF-like	ASF-PAM1-F[a]	GGT GCT GGA GGA AGT GCT T	64
	ASF-PAM2-F[a]	ACC GCC ATC ACC ACC ATA	60
	ASF-PAU-F[a]	CAC CGC CAC AAC ATC CAC TC	58
	ASF-PCJC17a6-F[a]	ACT ATC TCC TAT GAT ACA CT	54
	ASF-PC643e5-F[a]	TCC TAT GCT ACA CAC TAA C	60
	ASF-PCJC17c6-F[a]	GAT ACA GTG TAG CAC CAT C	58
*GPA*1	GPA-PAM1-R[b]	CTC GCA GCT CCC ACT GCG AG	60
	GPA-PAM2-R[b]	ACA GCA CCA AAC AAA GTA CC	60
	GPA-PAU-R[b]	TCC CAC TAT AAA CAT GTC A	54
RAS-Ypt	RAS-PAM1-F	AGA GGG ATA TAT TTG AGG TT	60
	RAS-PAM1-R	GTT GGA CCC GGG ACG GTC TTC	60
	RAS-PAM2-F	AGA GGG ATA TAT TTG CGG CT	60
	RAS-PAM2-R	TCA GCA ATC GGA GAG CAA GCT	60
	RAS-PAU-F	A TTT ACT TGC AGC CGC AGG CT	58
	RAS-PAU-R	ACC TAG GGC AGA CAA GCT AGT C	58
	RAS-PC643g1-F	AGC GGT AGA CTG ACC ACA CCG	60
	RAS-PC643g1-R	GCC TGG AGG TCA AAA CTT AG	60
	RAS-PC643a2-F	GCT GCT AAC AGA CAG CAG AC	60
	RAS-PC643a2-R	ATG AAG CAC TCC GAA CCG GT	60
	RAS-PFR109h1-F	TGT CGA GAG TGA TTT ATT	60
	RAS-PFR109h1-R	AA TGG CAA GGC TAG TTA CTA	60
*TRP*1	TRP-PAM1-R[c]	CCT GTA GCA ACA GAG CAA TG	60
	TRP-PAM2-F	CCC GTT GCT GCG GCT GGC	62
	TRP-PAM2-R	GGT CGC CTA CAC CGC GTG	62
	TRP-PAU-F	GTG CGT CGC TAG CCC ATC A	60
	TRP-PAU-R	CGC CTA CAG AGC ATC ATA G	60
	TRP-PCJC17c3-F	TGG ACG TAG AAG CCG CCA AG	60
	TRP-PCJC17c3-R	CAG GCA TAT ACC GTT TCC AC	60
	TRP-PFF309a9-F	CTA CCT CCC TAA GCT TAT CA	60
	TRP-PFF309a9-R	ACG CAG CAT CAT AGA AAA T	60

[a] Used in combination with gene-specific ASF-E2-2R as reverse primer.
[b] Used in combination with gene-specific GPA-E1-1F as forward primer.
[c] Used in combination with gene-specific TRP-E1-1F as forward primer.

translated and compared by sequence alignment to the published reference sequence(s) in order to check the identity of the cloned sequences (data not shown). The sequences obtained from the mitochondrial genes were compared using BLASTn software with the series of sequences published by Kroon et al. (2004) and shown to represent orthologs of *cox*1 and *nadh*1, as expected (data not shown).

3.3. Sequence analysis for the nuclear genes

Separate phylogenetic analyses were conducted for individual nuclear genes using all of the sequences obtained from the 15-isolate panel, and the retrieved ortholog sequences from *P. ramorum* and *P. sojae* were considered as outgroups.

For each of the four nuclear genes, a single allele was found in all of the *Pau* isolates. By contrast, two different alleles were systematically observed for all the *Pam* isolates on our panel, whereas at least two, and sometimes three different alleles, were present for all the *Paa* isolates. All four phylogenetic trees showed the same clustering pattern regarding *P. alni* sequences (Fig. 2). Indeed, regardless of the gene considered, the different *P. alni* alleles could be split into three clusters, respectively, designated here as PAM1, PAM2, and PAU. For each of the four genes, PAM1 and PAM2 clusters only contained sequences originating from *Paa* and *Pam* isolates, whereas PAU clusters contained sequences originating from *Paa* and *Pau* isolates.

Single alleles were observed for the four studied genes for *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* isolates. By contrast, one or two different alleles of *GPA*1, *TRP*1, and *RAS-Ypt* genes were identified for each *P. cambivora* isolate, whereas up to four different alleles of the *ASF*-like gene could be observed (Fig. 2A).

Some *P. cambivora* alleles were closely related to the PAU cluster but, except for ASF-like, were not included in this PAU cluster. The alleles identified for the two different varieties of *P. fragariae* clustered together in another group, different from those containing *P. alni* or *P. cambivora* sequences. Nevertheless, for the ASF-like gene, the respective alleles for the two varieties were separated (Fig. 2A).

3.4. Allele-specific PCRs for the nuclear genes

Sequence alignments using all the data collected for each nuclear gene and for each isolate showed that polymorphism mainly occurred in the intronic regions. These

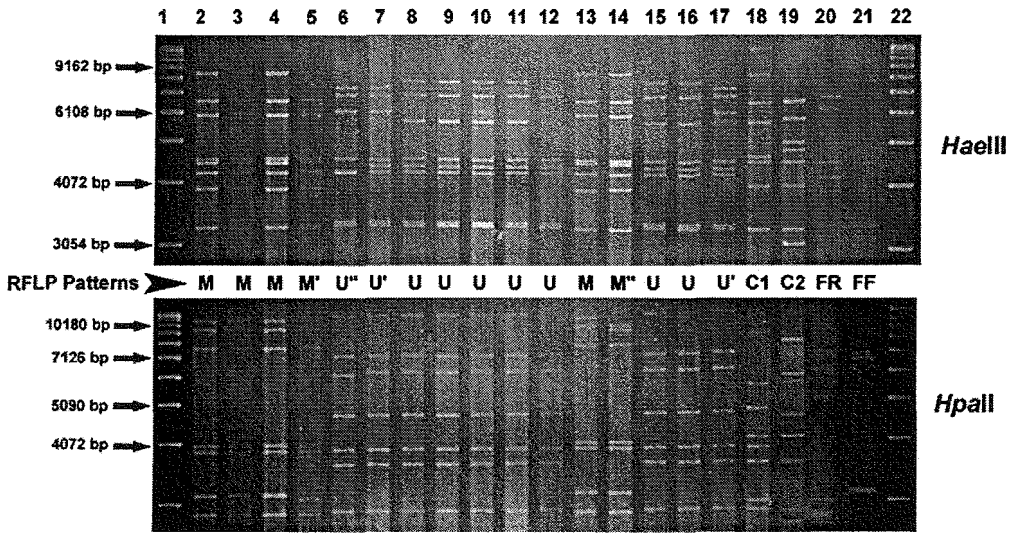

Fig. 1. Combination of the restriction patterns obtained by independent *Hae*III and *Hpa*II digestions of total DNA from a series of *P. alni* subsp. *multiformis* (*Pam*), *P. alni* subsp. *uniformis* (*Pau*), *P. alni* subsp. *alni* (*Paa*), *P. cambivora* (*Pc*), *P. fragariae* var. *rubi* (*Pfr*), and *P. fragariae* var. *fragariae* (*Pff*) isolates. Six different combinations were resolved for *P. alni* and named M, M′, M″, U, U′, and U″. Two different combinations were resolved for *Pc*, i.e., C1 and C2, respectively, whereas a single combination was resolved for *Pfr* (FR) and for *Pff* (FF). Lanes 1 and 22, molecular standard 1 kb DNA Ladder (Invitrogen, Cergy Pontoise, France); lanes 2–5, *Pam* isolates PAM54, PAM71, PAM73, and PAM186; lanes 6–11, *Pau* isolates PAU60, PAU84, PAU87, PAU89, PAU142, and PAU188; lanes 12–17, *Paa* isolates PAA129, PAA130, PAA143, PAA151, PAA162, and PAA141; lanes 18 and 19, *Pc* isolates PC643 and PCJC17; lane 20, *Pfr* isolate PFR109; lane 21, *Pff* isolate PFF309.

polymorphisms were used to design a series of allele-specific PCR primers for each nuclear gene (Figs. 3–6). For each nuclear gene, it was possible to design pairs of primers specific to each of the three clusters of *P. alni* alleles, i.e., PAM1, PAM2, and PAU, respectively (Table 4). Additionally, for the two related taxa, *P. cambivora* and *P. fragariae*, a series of PCR primers targeted regions that were specific to sequences obtained from at least one isolate and were named according to the clone they were designed from (Table 4). Unfortunately, it was sometimes impossible to design PCR primers from some of the sequences obtained from *P. cambivora* or *P. fragariae* because of possible cross-reactions with other sequences. Such sequences are indicated in italics in Table 5. Likewise, it was not possible to design primers that only targeted sequences present in one or the other variety of *P. fragariae* because of the very strong similarity between the sequences of the genes for the two varieties.

All the allele-specific primer pairs successfully yielded an amplicon of the expected size when tested with the DNA of the isolate the primers were designed from, confirming the reliability of the sequences. The testing of our Europe-wide collection of *P. alni* with the series of allele-specific PCR primers showed that, for each of the four nuclear genes, all the *Paa* isolates possessed three alleles, referred to as PAM1, PAM2, and PAU, whereas all the *Pam* possessed two alleles, PAM1 and PAM2 (Table 5). By contrast, all the *Pau* isolates possessed only single alleles (PAU). These results confirm the previous results obtained by sequencing with the 15-isolate panel.

Based on these specific PCRs, single isolates of *P. cambivora* were shown to possess more alleles for each gene than previously derived from sequencing and displayed a more complex allelic pattern than expected for this species (Table 5). The occurrence of two different alleles of both *TRP*1 and *RAS-Ypt* genes, of three alleles of the *GPA*1 gene, and of two to four alleles for the *ASF*-like gene, could be derived from allele-specific PCRs.

All the isolates of *P. fragariae* var. *rubi* and *P. fragariae* var. *fragariae* yielded only a positive signal with the primers derived from *Pff* or *Pfr* sequences, showing that these two varieties possessed single alleles of each of the four nuclear genes as well.

Finally, the series of allele-specific primers was tested with the collection of *Phytophthora* spp. and *Pythium* spp. listed in Table 1. None of the isolates yielded any PCR product with the allele-specific PCR primers, showing that the series of primers designed was specific to some of the three species studied here, *P. alni*, *P. cambivora*, and *P. fragariae*.

3.5. Sequence analysis for the mitochondrial genes

The mitochondrial gene sequences obtained with the limited six-isolate panel were aligned with the sequences of *cox*1 and *nadh*1 previously published by Kroon et al. (2004) for *P. alni* subsp. *multiformis* (isolate PD92/1471, formerly designated as *P.* hybrid-Dutch variant) (GenBank Accession Nos. AY564168 and AY563995, respectively), *P. fragariae* var. *fragariae* (GenBank Accession Nos. AY564177 and AY564178, and AY564004 and AY564005, respectively), and *P. fragariae* var. *rubi* (GenBank Accession Nos. AY564179 and AY564180, and AY564006 and AY564007, respectively).

Separate phylogenetic analyses were conducted for each mitochondrial gene using all the sequences obtained with *Paa*, *Pam*, *Pau*, and *Pc* and the retrieved sequences from

Pam, *Pff*, and *Pfr*. For the two mitochondrial genes, either a single or two nearly identical (1–3 substitutions) sequences were obtained for each individual isolate. For the two mitochondrial genes, the clusters regarding *P. alni* sequences were the same (Figs. 7A and B). Sequences from PAM54 and PAA130, if not identical, clustered together in the mtPAM cluster, along with sequences of *Pam* isolate PD92/1471 (Kroon et al., 2004), whereas another cluster called mtPAU gathered the sequences from PAU60 and PAA129. Sequences obtained from *P. cambivora* isolates significantly differed from those forming the two *P. alni* clusters. Moreover, sequences of isolates PC643 and PCJC17 were clearly separated in both phylogenetic trees. Sequences of *P. fragariae* retrieved from GenBank (Kroon et al., 2004) did not cluster with either *P. alni* or *P. cambivora*'s sequences.

4. Discussion

4.1. Allelic diversity within P. alni

This study performed on a large Europe-wide collection of *P. alni* isolates demonstrated that three different alleles of each of the four studied nuclear genes were present in the *P. alni* subsp. *alni* (*Paa*) genome. Two of these alleles, PAM1 and PAM2, were the same as those found in all the studied *P. alni* subsp. *multiformis* (*Pam*) isolates, whereas the third allele of each gene, i.e., PAU, corresponded to the single alleles found in all of the *P. alni* subsp. *uniformis* (*Pau*) isolates.

The presence of single alleles in all the *Pau* isolates and the low level of polymorphism observed suggest that this subspecies is probably close to homozygosity for the nuclear genes we studied. These data are consistent with this subspecies being near-diploid and with its high phenotypic and genetic uniformity (Brasier et al., 1999).

By contrast, two significantly divergent alleles (PAM1 and PAM2) of the four nuclear genes were observed in all the *Pam* isolates we studied. If, by extension, two alleles of most of the nuclear genes are present in *Pam*, then this subspecies should normally be near-tetraploid, which would be in disagreement with the ploidy levels ($2n+4$ to $2n+7$) determined with the acetoorcein method by Brasier et al. (1999). A first explanation is that *Pam* is homoploid, i.e., near-diploid for the genus *Phytophthora*, but heterozygous with two alleles, PAM1 and PAM2, as diploid orthologs, probably on homolog chromosomes. As a result, *Pam* could be a cryptic polyploid hybrid species

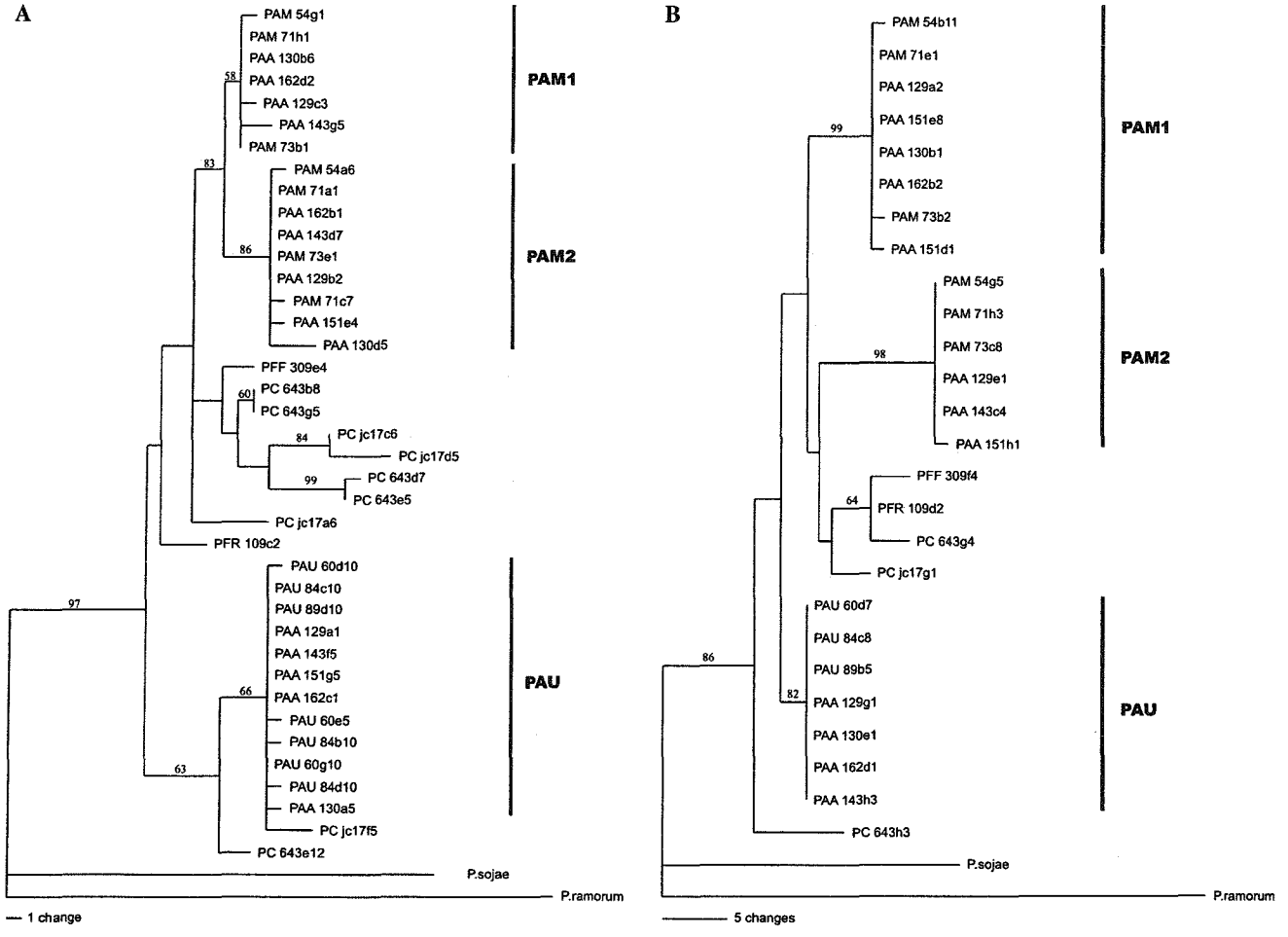

Fig. 2. Phylogenetic trees constructed using the parsimony method for each individual nuclear gene: *ASF*-like (A), *GPA1* (B), *RAS-Ypt* (C), and *TRP1* (D). Values given above the branches represent the bootstrap values from 1000 replicates; only values greater than 50% are shown. Clusters of similar *P. alni* sequences are defined on the right side of each tree.

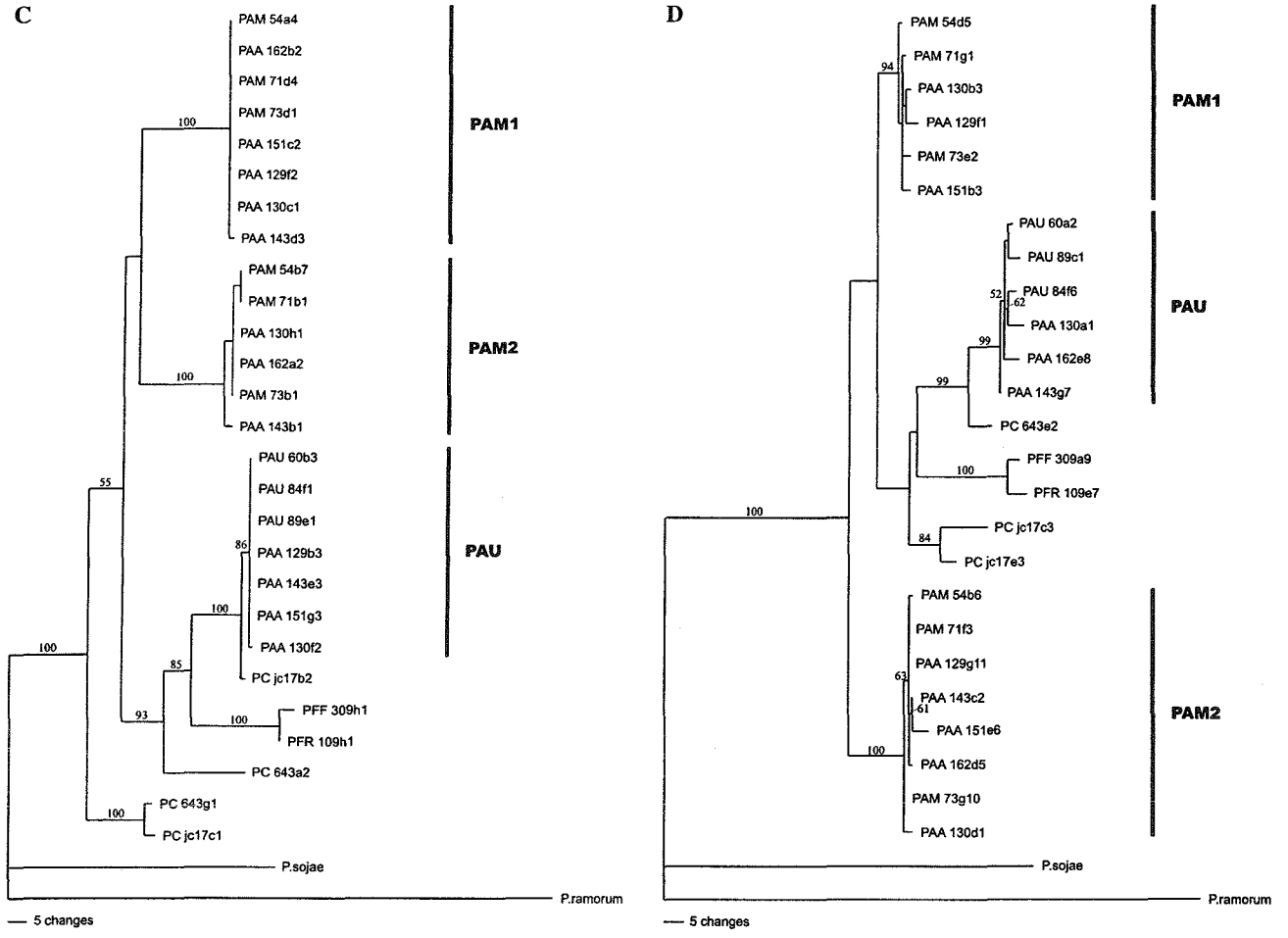

Fig. 2 (*continued*)

that regained a level of ploidy close to diploidy. However, *Pam* is homothallic and constant selfing should normally drive homothallic species to a homozygous state (Goodwin, 1997). Nevertheless, despite the homothallic nature of *Pam*, germinating oospores were not observed for any of the *P. alni* subspecies during an in vitro study (Delcan and Brasier, 2001) and, therefore, these species could be reproducing only asexually, which would maintain a high level of heterozygosity. A second hypothesis would be that the occurrence of two alleles of the studied nuclear genes in *Pam* has been generated by autopolyploidization. Indeed, the studied nuclear genes are not physically linked since they are located on different scaffolds in the *P. sojae* or *P. ramorum* genomes (Table 2). Therefore, the occurrence of multiple polymorphic sequences might not have arisen by gene duplication, generating paralogs, because the simultaneous duplication of four physically unlinked genes seems very unlikely. Finally, it cannot be inferred from our data whether or not the presence of two alleles of the four studied nuclear genes arose from an ancient autopolyploidization generating series of homologous alleles in a *Pam* ancestor, followed by divergent evolution between the two respective genes, or was generated by an ancient reticulation event such as interspecific hybridization.

The presence of three alleles in *Paa* and the level of divergence between their respective sequences, as high as the level of divergence between the ortholog sequences for each of the studied genes in *P. cambivora* and *P. fragariae*, are consistent with *Paa* being allopolyploid, as demonstrated by Brasier et al. (1999). Indeed, intraspecific polyploidization might not have generated three such divergent alleles and, therefore, the occurrence of multiple alleles in *Paa* would be the result of at least one reticulation. The three alleles are probably located on homoelogue chromosomes, following a reticulation event. Overall, the data from the nuclear gene are in full agreement with previous research using isozyme analysis. Nagy et al. (2003), as well as Brasier et al. (2004), revealed a trisomic state at the glucose-6-phosphate isomerase (*Gpi*) locus for *Paa*, which is consistent with the co-occurrence of three different alleles of the four nuclear genes. Likewise, heterozygosity at the *Gpi* locus for *Pam* and homozygosity at three different enzyme loci, including *Gpi*, for *Pau* (Brasier et al., 2004), are consistent with our findings of two and single alleles for *Pam* and *Pau*, respectively, for the four nuclear genes.

This multilocus analysis conducted on a Europe-wide collection of hybrids showed that the genome of the parental species has been conserved to a high degree in *Paa* and *Pam*. Thus, the co-existence of two and three alleles of most

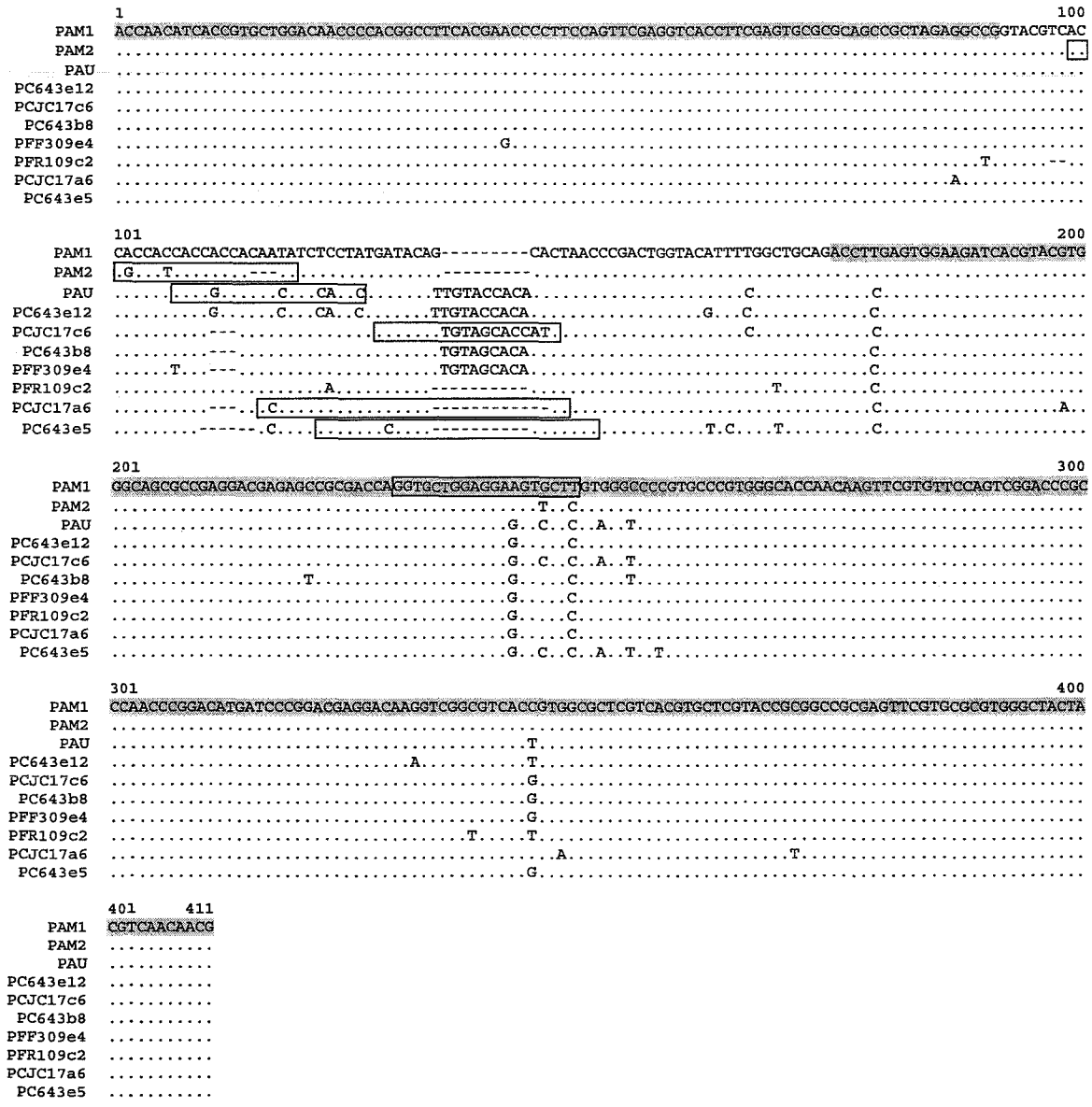

Fig. 3. Sequence alignment using the different groups of sequences collected from the 15-isolate panel for the *ASF*-like gene. Sequences are designated according to the clusters designed from the phylogenetic trees or the clone from which they are derived (see Fig. 2A). Boxed characters indicate the regions from which allele-specific primers could be designed. Shaded regions correspond to exons.

of the nuclear genes, probably located on respective homoelogue chromosomes, may be hypothesized. This co-existence does not mean that each allele is actually expressed, but may be an explanation why *Pam* and *Paa* are reported to be phenotypically more unstable than *Pau* (Delcan and Brasier, 2001; De Merlier et al., 2005), in which only single alleles were found. *Paa* is the most frequent subspecies in Europe at this time (Brasier, 2003; Ioos, unpublished results) and seems to propagate essentially by vegetative reproduction through the dissemination of zoospores. This subspecies cannot diversify by outcrossing, nor can it use meiosis to mitigate the accumulation of deleterious mutations known as Muller's ratchet (Muller, 1964). However, genetic redundancy in this hybrid and in *Pam*, to a lesser extent, might mask some deleterious mutations and may also enable the hybrid to be more fit in its ecological niche, i.e., to maintain itself as an aggressive pathogen on alder. For example, this type of selective advantage of hybrid species was demonstrated for a fungal endophyte species, *Neotyphodium coenophialum*, which also has three ancestors involved in two separate hybridization events (Moon et al., 2004). Based on the allelic distribution of the studied nuclear genes, it appears that (i) either the *Paa* genome contributed to the genomes of *Pam* and *Pau* by descent, or (ii) conversely, that *Pam* and *Pau* contributed to the genome of *Paa* by hybridization.

4.2. Dichotomous mitochondrial pattern within P. alni and relationship between the three subspecies

The mitochondrial DNA features combined with the different nuclear allelic patterns found indicate that *Pau*

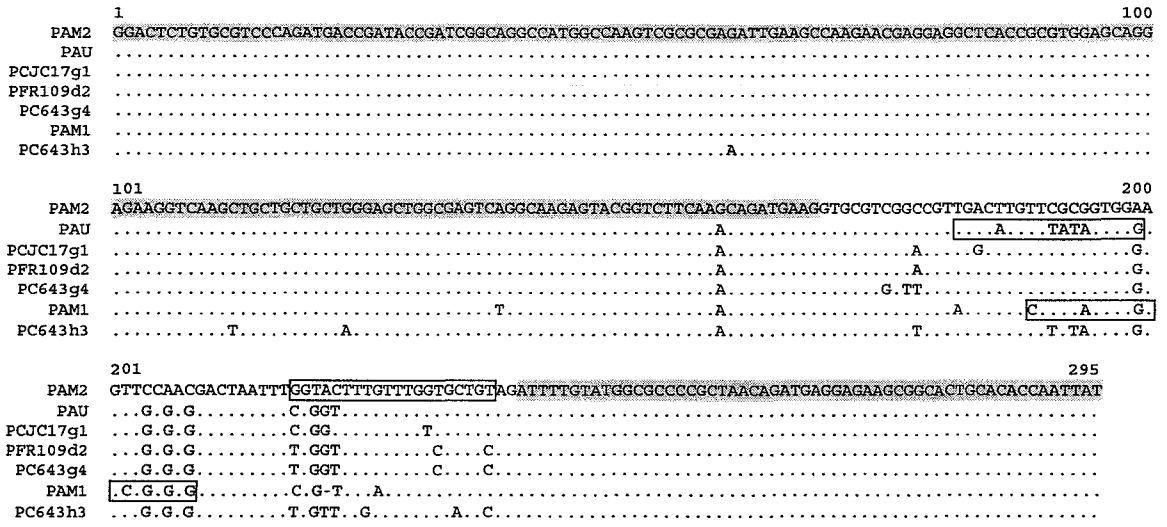

Fig. 4. Sequence alignment using the different groups of sequences collected from the 15-isolate panel for the *GPA1* gene. Sequences are designated according to the clusters designed from the phylogenetic trees or the clone from which they are derived (see Fig. 2B). Boxed characters indicate the regions from which allele-specific primers could be designed. Shaded regions correspond to exons.

and *Pam* might not have arisen from the genetic breakdown of *Paa*, as previously hypothesized by Delcan and Brasier (2001). Indeed, *Paa* isolates have all three different alleles of the nuclear genes we studied, and all display either an M/M′/M″ or a U/U′ mtDNA pattern. On the other hand, all the *Pam* isolates possess two of these alleles (PAM1 and PAM2) and display two closely related mtDNA patterns, M or M′, whereas all the *Pau* isolates possess single alleles (PAU), and display three closely related mtDNA patterns, U, U′, or U″. If *Pam* and *Pau* had originated from *Paa* through segregation, then other combinations of nuclear and mitochondrial DNA would be expected, which has not been the case so far.

The mitochondrial DNA analysis showed that all the *Paa* isolates tested displayed either a pattern identical to *Pam* isolates (M/M′/M″) or to *Pau* isolates (U/U′). The two mitotypes are present throughout Europe with no obvious geographical or sampling time pattern (Table 1). Moreover, further analyses carried out on additional French *Paa* isolates showed that the mitotypes M′ and U′ were also present in France (Ioos, unpublished data). Furthermore, the sequencing of the *cox*1 and *nadh*1 mitochondrial genes demonstrated the occurrence of a unique mitotype within *P. alni* isolates. Moreover, the sequences of individual *Paa* isolates, i.e., PAA129 or PAA130, cluster either with those of *Pau* (PAU60) or with those of *Pam* (PAM54), respectively, which is therefore fully consistent with the dichotomous distribution of the mtDNA patterns resolved by RFLP (see Table 1).

Some of the mtDNA RFLP patterns obtained with *Hae*III were identical to those obtained by Nagy et al. (2003) on the same subspecies; however, we obtained more patterns than previously described because we screened a broader collection of isolates. The occurrence of several closely related mtDNA patterns within each subspecies of *P. alni*, i.e., M, M′, and M″, on the one hand, and U, U′, and U″, on the other hand, is not surprising. Indeed, these patterns only differed by the size of one or two restriction fragments, which could be explained by insertion–deletion events and seems consistent with the level of intraspecific polymorphism already observed in other species such as *P. infestans* (Goodwin, 1991) or *P. parasitica* (Lacourt et al., 1994). By contrast, the two mitotype groups, M/M′/M″ and U/U′/U″, differed from each other by at least six restriction fragments for each enzyme, indicating a large divergence between the two mitotype groups.

Finally, the co-segregation of mtDNA along with nuclear patterns could also be explained either by linkage between mtDNA and the nuclear genes studied or by a poorer viability of the "missing" hybrids combining U/U′/U″ mtDNA and PAM1/PAM2 nuclear DNA or M/M′/M″ mtDNA and PAU nuclear DNA. However, linkage between mtDNA and four physically unrelated nuclear genes seems very unlikely, whereas poorer viability of the "missing" hybrids remains possible but difficult to demonstrate experimentally. Whittaker et al. (1994) proved that mtDNA was uniparentally inherited from A1 x A2 matings in *P. infestans* and that there was no evidence for biparental inheritance, recombination or segregation of mitotypes, regardless of the ages of the crossings. Later, Man in't Veld et al. (1998) demonstrated that only mtDNA from *P. nicotianae* was present in the natural hybrid *P. nicotianae* x *P. cactorum* isolates. Accordingly, we favor the most parsimonious hypothesis that *Paa* may have arisen via hybridization of two taxa close to *Pau* and *Pam*, if not *Pau* and *Pam* themselves. This hypothesis is well supported by other investigations conducted on a series of elicitin genes, which belong to a multigenic family encoding polypeptides specific to the genus *Phytophthora* and a few *Pythium* species (Ioos et al., in preparation). Moreover, since the

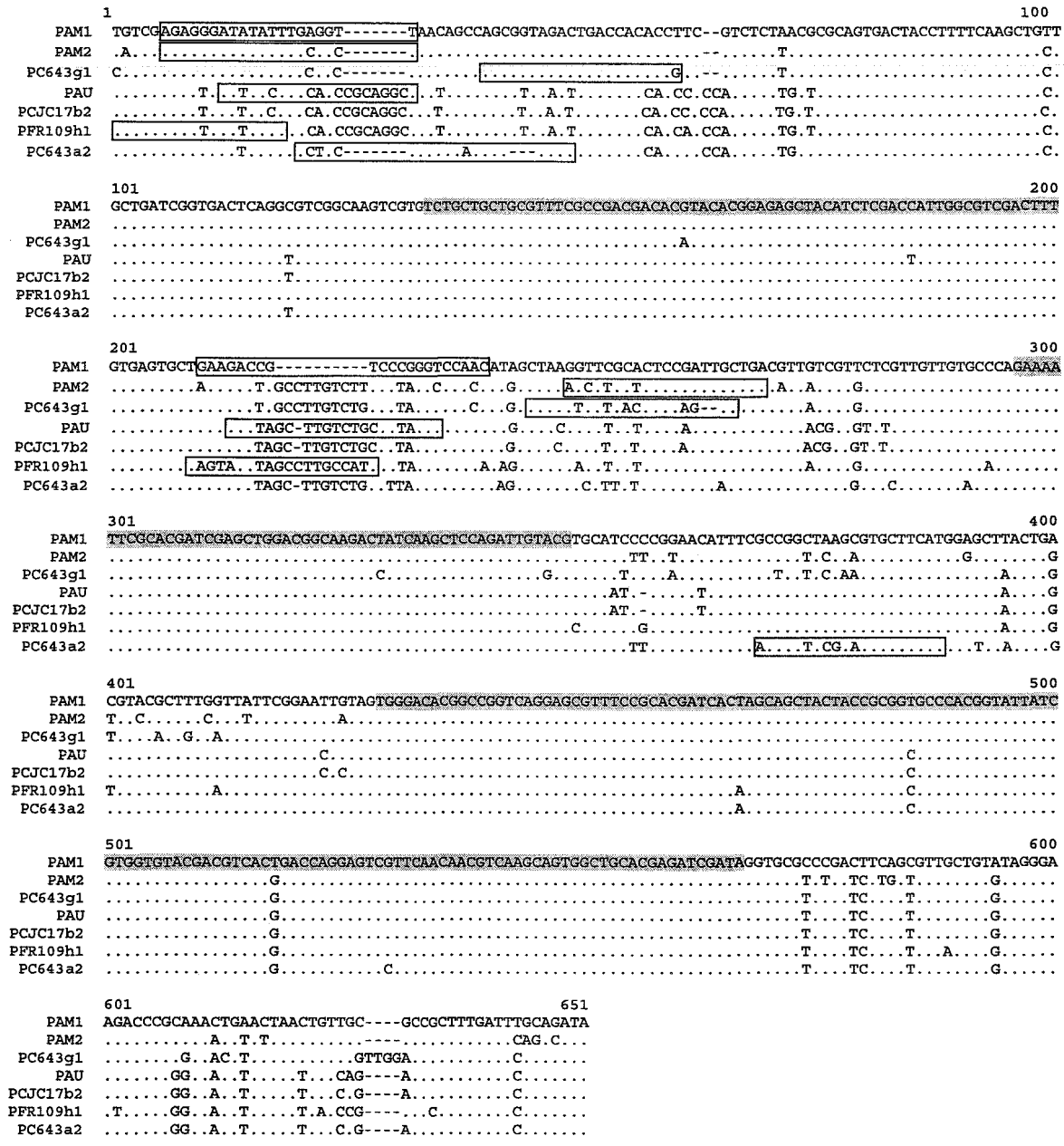

Fig. 5. Sequence alignment using the different groups of sequences collected from the 15-isolate panel for the *RAS-Ypt* gene. Sequences are designated according to the clusters designed from the phylogenetic trees or the clone from which they are derived (see Fig. 2C). Boxed characters indicate the regions from which allele-specific primers could be designed. Shaded regions correspond to exons.

mitochondrial DNA is only uniparentally inherited following crossing, this hypothesis might explain why *Paa* isolates display different mitochondrial DNA patterns, whereas they all possess the same three alleles for each of the four nuclear genes. From this point of view, sexual, rather than somatic, hybridization is more likely to have occurred.

Furthermore, the occurrence of significantly different mtDNA patterns among *Paa* isolates and the fact that these patterns are shared either with *Pau* or *Pam* also implies that different hybridization events might have occurred. Moreover, the uniparental inheritance of mtDNA and the occurrence of two groups of mitotypes in *Paa* suggest that the hybridization events may have occurred in both directions. Furthermore, different mtDNA patterns combined with intraspecific variation were observed among the *Paa* isolates that we gathered from different isolation dates. This suggests that the spread of this subspecies, within different countries and throughout Europe, might not be attributable to a single clone, as hypothesized by Brasier et al. (1995) and Nagy et al. (2003). The occurrence of multiple hybridization events has already been demonstrated for other natural *Phytophthora* hybrids between *P. nicotianae* and *P. cactorum*, probably generated in hydroponic systems of greenhouses in the Netherlands (Bonants et al., 2000).

Whether or not *Pau* and *Pam* are the direct progenitors of *Paa* cannot be inferred from our nuclear and

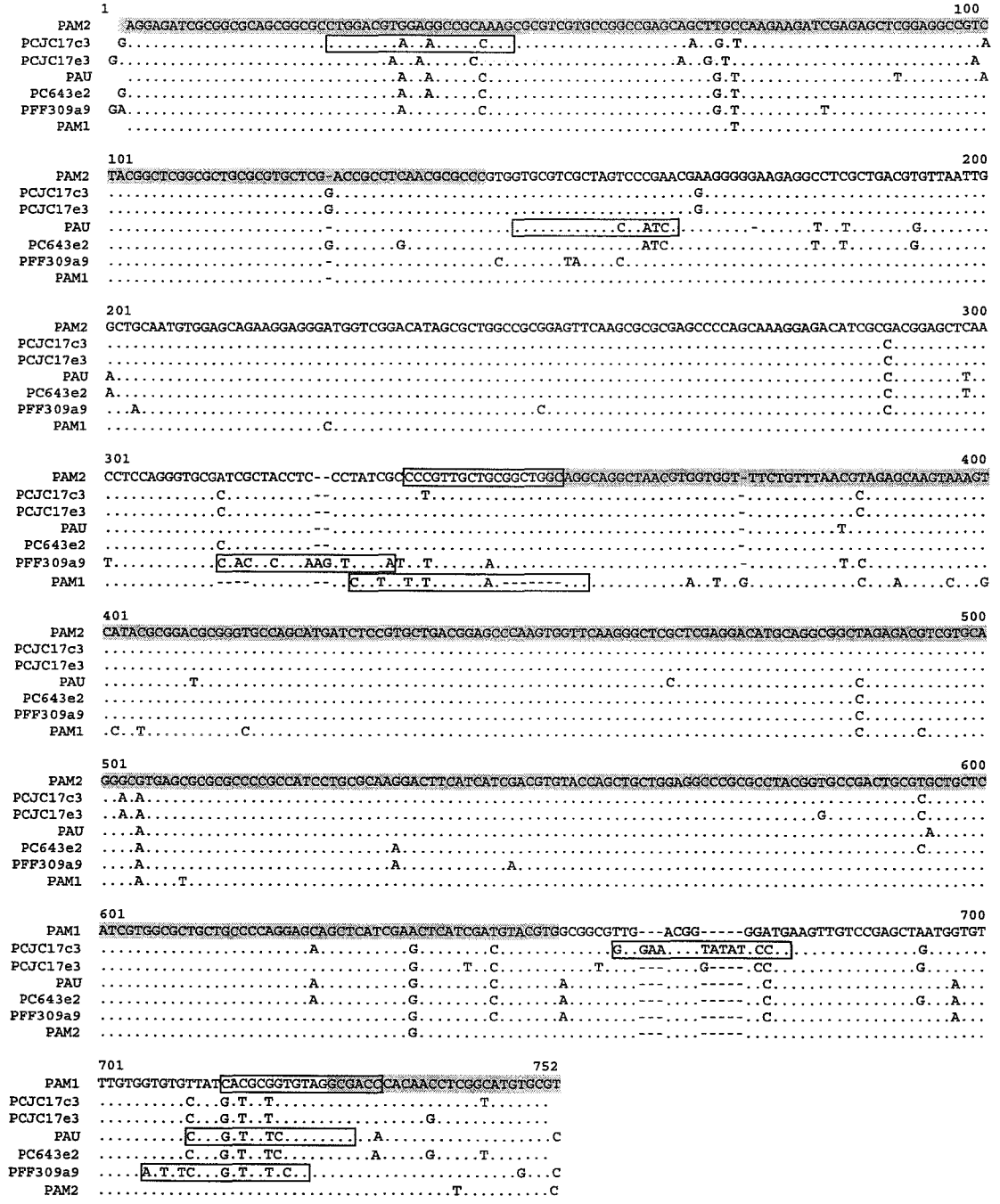

Fig. 6. Sequence alignment using the different groups of sequences collected from the 15-isolate panel for the *TRP1* gene. Sequences are designated according to the clusters designed from the phylogenetic trees or the clone from which they are derived (see Fig. 2D). Boxed characters indicate the regions from which allele-specific primers could be designed. Shaded regions correspond to exons.

mitochondrial investigations. *Pam* and *Pau* are far less frequently isolated from alder lesions than *Paa*, regardless of their geographical origins (Brasier et al., 2004) and have proved to be significantly less aggressive on alder bark than *Paa* (Brasier and Kirk, 2001). One of the first *Pam* isolates sampled in the Netherlands was found in 1995 in soil in a natural alder stand where none of the alders showed any symptoms of *Phytophthora* disease (Streito, 2003), while Santini et al. (2003) reported the isolation of *Pau* from asymptomatic alder seedlings. Therefore, *Pam* and *Pau*, or *Pam*- and *Pau*-like species, might have existed for a long time on—or in the vicinity of—alder trees before the recent emergence of large-scale decay in the European alder population. The occurrence of these species in the past might not have been noticed because of the lack of conspicuous symptoms or declines of whole trees.

Considering the wide extant hybrid zone where the different subspecies of *P. alni* disseminate, the introgression may presumably continue through further reticulation

Table 5
Occurrence and distribution of the different alleles for the four nuclear genes in the genome of the different subspecies of *P. alni*, *P. cambivora*, and the two varieties of *P. fragariae*, as inferred from allele-specific PCR tests

Species	*ASF*-like	*GPA1*	*RAS-Ypt*	*TRP1*
P. alni subsp. *alni*	ASF-PAM1 ASF-PAM2 ASF-PAU	GPA-PAM1 GPA-PAM2 GPA-PAU	RAS-PAM1 RAS-PAM2 RAS-PAU	TRP-PAM1 TRP-PAM2 TRP-PAU
P. alni subsp. *multiformis*	ASF-PAM1 ASF-PAM2	GPA-PAM1 GPA-PAM2	RAS-PAM1 RAS-PAM2	TRP-PAM1 TRP-PAM2
P. alni subsp. *uniformis*	ASF-PAU	GPA-PAU	RAS-PAU	TRP-PAU
P. cambivora	ASF-PAU ASF-PCJC17a6[a] ASF-PC643e5[b] ASF-PCJC17c6[a] *ASF-PC643b8*[c]	*GPA-PCJC17g1* *GPA-PC643h3* *GPA-PC643g4*	RAS-PAU[a] RAS-PC643g1 RAS-PC643a2[b]	TRP-PAU[b] TRP-PCJC17c3[a] *TRP-PCJC17e3*
P. fragariae var. *fragariae*	*ASF-PFF309e4*	*GPA-PFF309g4*	RAS-PFR109h1	TRP-PFF309a9
P. fragariae var. *rubi*	*ASF-PFR109c2*	*GPA-PFR109d2*	RAS-PFR109h1	TRP-PFF309a9
Phytophthora spp.	n.p.s.	n.p.s.	n.p.s.	n.p.s.
Pythium spp.	n.p.s.	n.p.s.	n.p.s.	n.p.s.

n.p.s., no positive signal when tested with all the available allele-specific primer pairs.
[a] Except isolate PC643.
[b] Positive only for isolates PC643 and PC463.
[c] Italics indicate alleles of genes obtained by cloning and sequencing, and for which PCR primers could not be designed.

events or backcrossings. Despite the fact that the *Paa*, *Pam*, and *Pau* isolates we tested only displayed a limited level of intraspecific nuclear polymorphism, the occurrence of new genotypes cannot be ruled out as a possibility. For instance, Jung and Blaschke (2004) and Brasier et al. (2004) reported the occurrence of additional major variants of *Pam* and minor variants of *Pau*, presumed to have been generated either by backcrossing or reassortments of the hybrid genome.

4.3. Relationship between the closely related P. alni, P. cambivora, and P. fragariae

Since no allele present in the two varieties of *P. fragariae* for the four nuclear and the two mitochondrial genes we studied was observed in any *P. alni* subspecies, our results confirm that this species is not among the parental species of *P. alni*, as previously suggested by Brasier (2003). Suggested to be one of the progenitors of *P. alni* by Brasier et al. (1999, 2004), *P. cambivora* was included in this study in order to unravel the relationship between the three subspecies of *P. alni*, only pathogenic on alder, and *P. cambivora*, pathogenic on numerous deciduous trees, but not on alder (Brasier and Kirk, 2001; Santini et al., 2003). Our results do not support the hypothesis that *P. cambivora* is directly involved as a parental species for *P. alni*. Although *P. cambivora* is undoubtedly close to *Pau* and *Paa*, with at least one allele of *P. cambivora* close to the allele shared by *Paa* and *Pau* for the four nuclear genes, there is never complete identity. Since the hybrid is believed to be in its nascent state (Brasier et al., 1999), one might expect to find alleles of the parental species for each nuclear gene in the hybrid genome. The series of PAU allele-specific primers designed in this study yielded a PCR product when tested with several isolates of *P. cambivora*. Nevertheless, sequencing of genes showed that within the PAU cluster, polymorphism occurred between the target regions of the two primers. In particular, *P. cambivora* sequences could be differentiated from *P. alni* sequences by several substitutions but, at the same time, could not be discriminated by PAU-specific PCRs. Therefore, our sequence data suggest that *P. cambivora* may not be directly involved as a parental species but it could still be one parental species of an ancient *Pau*-like species that has evolved. Nevertheless, considering the unexpected genetic variability among French isolates of *P. cambivora*, the involvement of another particular genotype of *P. cambivora*, either exotic or endemic to Europe, cannot be ruled out as a possibility. Further sequencing studies using the allele-specific primers designed in this study with a broader collection of *P. cambivora* isolates could be of great interest in order to validate such a hypothesis. *P. cambivora* isolates exhibited more alleles than expected for a typical diploid species, for example, with up to four different alleles of the *ASF*-like gene and two significantly different alleles of the *RAS-Ypt* gene. This is in agreement with previous observations based on ribosomal DNA where ITS polymorphisms were revealed within individual isolates (Brasier et al., 1999; Cooke and Duncan, 1997) and suggests that independent gene duplications generating paralogs might have occurred for that species and/or, that *P. cambivora* might have been involved in reticulation events in the past.

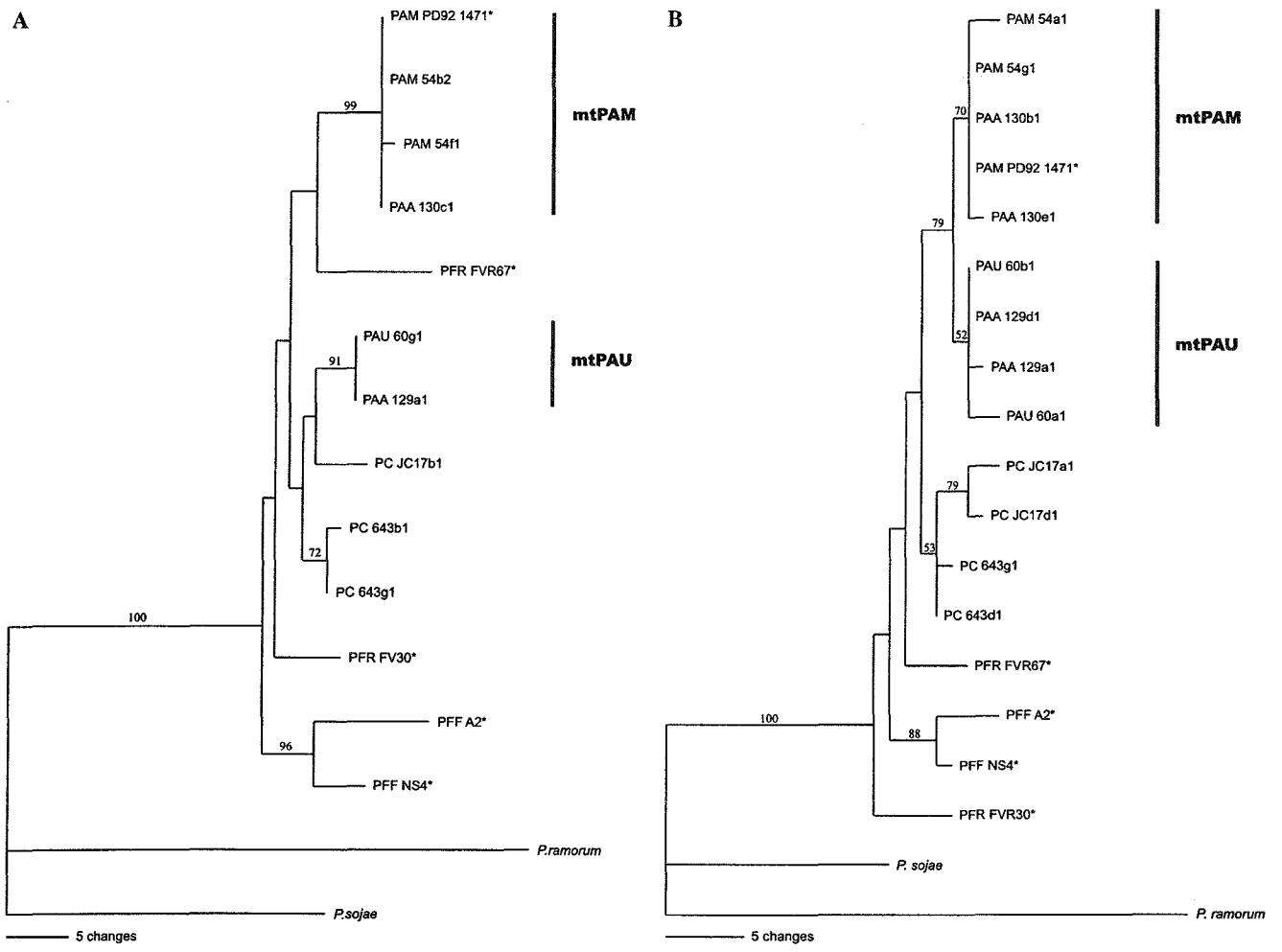

Fig. 7. Phylogenetic trees constructed using the parsimony method for each individual mitochondrial gene: *cox*1 (A) and *nadh*1 (B). Values given above the branches represent the bootstrap values from 1000 replicates; only values greater than 50% are shown. Sequences labeled with an asterisk are retrieved from the work of Kroon et al. (2004) and correspond to *cox*1 sequences for *Pam* isolate PD92/1471 (AY564168), *Pff* isolate A2 (AY564177), *Pff* isolate NS4 (AY564178), *Pfr* isolate FVR67 (AY564179), and *Pfr* isolate FVR30 (AY564180) (A), and to *nadh*1 sequences for *Pam* isolate PD92/1471 (AY563995), *Pff* isolate A2 (AY564004), *Pff* isolate NS4 (AY564005), *Pfr* isolate FVR67 (AY564006), and *Pfr* isolate FVR30 (AY564007) (B).

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References

Arnold, M.L., 2004. Natural hybridization and the evolution of domesticated, pest and disease organisms. Mol. Ecol. 13, 997–1007.

Bonants, P.J.M., Hagenaar-de Weerdt, M., Man in't Veld, W.A., Baayen, R.P., 2000. Molecular characterization of natural hybrids of *Phytophthora nicotianae* and *P. cactorum*. Phytopathology 90, 867–874.

Brasier, C.M., 2003. The hybrid alder *Phytophthora*: genetic status, pathogenicity, distribution and competitive survival. In: Gibbs, J.N., Van Dijk, C., Webber, J.F. (Eds.), *Phytophthora* Disease of Alder in Europe. Forestry Commission Bulletin No. 126. HMSO, Edinburgh, pp. 39–54.

Brasier, C.M., Kirk, S., 2001. Comparative aggressiveness of standard and variant hybrid alder Phytophthoras, *Phytophthora cambivora* and other *Phytophthora* species on bark of *Alnus*, *Quercus* and other woody hosts. Plant Pathol. 50, 218–229.

Brasier, C.M., Cooke, D.E.L., Duncan, J.M., 1999. Origin of a new *Phytophthora* pathogen through interspecific hybridization. Proc. Natl. Acad. Sci. USA 96, 5878–5883.

Brasier, C.M., Kirk, S.A., Delcan, J., Cooke, D.E.L., Jung, T., Man in't Veld, W.A., 2004. *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. Mycol. Res. 108, 1172–1184.

Brasier, C.M., Rose, J., Gibbs, J.N., 1995. An unusual *Phytophthora* associated with widespread alder mortality in Britain. Plant Pathol. 44, 999–1007.

Burnett, J.H., 1983. Speciation in fungi. Trans. Br. Mycol. Soc. 81, 1–14.

Chen, Y., Roxby, R., 1996. Characterization of a *Phytophthora infestans* gene involved in vesicle transport. Gene 181, 89–94.

Cooke, D.E.L., Duncan, J.M., 1997. Phylogenetic analysis of *Phytophthora* species based on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. Mycol. Res. 101, 667–677.

Cooke, D.E.L., Duncan, J.M., Williams, N.A., Hagenaar-de-Weerdt, M., Bonants, P.J.M., 2000. Identification of *Phytophthora* species on

the basis of restriction enzyme fragment analysis of the internal transcribed spacer regions of ribosomal RNA. EPPO Bull. 30, 519–523.

Delcan, J., Brasier, C.M., 2001. Oospore viability and variation in zoospore and hyphal tip derivatives of the hybrid alder Phytophthoras. Forest Pathol. 31, 65–83.

De Merlier, D., Chandelier, A., Debruxelles, N., Noldus, M., Laurent, F., Dufays, E., Claessens, H., Cavelier, M., 2005. Characterization of alder *Phytophthora* isolates from Wallonia and development of SCAR primers for their specific detection. J. Phytopathol. 153, 99–107.

Gibbs, J.N., 1995. *Phytophthora* root disease of alder in Britain. EPPO Bull. 25, 661–664.

Gibbs, J., Van Dijk, C., Webber, J., 2003. *Phytophthora* disease of alder in Europe. Forestry Commission Bulletin 126, Edinburgh, 82 pp.

Goodwin, S.B., 1991. DNA polymorphisms in *Phytophthora infestans*: the Cornell experience. In: Lucas, J.A., Shattock, R.C., Shaw, D.S., Cooke, L.R. (Eds.), *Phytophthora*. Cambridge University Press, Cambridge, pp. 256–271.

Goodwin, S.B., 1997. The population genetics of *Phytophthora*. Phytopathology 87, 462–473.

Ioos, R., Husson, C., Andrieux, A., Frey, P., 2005. SCAR-based PCR primers to detect the hybrid pathogen *Phytophthora alni* and its subspecies causing alder disease in Europe. Eur. J. Plant Pathol. 112, 323–335.

Ioos, R., Panabières, F., Andrieux, A., Frey, P. Overlapping elicitin genes patterns resolved within the hybrid oomycete *Phytophthora alni* and the related species *P. cambivora* and *P. fragariae*, in preparation.

Jung, T., Blaschke, M., 2004. *Phytophthora* root and collar rot of alders in Bavaria: distribution, modes of spread and possible management strategies. Plant Pathol. 53, 197–208.

Karlowsky, P., Prell, H.H., 1991. The TRP1 gene of *Phytophthora parasitica* encoding indole-3-glycerolphosphate synthase-*N*-(5′-phosphoribosyl) anthranilate isomerase: structure and evolutionary distance form homologous fungal genes. Gene 109, 161–165.

Kroon, L.P.N.M., Bakker, F.T., van den Bosch, G.B.M., Bonants, P.J.M., Flier, W.G., 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. Fungal Genet. Biol. 41, 766–782.

Lacourt, I., Panabières, F., Marais, A., Venard, P., Ricci, P., 1994. Intraspecific polymorphism of *Phytophthora parasitica* revealed by analysis of mitochondrial DNA restriction fragment length polymorphism. Mycol. Res. 98, 562–568.

Laxalt, A.M., Latijnhouwers, M., van Hulten, M., Govers, F., 2002. Differential expression of G protein alpha and beta subunit genes during development of *Phytophthora infestans*. Fungal Genet. Biol. 36, 137–146.

Man in't Veld, W.A., Veenbaas-Rijk, W.J., Ilieva, E., De Cock, A.W.A.M., Bonants, P.J.M., Pieters, R., 1998. Natural hybrids of *Phytophthora nicotianae* and *P. cactorum* demonstrated by isozyme analysis and random amplified polymorphic DNA. Phytopathology 88, 922–929.

Miller, P.M., 1955. V-8 juice agar as a general purpose medium for fungi and bacteria. Phytopathology 45, 461–462.

Moon, C.D., Craven, K.D., Leuchtmann, A., Clement, S.L., Schardl, C.L., 2004. Prevalence of interspecific hybrids amongst asexual fungal endophytes of grasses. Mol. Ecol. 13, 1455–1467.

Muller, H.J., 1964. The relation of recombination to mutational advance. Mutat. Res. 1, 2–9.

Munakata, T., Adachi, N., Yokoyama, N., Kuzuhara, T., Horikoshi, M., 2000. A human homologue of yeast anti-silencing factor has histone chaperone activity. Genes Cells 5, 221–233.

Nagy, Z.A., Bakonyi, J., Ersek, T., 2003. Standard and Swedish variant types of the hybrid alder *Phytophthora* attacking alder in Hungary. Pest Manag. Sci. 59, 484–492.

Olson, A., Stenlid, J., 2002. Pathogenic fungal species hybrid infecting plants. Microbes Infect. 4, 1353–1359.

Pinar, A., Ahkee, S., Miller, R.D., Ramirez, J.A., Summersgill, J.T., 1997. Use of heteroduplex analysis to classify Legionellae on the basis of 5S rRNA gene sequences. J. Clin. Microbiol. 35, 1609–1611.

Robin, C., Desprez-Loustau, M.L., Capron, G., Delatour, C., 1998. First record of *Phytophthora cinnamomi* on cork and holm oak in France and evidence of pathogenicity. Ann. Sci. For. 55, 869–883.

Santini, A., Barzanti, G.P., Capretti, P., 2003. Susceptibility of some mesophilic hardwoods to alder *Phytophthora*. J. Phytopathol. 151, 406–410.

Spitzer, E.D., Lasker, B.A., Travis, S.J., Kobayashi, G.S., Medoff, G., 1989. Use of mitochondrial and ribosomal DNA polymorphisms to classify clinical and soil isolates of *Histoplasma capsulatum*. Infect. Immun. 57, 1409–1412.

Streito, J.-C., 2003. Phytophthora disease of alder: identification and distribution. In: Gibbs, J.N., Van Dijk, C., Webber, J.F. (Eds.), *Phytophthora* Disease of Alder in Europe. Forestry Commission Bulletin No. 126. HMSO, Edinburgh, pp. 25–38.

Swofford, D.L., 2002. PAUP. Phylogenetic Analysis Using Parsimony. Sinauer Associates, Sunderland, MA.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Whittaker, S.L., Assinder, S.J., Shaw, D.S., 1994. Inheritance of mitochondrial DNA in *Phytophthora infestans*. Mycol. Res. 98, 569–575.

II.1.2. – Etude de la diversité et de l'expression de gènes de la famille des élicitines.

Les élicitines constituent un groupe d'éliciteurs protéiques sécrétés par la plupart des espèces du genre *Phytophthora* et quelques espèces de *Pythium* (Panabières et al., 1997). Elles ont la propriété d'induire une réaction d'hypersensibilité chez le tabac. Les gènes codant pour les élicitines sont parmi les gènes les plus exprimés chez *Phytophthora* (Kamoun et al., 1999 ; Panabières et al., 2005). La séquence des gènes d'élicitines de la classe I (« acides » et « basiques » ; Ponchet et al., 1999) et plus particulièrement leur partie 3' non codante (3'UTR , 3' UnTranslated Region) est considérée comme une signature de l'espèce chez *Phytophthora* (Panabières et al., 1997). Elle a par ailleurs été utilisée avec succès pour définir des amorces de PCR spécifiques de *P. nicotianae* (Lacourt et Duncan, 1997). Cette propriété de spécificité apparaissait donc prometteuse en termes taxonomiques pour étudier un phénomène d'hybridation. De plus, s'agissant de gènes fortement exprimés, il était intéressant d'étudier l'expression de ces gènes dans différents contextes génomiques d'hétéroploïdie. Dans cette deuxième approche, nous avons donc entrepris d'étudier la présence et la distribution de gènes codant pour des élicitines de classe I et II via le séquençage d'ARNm et une série de tests PCR 3'UTR sur une collection d'isolats de *Paa*, *Pam*, *Pau*, *P. cambivora* et *P. fragariae*.

Ce travail est résumé dans l'article suivant, soumis à publication :

<u>Publication 4</u> :

Ioos R., Panabières F., Industri B., Andrieux A., and Frey P. Overlapping elicitin genes patterns resolved within the hybrid oomycete *Phytophthora alni* and the related species *P. cambivora* and *P. fragariae*. Soumis à publication.

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Overlapping elicitin genes patterns resolved within the hybrid oomycete *Phytophthora alni* and the related species *P. cambivora* and *P. fragariae.*

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Abstract.

Phytophthora alni subsp. *alni* (*Paa*), *P. alni* subsp. *multiformis* (*Pam*) and *P. alni* subsp. *uniformis* (*Pau*) are responsible for alder disease in Europe. Class I and II elicitin genes patterns of *Paa*, *Pam*, *Pau* and the phylogenetically close species *P. cambivora* (*Pc*) and *P. fragariae* (*Pf*) were studied through mRNA sequencing and 3' untranslated region (3'UTR)-specific PCRs. The complementary elicitin patterns resolved confirmed the possible involvement of *Pam* and *Pau* or *Pam*- and *Pau*-like taxa in the genesis of the hybrid species *Paa*. The occurrence of multiple and common elicitin genes sequences throughout *Pc*, *Pf* and *P. alni*, not observed in other *Phytophthora* species, suggests that duplication of these genes occurred before the radiation of these species. The observation of multiple 3'UTR sequences associated to identical elicitin encoding sequences indicates duplication/recombination events. Last, the mRNA pattern displayed by *Paa* demonstrated that in this allopolyploid species, elicitin genes from all the parental genomes are actually expressed.

Index descriptors: acidic elicitin, basic elicitin, highly acidic elicitin, hybridization.

1. Introduction

The Oomycete *Phytophthora alni* is a recently described pathogen, specific to alder trees (*Alnus* spp.), that is spreading all over Europe, especially along rivers (Gibbs *et al.* 2003). *P. alni* is reported to be able to kill an alder tree within three years (Streito *et al.*, 2002). Once introduced in a healthy area, nothing can be done to prevent disease development, except cutting and burning the infested trees (Jung and Blaschke, 2004). Previously identified as a taxon similar to *P. cambivora* (Gibbs, 1995), this pathogen was further hypothesized to be a hybrid species involving *P. cambivora* and a taxon close to *P. fragariae* as putative parents (Brasier *et al.*, 1999). According to morphological, serological and genotypic traits, Brasier *et al.* (2004) formally described this new pathogen as *Phytophthora alni*, and recognized three subspecies: *P. alni* subsp. *alni* (*Paa*), *P. alni* subsp. *uniformis* (*Pau*) and *P. alni* subsp. *multiformis* (*Pam*), respectively. Furthermore, both Amplification Fragment Length Polymorphism (AFLP) and ITS analyses demonstrated that the different subspecies of *P. alni* were close to or lying between *P. cambivora* and *P. fragariae* (Brasier *et al.*, 1999). However, it was recently inferred from nuclear and mitochondrial characterization that *Paa* could actually have originated from hybridization between *Pau* and *Pam* or *Pau*- and *Pam*-like species (Ioos et al., 2006). *Phytophthora* species are typically diploid organisms but *P. alni* subsp. *alni* was shown to be near tetraploid (4n+2, n=18-22 chromosomes) (Brasier *et al.* 1999) and to hold an allopolyploid genome (Ioos et al., 2006). On the other hand, the respective ploidies for the two other subspecies, i.e. *Pau* and *Pam*, range from 2n+2 to 2n+7 (Brasier *et al.* 1999). Recent studies conducted on four independent single copy nuclear genes demonstrated that in *Paa* three different alleles of each gene are regularly observed, two of them being present in *Pam* while the third matched the single allele observed in *Pau* (Ioos et al., 2006). However, whether these alleles co-occurring in the allopolyploid *Paa* and the potentially homoploid *Pam* are actually expressed remains unknown.

Analysis of a collection of expressed sequence tags (ESTs) generated from *Phytophthora parasitica*, a worldwide polyphageous species, showed that a significant portion of these sequences corresponds to different members of the elicitin family (Panabières et al., 2005). Elicitin genes encode proteins restricted to the Oomycete genus *Phytophthora* and a few *Pythium* species (Panabières *et al.*, 1997; Jiang *et al.*, 2005). These proteins were first characterized as potential avirulence factors on the non-host tobacco (for a review, see Ponchet et al., 1999). Elicitins comprise a large family of proteins grouped in at least eight classes, whose intrinsic function remains largely unknown (Panabières et al., 2005). However, proteins of the class I, constituting by far the most abundant secreted elicitins, are known to bind lipids and sterols (Mikes et al., 1998; Osman et al., 2001). To date, more than 30 species of *Phytophthora* have been found to secrete class I elicitins (Ponchet *et al.* 1999). Although elicitin genes are not appropriate for phylogenetic studies, they can be used as a tool for identification purposes since the amino acid sequence of a given elicitin may provide a signature at the species level (Panabières *et al.*, 1997). In addition, the 3' untranslated regions (3'UTR) of class I elicitin genes are strictly conserved within an individual species, but diverge between species to such extent that sequence alignment is almost impossible (Duclos et al., 1998; Becker et al., 2000; Colas *et al.*, 2001; Panabières *et al.*, unpublished results).

The aim of the present work was first to confirm the hypothesis of involvement of *Pam* and *Pau* in the genesis of *Paa* using a new set of markers which were proven to be valuable for *Phytophthora* species discrimination. In consequence, we studied the occurrence and the distribution of members of class I and II elicitin genes among the different *P. alni* subspecies as well as in the phylogenetically close species *P. cambivora, P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi.* Second, these investigations on a gene family that is highly expressed in the *Phytophthora* genome provide data about the behaviour of additional genomes in natural allopolyploid *Phytophthora* species.

2. Materials and Methods.

2.1. *Phytophthora* isolates and culture.

French isolates of *Phytophthora alni* and other *Phytophthora* spp. were obtained by isolation from naturally infected tissues on PARBHY medium (Robin *et al.*, 1998). Foreign isolates of *P. alni* and *Phytophthora* spp. were obtained from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) or from collaborative researchers (Table 1). All the cultures were kept at 10°C in the dark on V8 agar slants (Miller, 1955) and as small V8 agar blocks flooded with sterile distilled water. Five isolates of *P. alni* subsp. *alni* (PAA129, PAA130, PAA143, PAA151, PAA162), three isolates of *P. alni* subsp. *uniformis* (PAU60, PAU84, PAU89), three isolates of *P. alni* subsp. *multiformis* (PAM54, PAM71, PAM73), two isolates of *P. cambivora* (PC643, PCjc17), one isolate of *P. fragariae* var. *fragariae* (PFF309) and one isolate of *P. fragariae* var. *rubi* (PFR109), chosen from different geographical locations, were used for *in vitro* elicitin production and mRNA studies (see Table 1).

2.2. Nucleic acid manipulation.

Genomic DNA was extracted from 5 day-old cultures grown in shake culture in liquid V8-juice medium (Miller, 1955) at 20°C, using a plant DNA extraction kit (DNeasy plant mini kit®, Qiagen, Courtaboeuf, France) and following the manufacturer's instructions with slight modifications. Briefly, ca 200 mg of fresh mycelium were harvested and mixed in a 2 ml tube with 400 µl of lysis buffer and 4 µl of the RNase provided with the kit. The mixture was ground for 2 min with two 3-mm tungsten carbide beads at a frequency of 30 Hz, using a mixermill grinder (Tissuelyser® Qiagen, Courtaboeuf, France). The ground solution was subsequently centrifuged for 5 min at 14000 rpm to compact the debris and the resulting supernatant was treated following the manufacturer's instructions. Genomic DNA was stored at -20°C until used for PCR tests.

To enhance elicitin mRNA synthesis, Oomycete cultures were grown for 3 days in shake culture in liquid elicitin secretion medium (ESM, Marília Horta, Algarve University, Portugal, pers. comm.) at 20°C. The composition of the ESM was: 0.05% (w/v) KH_2PO_4; 0.025% (w/v) $MgSO_4.7H_2O$; 0.1% (w/v) asparagine; 1 mg/l thiamine; 0.05% (w/v) yeast extract; 2% (w/v) glucose. The medium was sterilized by filtration through a 0.2 µm membrane. Messenger RNA was extracted using a QuickPrep micro mRNA purification kit with oligo (dT) cellulose (Amersham Biosciences, Orsay, France) following the

manufacturer's instructions, resuspended in 40 µl of DEPC-treated molecular biology grade water and stored at -80°C until RT-PCR.

2.3. RT-PCR, cloning and sequencing of elicitin mRNA.

Polyadenylated RNA was reverse-transcribed using a SuperScript™ First-Strand Synthesis System (Invitrogen, Cergy Pontoise, France), using the *Not*I-oligo(dT) (5'-ATTCGCGGCCGCAGGA$(T)_{16}$-3'). A PCR was performed on the cDNA-template using a combination of the *Not*I-oligo(dT) primer and the degenerate primer 1 (5'ATGAACTTCCGCGCTCTSYTYGC-3'), initially designed from conserved sequences of class I elicitins located in the peptide signal region (Panabières *et al.*, 1997). This primer was assumed to efficiently anneal to the peptide signal region of any elicitin class I gene unravelled until now. PCRs were carried out in a 20 µl PCR mixture containing 1X polymerase buffer (Sigma-Aldrich, L'Isle d'Abeau, France), 0.9 mM $MgCl_2$, 0.3 µM of each primer, 180 µM dNTPs, 0.6 unit of *Taq* DNA Polymerase (Sigma-Aldrich), and 2 µl of template cDNA. Molecular biology grade water was added to 20 µl. For each isolate, PCR amplifications were also performed using genomic DNA as a negative control to test potential amplification of genomic DNA in cDNA amplification. The amplification reactions were carried out with a Genamp 9700 thermalcycler (Applied Biosystems, Foster City, California). The cycling profile for PCR included an initial denaturation step at 95°C for 2 min followed by 35 cycles of denaturation, annealing and elongation for respectively 20 s at 94°C, 30 s at 60°C and 1 min at 72°C, and a final extension step at 72°C for 7 min. PCR fragments were separated, together with a 100 bp DNA ladder (Invitrogen, Cergy Pontoise, France), by a 1 hr electrophoresis, on a 1% agarose gel at 4 $V.cm^{-1}$. Gels were stained with ethidium bromide and images were recorded with a CCD camera and a GELDOC 2000® system (Biorad, Marne-La-Coquette, France).

The PCR products generated with cDNA were cloned for each of the 15 isolates tested using the pCR® 4-TOPO® - TA cloning kit (Invitrogen). Five microlitres of the PCR product were transferred into a sterile 1.5 ml microcentrifuge tube and the amplicons were ligated to a TOPO® vector (Invitrogen) and used to transform TOP 10® competent cells (Invitrogen) according to the manufacturer's instructions. Positive clones were selected by PCR amplifications of inserts with M13 sequencing primers. The PCRs were carried out directly with a suspension of transformed bacteria in ultrapure water. Positive clones were selected according to their expected PCR product size, corresponding to class I elicitin transcripts. PCR products were purified by centrifugation using a PEG-8000 solution and according to Rosenthal *et al.* (1993). Double-strand DNA sequencing was performed by the di-deoxy-chain termination method using a T3-T7 sequencing kit on a CEQ 2000 XL DNA sequencer (Beckman, Fullerton, California). Sequences were edited with Sequencher software (Gene Codes, Ann Arbor, Michigan) and aligned using ClustalW (Thompson *et al.*, 1994). Messenger RNA sequences were deposited on Genbank (Accessions DQ012508 to DQ012535, Table 1). cDNA sequences were translated using an on-line translation software (http://www.infobiogen.fr/services/analyseq/cgi-bin/forpub_in.pl). The isoelectric point of the deduced proteins was calculated using MWCALC on-line software (http://www.infobiogen.fr/services/analyseq/cgi-bin/mwcalc_in.pl). Multiple amino acid sequence

alignments with hierarchical clustering were performed using MultAlin program version 5.3.3 (Corpet, 1988), with Blossum 62 as symbol comparison table. Phylogenetic analyses and the construction of an unrooted phylogram tree were performed with PAUP version 4.0 (Swofford, 2002), using the Neighbor-Joining method.

2.4. 3'UTR-specific primer design and 3'UTR-specific PCR detection

Based on cDNA sequence alignment, a set of reverse primers was designed to target 3'UTR-specific regions among elicitin-encoding sequences (Table 2). Primers were synthesized by Invitrogen (Cergy Pontoise, France) and tested on the 15-isolate panel, then on the entire *Phytophthora* and *Pythium* collection listed on Table 1.

The 3'UTR PCR tests were carried out in a 20 µl mixture containing 1X polymerase buffer (Sigma-Aldrich), 1.8 mM $MgCl_2$, 0.45 µM of degenerate primer 1, 0.45 µM of 3'UTR specific primer, 180 µM dNTPs, 0.7 $\mu g.\mu l^{-1}$ Bovine Serum Albumin (BSA, Sigma-Aldrich), 0.6 unit of *Taq* DNA Polymerase (Sigma-Aldrich) and 2 µl of template genomic DNA or cDNA. Molecular biology grade water was added to 20 µl. PCR parameters were as indicated above except that the annealing temperature was lowered to 58°C. PCR products were resolved by agarose gel electrophoresis as described above.

3. Results

3.1. Cloning, sequencing and classification of elicitin-encoding sequences

A subset of 15 *Phytophthora* isolates (Table 1) was selected to study the elicitin pattern through mRNA analysis. Using a combination of oligo d(T) primer and a degenerated oligonucleotide designed after the 5' end of the elicitin coding sequence, RT-PCR of the mRNA extracted for each of these strains showed a strong smeared signal corresponding to a 450-550 bp product (Figure 1). For each isolate, the entire amplicon was cloned and 32 individual clones were randomly chosen and checked for insert size. Inserts ranged from ca 480 to 570 bp. Therefore, from each isolate, one to three clones of different sizes were selected for sequencing. A total of 28 different sequences were obtained with clones generated from the 15 isolates and were aligned along with two unpublished class I elicitin sequences from *P. cambivora* , isolate 143 (*PC_cam1*) and *P. fragariae* var. *rubi* isolate 486 (*PFR_fra1*) (F. Panabières, unpublished data).

Translation of the 28 cDNA sequences resolved 5 distinct proteins, which belonged to the class I (98 aa) and class II elicitins (99 aa)(Figure 2), according to the classification of Ponchet et al. (1999).

Two protein sequences were deduced from five cDNA clones, including *PC_cam1*, and were both identified as basic elicitins, after searching for homologies in public databases. They displayed a predicted isoelectric point (pI) of 8.22 and several key signatures such as the K13 frequently observed in basic elicitins (Ponchet et al., 1999). The two proteins only differed by a single A84V substitution and were therefore designated as Pa_BE1 and Pa_BE2 (*Phytophthora alni* Basic Elicitin), respectively. Pa_BE1 was identified in cDNAs from isolates PAM71, PAM73 and PAA162, in addition to *PC_cam1*, whereas Pa_BE2 was deduced from a single cDNA originating from isolate PAA151.

Limiting the sequence alignment to the 98 aa core-region that corresponds to the mature elicitin (excluding the 20 aa peptide signal sequence for secretion) allowed the identification of two other proteins differing by a single S40A substitution, among a set of 23 sequences. From alignment with published sequences, and from their predicted pI of 4.99, they were clearly identified as acidic elicitins, and named as Pa_AE1 and Pa_AE2, after the nomenclature used for the basic elicitin. Pa_AE1 and Pa_AE2 were encoded by 18 and five cDNA clones, respectively. Extending the comparison to the entire amino acid sequence further resolved Pa_AE1 into two proteins, which differed by a single S17F mutation in the peptide signal sequence, and were designed Pa_AE1.1 and Pa_AE1.2, respectively. Pa_AE1.1 was deduced from nine cDNA sequences obtained from isolates PAM54, PAU84, PAU89, PAA129, PCjc17, PC643, and PFR109. It also corresponded to the *PFR_fra1* sequence already determined (F. Panabières et al., unpublished). Pa_AE1.2 was only encoded by seven cDNA clones from *Paa* isolates, namely PAA130, PAA143, PAA151 and PAA162. Lastly, Pa_AE2 was deduced from cDNA clones obtained from isolates PAM71, PAM73, PAA129 and PFF309.

Finally, a third type of elicitin protein was identified. The deduced peptide would comprise 119 aa, including the peptide signal sequence, and displayed a predicted pI of 3.95. From alignment with known elicitin sequences, it was considered to belong to the class II of Highly Acidic Elicitins and was accordingly called Pa_HAE1. Pa_HAE1 was deduced from two cDNA clones found in isolates PAM54 and PAU89.

The five protein sequences were aligned with a set of representative elicitin sequences publicly available. The subsequent phylogenetic reconstruction confirmed the classification into acidic, basic and highly acidic elicitins (Figure 3). However, the three acidic elicitins possess a histidine residue at position 89, that is unprecedented in class I and class II elicitins identified so far, with the exception of an elicitin-related sequence in the close relative *Pythium oedochilum* (Panabières et al., 1997). To date, highly acidic elicitin genes have been only described as clustered with basic elicitin genes in *P. cryptogea* (Panabières et al., 1995) and *P. cinnamomi* (Duclos et al., 1998). They would encode 102 or 103 residues, and were characterized by an important negative net charge (-7 to -10). The HAE peptide identified in *P. alni* is only 99 aa long, and displays a net charge of -5. However, this is the first report of a gene of this class to be expressed.

3.2. Elicitin-encoding mRNAs display important 3'UTR variability

The diversity of elicitin-encoding sequences was investigated at the nucleotide level, both on the coding regions (Figure 4) and on the 3' untranslated regions (Figure 5). The sequences of 3'UTR were shown to be more variable than coding sequences, and are considered as a signature of a given species (Ponchet et al. 1999).

First, the two sequences encoding the putative HAE elicitin were identical, including the 3'UTR. This group of sequences was consequently designated as ha1 (Figure 5).

Second, sequences encoding basic elicitins were highly similar, diverging by 1-4 synonymous substitutions and by a C/T transversion leading to the A84V substitution in the coding region. In addition, the 3'UTRs of the different clones derived from *P. alni* were strictly identical over the

entire168 bp region, with the exception of a single G/C transversion in the transcript obtained from isolate PAA162. The *PC_cam1* sequence was more divergent, displaying 8 synonymous substitutions in the coding region, and 14 substitutions as well as a 5 bp deletion in the 3'UTR. This 3'UTR sequence group was called b2, to distinguish it from the group of 3'UTR sequences identified in *P. alni*, which was called in return b1 (Figure 5). It has to be noted that clones grouped as b1 were identical at the nucleotide level, but display variation in length, with respect to the location of the poly (A) tail. As this variation may be an outcome of experimental conditions during cDNA synthesis, it was not considered further in subsequent analyses, and these sequences were considered as corresponding to a single 3'UTR.

Last, for acidic elicitins, 11 clones encoding Pa_AE1.1 and the seven cDNA clones encoding Pa_AE1.2 were examined. They displayed extensive conservation in the coding region, as only three synonymous mutations further differentiated the two groups, while an additional transversion was observed in some, but not all, cDNA clones encoding Pa_AE1.1. Finally, only six variable sites were observed over a 357 bp-length, which could be interpreted as either sporadic mutations or base-calling events. Alignment of the five cDNA sequences encoding Pa_AE2 clearly separated the three *P. alni*-derived sequences and the two sequences observed for *P. fragariae*. These latter clones differed from *P. alni* by 10 synonymous mutations in the 357 bp- coding region, while the diversity within *P. alni* was only one or two synonymous mutations. The situation was quite different for the 3'UTRs. As a rule, they were more diverse, and could be classified into 11 groups, called a1 to a11. The diversity was the outcome of frequent insertions and deletions (indels) as well as single nucleotide polymorphisms (SNPs). Sequences generated from *P. cambivora*, *P. fragariae* and from the different *P. alni* subspecies constituted distinct groups. The 3'UTRs from *P. alni* fell into five groups. Group a1 was found in five cDNA clones from all subspecies of *P. alni*, and a3 was found in two cDNA clones from *Paa* and a single cDNA from *Pam*. Group a2 was found in four cDNA clones from *Paa* isolates, while two sequences isolated from one *Pam* and one *Paa* isolates were designated as a4 and a5, respectively. The four sequences from *P. cambivora* were designated as a6, a7 and a8, while those obtained in *P. fragariae* were designated as a9, a10 and a11, respectively (Figure 5).

Overall, there was no obvious correlation between the classification of 3'UTRs and the protein sequence deduced from the coding region (Figure 5). Hence, Pa_AE2 was deduced from cDNA clones possessing 3'UTRs classified as a3, a4, a9 and a11. Similarly, transcripts encoding Pa_AE1.1 possessed 3'UTR classified as a1, a2, a6, a9 and a10, while Pa_AE1.2 possessed 3'UTR classified as a1, a2, a3, a4 and a5. As a consequence, a given 3'UTR may be associated with two different protein sequences. As an example, a1 and a2 3'UTR were associated with both Pa_AE1.1 and Pa_AE1.2; a3 was associated with Pa_AE1.2 and Pa_AE2, a9 was associated with Pa_AE1.1 and Pa_AE2, while b1 was associated with both Pa_BE1 and Pa_BE2 (Figure 6).

3.3 Expression of the different elicitin genes in the 15-isolate panel

The large diversity observed among elicitin genes could reflect individual variation and subsequently the overall heterogeneity within *P. alni*. Alternatively, it may only reflect the complexity of the elicitin gene family in *P. alni*. As the sequences originated from different isolates, we intended to investigate

the occurrence of the whole set of genes revealed by RT-PCR in all isolates of the panel, through 3'UTR-specific amplifications. Specific primers could be designed for most, but not all, 3'UTR sequences and amplifications were carried out on the cDNAs from the 15 isolates of the panel (Table 3). For *P. alni*, amplification results were identical for all isolates of a given subspecies, suggesting that the diversity observed in the elicitin-encoding sequences does not correspond to individual variation, but rather to a high level of elicitin complexity among the subspecies of the complex.

A fragment corresponding to ha1 3'UTR was detected in all isolates of the *P. alni* complex, as well as *P. fragariae sensu lato.* In contrast, it was not amplified in any of the *P. cambivora* cDNAs. Using the cDNA from *Pam* isolates as templates, only b1 was detected, whereas b2, first characterized in a *Paa* strain (PAA151), was detected in all *Pc* isolates and in the *Pau* isolates. Both b1 and b2 were amplified from cDNAs of the *Paa* isolates.

The distribution of genes encoding acidic elicitins was variable between the different subspecies of *P. alni*. The *Pau* isolates appeared to express only genes containing a8 and a10, in addition to a1, which was identified through sequencing RT-PCR products. The a2, a3, a4, a5 and a8 3'UTR could be detected in all *Pam* isolates (an a1-specific primer combination could not be designed). All five isolates of *Paa* possessed a pattern that combined those observed in the *Pam* and the *Pau* isolates. In contrast with *P. alni*, analysis of *P. cambivor*a and *P. fragariae* isolates revealed a patchy distribution of the various acidic elicitin-related 3'UTRs. a8 3'UTR was detected in the two strains of *P. cambivora* (the sequence of a6 previously obtained from these strains did not allow the design of specific primers), but only isolate PCjc17 appeared to possess additional 3'UTRs, namely a2, a7 and a10 3'UTR. Similarly the strains from *Pff* and *Pfr* shared a2 and a9 3'UTR (whose sequence did not allow the design of specific primers), but only the *Pfr* isolate possessed an additional gene containing a10, while a11 appeared to be specific to the *Pff* isolate.

From these experiments, the *P. alni* complex appeared to display a high degree of genetic organization on the basis of the distribution and expression of elicitin genes, while *P. cambivora* and *P. fragariae* display a looser structure. Moreover, the pattern observed in *Paa* corresponds to the addition of *Pam* and *Pau* patterns.

3.4 Distribution of elicitin genes within *P. alni* and its relatives

As detection of various 3'UTR sequences was carried out on cDNAs, it only gave information on the nature of elicitin genes actually expressed in the standard culture conditions used (see methods), and did not reflect the exact nature of elicitin gene distribution. The analysis was then extended to a large set of 101 *P. alni*, *P. cambivora* and *P. fragariae* isolates, including the 15-isolate panel, as well as other *Phytophthora* and *Pythium* species (see Table 1). The 3'UTR-specific amplifications were performed on genomic DNA using the same primer combinations (Table 4). As a rule, these results are in total agreement with those obtained on the cDNAs from the 15-isolate panel, and the distribution of the various acidic elicitin-related 3'UTRs was identical in both experiments, indicating that the whole elicitin gene content is actually expressed in the current culture conditions. As a single exception, ha1 was amplified from the genomic DNA of *P. cambivora* isolates, whereas it was

not detected in the cDNA, indicating that this class of genes is not expressed during vegetative growth.

3'UTR-specific PCR did not give any positive signal in other *Phytophthora* or *Pythium* species, confirming that the primers designed in this study are specific of *P. alni*, *P. cambivora* and *P. fragariae*, and that these three species (or species complex) share particular evolutionary relationships, as already mentioned (Brasier et al., 1999; Ioos et al., 2006).

4. Discussion

In order to provide additional data concerning the hybrid status of *P. alni* subsp. *alni*, we studied the expression and distribution of elicitin genes within this taxon. The phylogenetically close *P. cambivora* and *P. fragariae* as well as the two other subspecies of *P. alni* were also included as they were hypothesized to represent the parental species of the hybrid (Brasier et al., 1999; Ioos et al., 2006). Elicitin genes and especially their 3' untranslated region have been reported as valuable species-specific markers (Panabières et al., 1997; Ponchet et al., 1999). In this respect, the occurrence and the distribution of these genes were expected to reflect the relationships between these three species.

4.1. Elicitin diversity observed and comparison to other *Phytophthora* species

In this study, different elicitin genes from *P. alni*, *P. cambivora* and *P. fragariae* were identified following cloning and sequencing of corresponding cDNAs. A first group of gene encodes slightly different proteins, called Pa_AE1.1, Pa_AE1.2 and Pa_AE2. They differ from each other by a single amino acid replacement. From their predicted isoelectric point and characteristics of their sequence, they appear to be typical class I, acidic elicitins (Kamoun et al., 1997; Ponchet et al., 1999). However, these three proteins share an unusual histidine residue which has been observed only once in an elicitin-encoding cDNA of *Pythium oedochilum* (Panabières et al., 1997). The restriction of these histidine-containing proteins to the *P. alni* complex, *P. cambivora* and *P. fragariae sensu lato* confirms the particular evolutionary relationships between these species, previously documented (Brasier et al., 2004; Ioos et al, 2006). A second group of genes encodes two slightly different proteins called Pa_BE1 and Pa_BE2, which are typical of class I basic elicitins. A third group of elicitin genes was found in all species and encodes a protein corresponding to the class II, highly acidic elicitins, according to their amino acid sequence and predicted isoelectric point. Highly acidic elicitin genes were first characterized in elicitin gene clusters of *P. cryptogea* (Panabières et al., 1995) and *P. cinnamomi* (Duclos et al., 1998). They have been recently identified in *P. sojae* (Jiang et al., 2005). They would encode 102 or 103 residues, were characterized by a strong negative net charge (-7 to -10), and are not expressed during vegetative growth (Panabières et al., 1995). They are physically linked to genes encoding basic elicitins in *P. cryptogea* and *P. cinnamomi* and appear to be absent from species that possess only acidic elicitins, which constitute the majority of *Phytophthora* species. As *P. sojae* has been shown to possess both basic and acidic elicitins (Mao and Tyler, 1996), a

hypothesis is that the occurrence of class II genes is correlated to the presence of basic elicitins. So, one might expect to find this class of genes in *P. cambivora*, but not in *P. fragariae*, which only possesses acidic elicitins (Ponchet et al., 1999). The presence of a highly acidic elicitin gene in *P. fragariae sensu lato*, and its expression in this species as well as *P. alni* is therefore surprising. However, the corresponding protein is only 99 aa long, and displays a net charge of -5. It may then represent an exception among the class II gene family.

Although elicitin amino acid sequences may be, as a general rule, a signature at the species level (Panabières et al., 1997), Ponchet et al. (1999) have shown that two phylogenetically related species, e.g. *P. cactorum* and *P. pseudotsugae*, share similar acidic elicitins (i.e. cactorein and pseudotsugaein, respectively). The present work extends this observation, as Pa_AE1.1 is present in the three subspecies of the *P. alni* complex, *P. cambivora* and *P. fragariae* var. *rubi*, while Pa_AE2 was found in *P. fragariae* var. *fragariae*, as well as in *Paa* and *Pam*. *P. cambivora* is akin to *P. fragariae* (Cooke et al., 2000) and Brasier et al. (2004) showed that *Paa* was close to or between these two species. The occurrence of common acidic elicitins, displaying some unusual features, reinforces the idea that these three species are tightly related.

4.2. Complementary and overlapping elicitin gene patterns among the *P. alni* complex.

The results of the 3'UTR-specific PCRs indicate that the elicitin gene patterns for the different subspecies of *P. alni* are overlapping. Overall, the genomic elicitin pattern of *Paa* represents the exact juxtaposition of those of *Pam* and *Pau*, and is fully consistent with the allelic distribution observed for four unlinked nuclear genes within the three subspecies (Ioos et al., 2006). These results reinforce the hypothesis that *Pam* and *Pau*, or *Pam*- and *Pau*-like taxa could be the progenitors of the hybrid *Paa* (Ioos et al., 2006). In addition, while *Pau* shared several 3'UTRs with *P. cambivora*, *Paa* and *Pam* possessed "private" group of sequences, e.g. a3, a4, and b1. These sequences were neither found in *P. cambivora* nor in *P. fragariae*. These findings confirm the close relationship between *P. cambivora* and *Pau* (Brasier et al., 1999; Ioos et al., 2006). Conversely, both *P. cambivora* and *P. fragariae* display specific sequences not found in *Paa*, in agreement with previous results rejecting the hypothesis that these species may have been the putative parents of the hybrid *P. alni* subsp. *alni* (Ioos et al., 2006).

4.3. *P. alni*, *P. cambivora* and *P. fragariae* share common elicitin genes

The 3'UTRs of class I elicitin genes are reported to display extensive variability among different species of *Phytophthora* (Duclos et al., 1998; Becker et al., 2000; Colas et al., 2001; Panabières et al., unpublished results). Mining the genomes of *P. sojae* (http://genome.jgi-psf.org/sojae1/sojae1.home.html) and *P. ramorum* (http://genome.jgi-psf.org/ramorum1/ramorum1.home.html) reveals that a unique 3'UTR is always associated with a single elicitin gene, even when present as multiple copies in the genome (data not shown). Recent data analyses of EST sequences from *P. sojae* and *P. infestans* also confirmed that duplication of certain class I elicitin genes did not affect the respective 3'UTR sequence (Jiang et al., 2005). In contrast with these observations, the analysis of the mRNAs produced by *P. cambivora*, *P. fragariae*,

and *P. alni* showed that a given coding region may be associated with an array of divergent 3'UTR sequences within the genome of a single species, but also within the genome of individual isolates (Figures 4, 5). Moreover, this study demonstrates for the first time that a given 3'UTR may be associated with several coding regions (Figure 5 and 6 and Table 4).

Genes encoding basic elicitins have been found to be restricted to a few, mainly nonpapillate species (Ponchet et al., 1999), now including *P. cambivora*, *P. fragariae* and *P. alni*. *P. cambivora* is a broad host range pathogen on woody plants, whereas the host range of *P. fragariae* var. *fragariae*, *P. fragariae* var. *rubi* and *P. alni* are restricted to single hosts or to a narrow host range, i.e., strawberry, *Rubus* spp. and *Alnus* spp., respectively. Our results confirm that the host specialization is not a phylogenetic criterion, and that the elicitin pattern is not related to the host range (Ponchet et al., 1999). On the other hand, the different subspecies of *P. alni*, *P. cambivora* and *P. fragariae* seem to have retained identical genes independently of the speciation. This has been partially shown for *P. cactorum* and *P. pseudotsugae* (Ponchet et al., 1999) but cactorein and pseudotsugaein genes possess distinct 3'UTR sequences (F. Panabières, unpublished data). Jiang et al. (2005) demonstrated that the main diversification events of elicitin genes occurred before *Phytophthora* radiation, and that elicitin genes of a given clade (such as those analyzed in the present work) are under purifying selection. In this respect, the duplication of elicitin genes creating paralogs, could also explain the multiplicity of divergent 3'UTR sequences for a given elicitin gene, with the assumption of a lower selection pressure on this part of the gene. It is likely that the diversity of elicitin genes in *P. cambivora*, *P. fragariae* and in *P. alni* reflects duplication events prior to radiation of these species from their common ancestor. It would also indicate that the radiation of these species is of particularly recent origin, which seems to be supported by the cross-amplification of microsatellite markers in all three species (Ioos et al., in preparation). However, the unexpected conservation of several 3'UTR regions for these different species (e. g. ha1, b2, a1, a2, a8, a10) could also be the outcome of reticulation or introgression events. Gene transfers after speciation should not be ruled out as a possibility to explain the elicitin gene patterns observed within this clade.

More unexpected, from an evolutionary point of view, is the association of a given 3'UTR with several coding regions, since a higher selection pressure is assumed to have been exerted on this latter part of the genes. In this respect, it may be hypothesized that duplication may have been followed by recombination with coding sequences from paralogs.

Beyond giving new insights on the genesis and evolution of the *P. alni* complex, the present work may have implications in diagnosis activities. The 3'UTR specific PCR primers designed from sequences common to *P. cambivora*, *P. fragariae* and *P. alni* did not lead to amplification in any other *Phytophthora* species. This specificity confirms the usefulness of elicitin genes for identification purposes, but also demonstrates that molecular tools developed from these genes should be carefully tested for specificity as we demonstrated here that the elicitin genes, including the 3'UTR, could be shared by phylogenetically close species.

4.4. Expression of the elicitin genes in an allopolyploid genome.

Taken as a whole and inferred both from sequence data and from 3'UTR specific PCR amplifications, the acidic elicitin gene family is more diverse in *Pam* than in *Pau*. This is consistent with the hypothesis that *Pam* is probably an ancient allo- or auto-polyploid species. In contrast, relatively high homozygosity was reported for *Pau* in a study conducted on single copy nuclear genes (Ioos et al., 2006). Likewise, the mRNA pattern observed in the allopolyploid hybrid *Paa* is a composite of the mRNA patterns from *Pam* and *Pau*. In this respect, we may hypothesize that at least at the elicitin gene level, the different genomes are currently expressed in the hybrid *Paa*. This observation is in good accordance with the probable recent origin of *Paa*. Garcia-Olmedo et al., (1978) and Volkov et al. (1999) showed that in the case of plant protein-coding genes, the loci of one parental species are mainly transcriptionally active in polyploid species, whereas loci from the other parent are gradually transformed into pseudogenes. If the recent origin of *Paa* is confirmed, which would be in good accordance with its recent emergence as an aggressive alder pathogen (Gibbs, 1995), its different genomes would be co-expressed, as we observed for the elicitin gene family. Likewise, *Pam* exhibited a more complex elicitin pattern than the diploid or nearly diploid species *P. fragariae* and *Pau*, respectively. In this respect, the present results are fully consistent with the hypothesis that *Pam* possesses a (allo)polyploid genome.

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References.

Becker, J., Nagel, S., and Tenhaken, R., 2000. Cloning, expression and characterization of protein elicitors from the soybean pathogenic fungus *Phytophthora sojae*. J. Phytopathol. 148, 161-167.

Brasier, C.M., Cooke, D.E.L., and Duncan, J.M., 1999. Origin of a new *Phytophthora* pathogen through interspecific hybridization. Proc. Natl. Acad. Sci. USA 96, 5878-5883.

Brasier, C.M., Kirk, S.A., Delcan, J., Cooke, D.E.L., Jung, T. and Man in't Veld, W.A., 2004. *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. Mycol. Res. 108, 1172-1184

Colas, V., Conrod, S., Venard, P., Keller, H., Ricci, P., and Panabières, F., 2001. Elicitin genes expressed *in vitro* by certain tobacco isolates of *Phytophthora parasitica* are down regulated during compatible interactions. Mol. Plant Microbe. Interact. 14, 326-335.

Cooke, D.E.L., Drenth, A., Duncan, J.M., Wagels, G., and Brasier, C.M., 2000. A molecular phylogeny of *Phytophthora* and related Oomycetes. Fungal Genet. Biol. 30, 17-32.

Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. Nucl. Acids Res. 16, 10881-10890.

Duclos, J., Fauconnier, A., Coelho, A.C., Bollen, A., Cravador, A., and Godfroit, E., 1998. Identification of a gene cluster in *Phytophthora cinnamomi*. J. Seq. Mapp. 9, 231-237.

Garcia-Olmedo, F., Carbonero, P., Aragancillo, C., and Salcedo, G., 1978. Loss of redundant gene expression after polyploidization in plants. Experentia 34, 332-333.

Gibbs, J.N., 1995. *Phytophthora* root disease of alder in Britain. EPPO Bull. 25: 661-664.

Gibbs, J., Van Dijk, C., Webber, J., 2003. *Phytophthora* disease of alder in Europe. Forestry Commission Bulletin 126, Edinburgh, 82 pp

Ioos, R., Andrieux, A., Marçais, B., Frey, P., 2006. Genetic characterization of the natural hybrid species *Phytophthora alni* as inferred from nuclear and mitochondrial DNA analyses. Fungal Genet. Biol. 43, 511-529.

Ioos, R., Barres, B., Andrieux, A., and Frey, P. Characterization of microsatellite markers in the hybrid *Phytophthora alni* subsp. *alni* and cross-amplification with related subspecies and species, *in preparation*.

Jiang, R.H.Y., Tyler, B.M., Whisson, S.C., Hardham, A.R., Govers, F., 2005. Ancient origin of elicitin gene clusters in *Phytophthora* genomes. Mol. Biol. Evol. 23, 338-351.

Jung, T., and Blaschke, M., 2004. *Phytophthora* root and collar rot of alders in Bavaria: distribution, modes of spread and possible management strategies. Plant Pathol. 53, 197-208.

Kamoun, S., Lindqvist, H., and Govers, F., 1997. A novel class of elicitin-like genes from *Phytophthora infestans*. Mol. Plant-Microbe Interact. 10, 1028-1030.

Mao, Y., Tyler, B.M., 1996. Cloning and sequence analysis of elicitin genes of *Phytophthora sojae*. Fungal Genet. Biol. 20,169-72.

Miller, P.M., 1955. V-8 juice agar as a general purpose medium for fungi and bacteria. Phytopathology 45, 461-462.

Mikes, V., Milat, M-L., Ponchet, M., Panabières, F., Ricci, P., Blein, J. P., 1998. Elicitins, proteinaceous elicitors of plant defense, are a new class of sterol carrier proteins. Biochem. Biophys. Res. Comm. 245, 133-139.

Osman, H., Mikes, V., Milat, M.-L., Ponchet, M., Marion, D., Prange, T., Maume, B., Vauthrin, S., Blein, J. P., 2001. Fatty acids bind to the fungal elicitor cryptogein and compete with sterols. FEBS Lett. 489, 55-58.

Panabières, F., Amselem, J., Galiana, E., Le Berre, J.-Y., 2005. Gene identification in the Oomycete pathogen *Phytophthora parasitica* during *in vitro* vegetative growth through expressed sequence tags. Fungal Genet. Biol. 42, 611-623.

Panabières, F., Marais, A., Le Berre, J.-Y., Penot, I., Fournier, D., and Ricci, P., 1995. Characterization of a gene cluster of *Phytophthora cryptogea* which codes for elicitins, proteins inducing a hypersensitive-like response in tobacco. Mol. Plant-Microbe Interact. 8, 996-1003.

Panabières, F., Ponchet, M., Allasia, V., Cardin, L., and Ricci, P., 1997. Characterization of border species among Pythiaceae: several *Pythium* isolates produce elicitins, typical proteins from *Phytophthora* spp. Mycol. Res. 101, 1459-1468.

Ponchet, M., Panabières, F., Milat, M.-L., Montillet, J.-L., Suty, L., Triantaphylides, C., Tirilly, Y., and Blein, J.-P., 1999. Are elicitins cryptogams in plant-Oomycete communications ? Cell. Mol. Life. Sci. 56, 1020-1047.

Robin, C., Desprez-Loustau, M.L., Capron, G., and Delatour, C., 1998. First record of *Phytophthora cinnamomi* on cork and holm oak in France and evidence of pathogenicity. Ann. Sci. For. 55, 869-883.

Rosenthal, A., Coutelle, O., and Craxton, M., 1993. Large-scale production of DNA sequencing templates by microtitre format PCR. Nucl. Acids Res. 21, 173 - 174.

Streito, J.-C., Legrand, P., Tabary, F. and Jarnouen de Villartay, G., 2002. *Phytophthora* disease of alder (*Alnus glutinosa*) in France: investigations between 1995 and 1999. Forest Pathol. 32, 179-191.

Swofford, D.L., 2002. PAUP. Phylogenetic Analysis Using Parsimony. Sinauer Associates, Sunderland, MA.

Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673-4680.

Volkov, R.A., Borisjuk, N.V., Panchuk, I.I., Schweizer, D., and Hemleben, V., 1999. Elimination and rearrangement of parental rDNA in the allotetraploid *Nicotiana tabacum*. Mol. Biol. Evol. 16, 311-320

Table 1: List of the *Phytophthora* spp. and *Pythium* spp. used in this study.

Species	isolate	Host	Geographical origin	Year	Isolator / supplier, réference	mRNA sequence accession number
P. alni* subsp. *alni	PAA2	*Alnus glutinosa*	France	2002	J.C. Streito (2N0685)	
	PAA20	*Alnus glutinosa*	France	1997	J.C. Streito (71T1)	
	PAA21	*Alnus glutinosa*	France	1997	J.C. Streito (77T4)	
	PAA23	*Alnus glutinosa*	France	1997	J.C. Streito (82T1A)	
	PAA24	*Alnus glutinosa*	France	1997	J.C. Streito (84T2)	
	PAA34	*Alnus glutinosa*	France	1998	J.C. Streito (98-7-5)	
	PAA35	*Alnus glutinosa*	France	1998	J.C. Streito (98-7-6)	
	PAA38	*Alnus glutinosa*	France	2002	J.C. Streito (2N0529)	
	PAA44	*Alnus glutinosa*	France	1998	J.C. Streito (DSFO98172)	
	PAA47	*Alnus glutinosa*	France	1999	J.C. Streito (AUL026/1)	
	PAA52	*Alnus glutinosa*	France	1999	J.C. Streito (9900783.4)	
	PAA53	*Alnus glutinosa*	France	2001	J.C. Streito (1R0152)	
	PAA58	*Alnus glutinosa*	France	2001	J.C. Streito (1N0201)	
	PAA100	*Alnus glutinosa*	France	2003	R. Ioos (P1bisa)	
	PAA103	*Alnus glutinosa*	France	2003	R. Ioos (P3a)	
	PAA107	*Alnus glutinosa*	France	2003	R. Ioos (Priva)	
	PAA108	*Alnus glutinosa*	France	2003	R. Ioos (Privb)	
	PAA109	*Alnus glutinosa*	France	2003	R. Ioos (P6-2)	
	PAA110	*Alnus glutinosa*	France	2003	R. Ioos (P6-1)	
	PAA111	*A. glutinosa* soil	France	2003	C. Husson (Ainvelle Sol)	
	PAA112	*Alnus glutinosa*	France	2003	C. Husson (2ALD03)	
	PAA113	*Alnus glutinosa*	France	2003	C. Husson (102-1)	
	PAA114	*Alnus glutinosa*	France	2002	C. Husson (Moselle)	
	PAA115	*Alnus glutinosa*	France	2002	C. Husson (370-2)	
	PAA116	*Alnus glutinosa*	France	2003	R. Ioos (3N10094-5a)	
	PAA118	*Alnus glutinosa*	France	2003	R. Ioos (3N10094-5c)	
	PAA120	*Alnus glutinosa*	France	2003	R. Ioos (3N10048-3a)	
	PAA121	*Alnus glutinosa*	France	2003	R. Ioos (3N10048-3b)	
	PAA125	*Alnus glutinosa*	France	2003	R. Ioos (3N10048-3f)	
	PAA126	*Alnus glutinosa*	France	2003	C. Husson (Ainvelle4-4)	
	PAA127	*Alnus glutinosa*	France	2003	C. Husson (Ainvelle1-2)	
	PAA128	*Alnus glutinosa*	France	2003	C. Husson (Ainvelle1-1)	
	PAA129*	*Alnus glutinosa*	France	2003	G. Capron (703)	DQ012517, DQ012718
	PAA130*	*Alnus glutinosa*	France	2003	R. Ioos (1429-6b)	DQ012519, DQ012520
	PAA131	*A. glutinosa* soil	France	2003	C. Husson (Sol A15)	
	PAA132	*A. glutinosa* soil	France	2003	C. Husson (Sol A1)	
	PAA133	*A. glutinosa* soil	France	2003	C. Husson (Sol A7)	
	PAA151*	*Alnus glutinosa*	France	2004	B. Thoirain (2051000-D12)	DQ012523, DQ012524, DQ012525
	PAA185	*Alnus glutinosa*	France	2004	R. Ioos (4N1605)	
	PAA29	*Alnus glutinosa*	Belgium	1999	J.C. Streito (9900715.6)	
	PAA86	*Alnus glutinosa*	Belgium	1999	D. De Merlier (2198[c])	
	PAA88	*Alnus glutinosa*	Belgium	2001	D. De Merlier (2295[c])	
	PAA70	*Alnus* sp.	The Netherlands	Unknown	W. Man in't Veld (PD2010953)	
	PAA74	*Alnus glutinosa*	Scotland	2000	G. Mackaskill (P1275)	
	PAA75	*Alnus viridis*	Scotland	2000	J. Gibbs (P1272)	
	PAA76	*Alnus glutinosa*	Scotland	2000	J. Gibbs (P1271)	
	PAA77	*Alnus glutinosa*	Scotland	2000	J. Delcan (P1270)	
	PAA78	*Alnus glutinosa*	England	1997	J. Delcan (P1960)	
	PAA79	*Alnus glutinosa*	England	1997	J. Delcan (P957[a])	
	PAA80	*Alnus glutinosa*	England	1997	J. Delcan (P950[a])	
	PAA81	*Alnus glutinosa*	England	1997	J. Delcan (P937)	
	PAA82	*Alnus glutinosa*	England	1996	S. Gregory (P850)	
	PAA85	*Alnus glutinosa*	England	Unknown	C. Brasier (P834[e])	
	PAA91	*Alnus glutinosa*	Hungary	2001	Z. Nagy (6[d])	
	PAA92	*A. glutinosa* soil	Hungary	2001	Z. Nagy (8[d])	
	PAA93	*A. glutinosa* soil	Hungary	2001	Z. Nagy (9[d])	
	PAA94	*A. glutinosa* soil	Hungary	2001	Z. Nagy (1a[d])	
	PAA95	*Alnus glutinosa*	Hungary	2001	Z. Nagy (4-2[d])	
	PAA134	*Alnus glutinosa*	Germany	2000	K. Kaminski (BBA 23/00)	
	PAA162*	*Alnus glutinosa*	Germany	2004	R. Ioos (9a)	DQ012526, DQ012527
	PAA168	*Alnus glutinosa*	Germany	2004	R. Ioos (8b)	
	PAA141	*Alnus glutinosa*	Austria	Unknown	T. Cech (Pucking B10)	
	PAA143*	*Alnus glutinosa*	Poland	2002	G. Skuta (PO 192)	DQ012521, DQ012522
	PAA144	*Alnus glutinosa*	Poland	2003	G. Skuta (PO 193)	
	PAA145	*Alnus glutinosa*	Poland	2004	G. Skuta (PO 203)	
	PAA146	*Alnus glutinosa*	Poland	2002	G. Skuta (PO 205)	
	PAA189	*A. glutinosa* soil	Poland	2004	L. Orlikowski (*P. alni* soil)	
	PAA190	*Alnus glutinosa*	Poland	2004	L. Orlikowski (*P. alni* 5-yo)	
P. alni* subsp. *uniformis	PAU60*	*Alnus glutinosa*	France	1999	J.C. Streito (AUL028)	
	PAU84*	*Alnus glutinosa*	Sweden	1997	C. Olsson (P875[a,b,c,f])	DQ012514
	PAU87	*Alnus glutinosa*	Belgium	2001	D. De Merlier (2271[c])	
	PAU187	*Alnus glutinosa*	Belgium	2001	D. De Merlier (2276[c])	
	PAU188	*Alnus incana*	Belgium	2001	D. De Merlier (2277[c])	
	PAU89*	*Alnus cordata*	Italy	2000	P. Capretti (CBS109280[e])	DQ012515, DQ012516
	PAU96	*Alnus glutinosa*	Hungary	1999	Z. Nagy (155-a[d])	

	PAU97	*A. glutinosa* soil	Hungary	1999	Z. Nagy (155-b[d])	
	PAU98	*A. glutinosa* soil	Hungary	1999	Z. Nagy (155-c[d])	
	PAU142	*Alnus glutinosa*	Slovenia	2003	A. Munda (Phy-A-Slo)	
P. alni* subsp. *multiformis	PAM54*	*Alnus glutinosa*	France	2000	J.C. Streito(DSFO/0125)	DQ012508, DQ012509
	PAM71*	*Alnus glutinosa* soil	The Netherlands	Unknown	W. Man in't Veld (W1139)	DQ012510, DQ012511
	PAM90	*Alnus glutinosa* soil	The Netherlands	Unknown	W. Man in't Veld (P972[a,c,f])	
	PAM73*	*Alnus glutinosa*	UK	1996	S. Gregory(P841[a,c,f])	DQ012512, DQ012513
	PAM186	*Alnus glutinosa*	Belgium	2001	D. De Merlier (2274[c])	
P. cambivora	PC463*	*Castanea sativa*	France	1994	INRA Bordeaux	DQ012528, DQ012529
P. cambivora	PC643	*C. sativa* soil	France	2000	INRA Bordeaux	
P. cambivora	PCjc17*	*Quercus* sp. soil	France	1999	C. Delatour	DQ012530, DQ012531
P. cambivora	PCGA1	*Quercus* sp. soil	France	1999	C. Delatour	
P. cambivora	PC99428	*Castanea sativa*	France	1999	R. Ioos	
P. cambivora	PCST3R1	*Quercus petraea*	France	1999	C. Delatour	
P. cambivora	PC627	*Castanea sativa*	Italy	2000	INRA Bordeaux	
P. cambivora	PC1A21	*Quercus* sp soil	France	1999	INRA Bordeaux	
P. cambivora	PC4N1425	*Castanea sativa*	France	2004	LNPV-UMAF	
P. cambivora	PC4N444	*Castanea sativa*	France	2004	LNPV-UMAF	
P. fragariae var. *fragariae*	PFF1	*Fragaria x ananassa*	UK	Unknown	K. Hughes	
P. fragariae var. *fragariae*	PFF209.46	*Fragaria x ananassa*	UK	1946	CBS (CBS209.46)	
P. fragariae var. *fragariae*	PFF309*	*Fragaria x ananassa*	UK	1962	CBS (CBS 309.62)	DQ012532, DQ012533
P. fragariae var. *rubi*	PFRVR 59	*Rubus* sp.	UK	Unknown	D. Cooke (FVR 59)	
P. fragariae var. *rubi*	PFR163-2	*Rubus* sp.	France	Unknown	A. Baudry (163-2)	
P. fragariae var. *rubi*	PFR2	*Rubus* sp.	UK	Unknown	K. Hughes	
P. fragariae var. *rubi*	PFR967.95	*Rubus* sp.	UK	1985	CBS (CBS967.95)	
P. fragariae var. *rubi*	PFR109*	*Rubus* sp.	UK	1991	CBS (CBS109.892)	DQ012534, DQ012535
P. cactorum	CAC4810/TJ	Unknown	France	Unknown	C. Delatour	
P. cinnamomi	DSFO2N0964	*Castanea sativa*	France	2002	J.C. Streito	
P. cinnamomi	DSFA970060	*Quercus suber*	France	1997	J.C. Streito	
P. cinnamomi	DSFO990050	*C. sativa* soil	France	1999	J.C. Streito	
P. cinnamomi	P382	*Nothofagus procera* soil	UK	1980	C. Brasier	
P. citricola	2N0750-171	Unknown	France	2002	J.C. Streito	
P. citricola	AUL 045 AP7	*Alnus glutinosa*	France	1999	J.C. Streito	
P. citricola	2AE5	*Quercus* sp. soil	France	1998	C. Delatour	
P. citricola	3N1345-17	*Alnus glutinosa*	France	2003	R. Ioos	
P. citrophthora	2N1021	*Rosa* sp.	France	2002	J.C. Streito	
P. cryptogea	990675	*Actinidia chinensis*	France	1999	J.C. Streito	
P. erythroseptica	960713	*Polygonum oberti*	France	1999	J.C. Streito	
P. europaea	AL5	*Quercus* sp. soil	France	1998	C. Delatour	
P. europaea	2AU2	*Quercus* sp. soil	France	1999	C. Delatour	
P. gonapodyides	Gonap 4	*Quercus* sp. soil	France	1998	C. Delatour	
P. gonapodyides	AB4	*Quercus* sp. soil	France	1998	C. Delatour	
P. humicola	3N1245-j	*A. glutinosa* soil	France	2003	R. Ioos	
P. ilicis	3N1245-l	*A. glutinosa* soil	France	2003	R. Ioos	
P. inundata	9500802	*A. glutinosa* soil	France	1995	J.C. Streito	
P. lateralis	98093.1-SPV	*Chamaecyparis* sp.	France	1998	J.C. Streito	
P. megasperma	3N1245-m	*A. glutinosa* soil	France	2003	R. Ioos	
P. megasperma	BK1	*Quercus* sp. soil	France	1998	C. Delatour	
P. megasperma	03-12	water under *Quercus* sp	France	1998	C. Delatour	
P. megasperma	mega 1	Unknown	Germany	1998	T. Jung	
P. megasperma	8RPOC3	*Quercus* sp. soil	France	1998	C. Delatour	
P. nicotianae	960579	*Nicotiana tabacum*	France	1996	J.C. Streito	
P. taxon forestsoil	8CARPPOC1	*Quercus* sp. soil	France	1998	C. Delatour	
P. palmivora	970423	*Hedera* sp.	France	1997	J.C. Streito	
P. parasitica	970029	*Lycopersicon esculentum*	France	1997	J.C. Streito	
P. taxonPgchlamydo	Haye,3,1	*Quercus* sp. soil	France	1998	C. Delatour	
P. pseudosyringae	EW5	*Quercus* sp. soil	France	1998	C. Delatour	
P. psychrophila	FF20	*Quercus* sp. soil	France	1998	C. Delatour	
P. quercina	FNA	*Quercus* sp. soil	France	1999	C. Delatour	
P. quercina	Mers2	*Quercus* sp. soil	France	1999	C. Delatour	
P. ramorum	2N0983	*Rhododendron* sp.	France	2002	C. Saurat	
P. ramorum	3N0003	*Viburnum* sp.	France	2002	C. Saurat	
P. sojae	443	*Glycine max*	Unknown	Unknown	F. Panabières	
P. syringae	2JZ2	*Quercus* sp. soil	France	1999	C. Delatour	
Pythium aphanidermatum	Ctsa	Unknown	France	2003	S. Verger	
Pythium sylvaticum	0675/a	Unknown	France	2003	S. Verger	
Pythium intermedium	02/84/1	Unknown	France	Unknown	S. Verger	
Pythium irregulare	02/57/1	Unknown	France	Unknown	S. Verger	
Pythium ultimum	433/3	Unknown	France	Unknown	S. Verger	
Pythium sp.	3N1345-11	*A. glutinosa* soil	France	2003	R. Ioos	

*Isolate used for mRNA production in this study and also studied by Ioos et al., 2006
[a]also studied by Delcan and Brasier, 2001
[b]also studied by Brasier *et al.*, 1999
[c]also studied by De Merlier *et al.*, 2005
[d]also studied by Nagy *et al.*, 2003
[e]also studied by Santini *et al.*, 2003
[f]also studied by Brasier and Kirk, 2001

Table 2: List and sequence of the 3'UTR-specific reverse primers designed in this study.

Class of elicitin	Code	Sequence (5'-3')
Class I, acidic	a2-R	AGG GTG GAT GGG GGA TTG CCA
	a3-R	CGA AGA CAC GTC GGT ATC CAT
	a4-R	GAC AAG TCG GCA TAA CAA AC
	a5-R	GCT CAG ACA ACA CTC AAG CT
	a7-R	GCTG AAA CAA TGC TCA AGA
	a8-R	GCT GAT CTG AAG ACG AGT C
	a10-R	GCT GCG TAC TTA GTC CAC GC
	a11-R	CTG CAT CGG AAT TCC AAC AAC
Class I, basic	b1-R	CTT CGA GTT AAT GGC GTA TTA
	b2-R	CCT TGA GTT TTA ATG GTA GA
Class II, highly acidic	ha1-R	GTG ACG TCG CGC CTG ATC CAG

Table 3: Occurrence and distribution of the different elicitin transcripts inferred from combination of sequencing and 3'UTR-specific PCR carried out on the cDNA libraries of the 15 isolate-panel. The occurrence of a specific 3'UTR is underlined when inferred from sequencing data.

Species	cDNA library	Acidic elicitin							3'UTR occurrence			Basic elicitin		HA elicitin
P. alni subsp. *uniformis*	PAU60	*nd*	-	-	-	-	*nd*	-	a8	*nd*	a10	-	-	b2	ha1
	PAU84	a1	-	-	-	-	*nd*	-	a8	*nd*	a10	-	-	b2	ha1
	PAU89	a1	-	-	-	-	*nd*	-	a8	*nd*	a10	-	-	b2	ha1
P. alni subsp. *multiformis*	PAM54	a1	a2	a3	a4	a5	*nd*	-	a8	*nd*	-	-	b1	-	ha1
	PAM71	*nd*	a2	a3	a4	a5	*nd*	-	a8	*nd*	-	-	b1	-	ha1
	PAM73	*nd*	a2	a3	a4	a5	*nd*	-	a8	*nd*	-	-	b1	-	ha1
P. alni subsp. *alni*	PAA129	*nd*	a2	a3	a4	a5	*nd*	-	a8	*nd*	a10	-	b1	b2	ha1
	PAA130	a1	a2	a3	a4	a5	*nd*	-	a8	*nd*	a10	-	b1	b2	ha1
	PAA143	a1	a2	a3	a4	a5	*nd*	-	a8	*nd*	a10	-	b1	b2	ha1
	PAA151	*nd*	a2	a3	a4	a5	*nd*	-	a8	*nd*	a10	-	b1	b2	ha1
	PAA162	*nd*	a2	a3	a4	a5	*nd*	-	a8	*nd*	a10	-	b1	b2	ha1
P. cambivora	PCjc17	*nd*	a2	-	-	-	a6	a7	a8	*nd*	a10	-	-	b2	-
	PC643	*nd*	-	-	-	-	a6	-	a8	*nd*	-	-	-	b2	-
P. fragariae var. *rubi*	PFR109	*nd*	a2	-	-	-	*nd*	-	-	a9	a10	-	-	-	ha1
P. fragariae var. *fragariae*	PFF309	*nd*	a2	-	-	-	*nd*	-	-	a9	-	a11	-	-	ha1

nd: could not be determined as no 3'UTR-specific PCR primer could be designed from these sequences

Table 4: Occurrence and distribution of the different elicitin encoding sequences and the associated 3'UTR in the genome of *P. cambivora*, *P. fragariae*, *P. alni* and the other *Phytophthora* spp. and *Pythium* spp. tested in this study. The occurrence of a specific 3'UTR is indicated in bold when inferred from a 3'UTR- specific PCR test and is underlined when inferred from the sequence of a corresponding mRNA for at least one isolate of the species/subspecies/variety. As sometimes two slightly different proteins were deduced from mRNAs with distinct coding sequence but sharing identical 3'UTR, it could not be determined whether the detection by PCR of a given 3'UTR was associated with one or the other protein and both are therefore indicated. Otherwise indicated, all the isolates from a given species/subspecies/variety gave similar results when tested by 3'UTR-specific PCR.

Species	Acidic elicitin (AE)	AE 3'UTR										Basic elicitin (BE)	BE 3'UTR		Highly acidic elicitin (HAE)	HAE 3'UTR
P. alni subsp. *uniformis*	Pa_AE1.1/1.2	a1	-	-	-	-	*nd*	-	**a8**	*nd*	**a10**	-	Pa_BE1	-	**b2**	Pa_HAE1	**ha1**
P. alni subsp. *multiformis*	Pa_AE1.1/1.2	a1	**a2**	**a3**	-	**a5**	*nd*	-	**a8**	*nd*	-	-	Pa_BE1/2	**b1**	-	Pa_HAE1	**ha1**
	Pa_AE2	*nd*	-	**a3**	**a4**	-	*nd*	-	-	*nd*	-	-					
P. alni subsp. *alni*	Pa_AE1.1/1.2	a1	**a2**	**a3**	-	**a5**	*nd*	-	**a8**	*nd*	**a10**	-	Pa_BE1	**b1**	**b2**	Pa_HAE1	**ha1**
	Pa_AE2	*nd*	-	**a3**	**a4**	-	*nd*	-	-	*nd*	-	-	Pa_BE2	**b1**	-		
P. cambivora	Pa_AE1.1/1.2	a1	**a2***	-	-	-	a6	**a7***	**a8**	*nd*	**a10**	-	Pa_BE1	-	**b2**	Pa_HAE1	**ha1**
P. fragariae var. *rubi*	Pa_AE1.1/1.2	*nd*	**a2**	-	-	-	*nd*	-	-	a9	**a10**	-	-	-	-	Pa_HAE1	**ha1**
P. fragariae var. *fragariae*	Pa_AE1.1/1.2	*nd*	**a2**	-	-	-	*nd*	-	-	*nd*	-	-	-	-	-	Pa_HAE1	**ha1**
	Pa_AE2	*nd*	-	-	-	-	*nd*	-	-	a9	-	**a11$**					
Phytophthora spp. (other)	-	*nd*	-	-	-	-	*nd*	-	-	*nd*	-	-	-	-	-	-	-
Pythium spp.	-	*nd*	-	-	-	-	*nd*	-	-	*nd*	-	-	-	-	-	-	-

nd: could not be determined as no 3'UTR-specific PCR primer could be designed from these sequences
*all *P. cambivora* isolates except for PC643 and PC463
$This sequence corresponds to the 3'UTR sequence "*PFR_fra1*" observed in a *P. fragariae* var. *rubi* isolate and provided by F. Panabières (unpublished data)

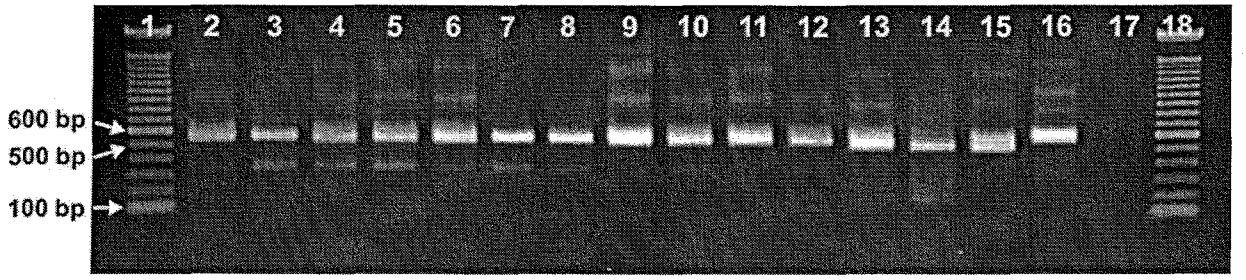

Figure 1: *In vitro* expression of elicitin-encoding genes in the 15-isolate panel. Reverse transcriptions were carried out using *Not*I-oligodT and polymerase chain reactions were performed using degenerate Primer1, located in the highly conserved peptide signal region, as forward primer in combination with *Not*I-oligodT as reverse primer. Lanes 1 and 18, 100 bp ladder; Lane 2 to 16, isolates PAA162, PAM54, PAM71, PAM73, PAU60, PAU84, PAU89, PAA129, PAA130, PAA143, PAA151, PCjc17, PC643, PFR109, PFF309, respectively; Lane 17, control (no RNA).

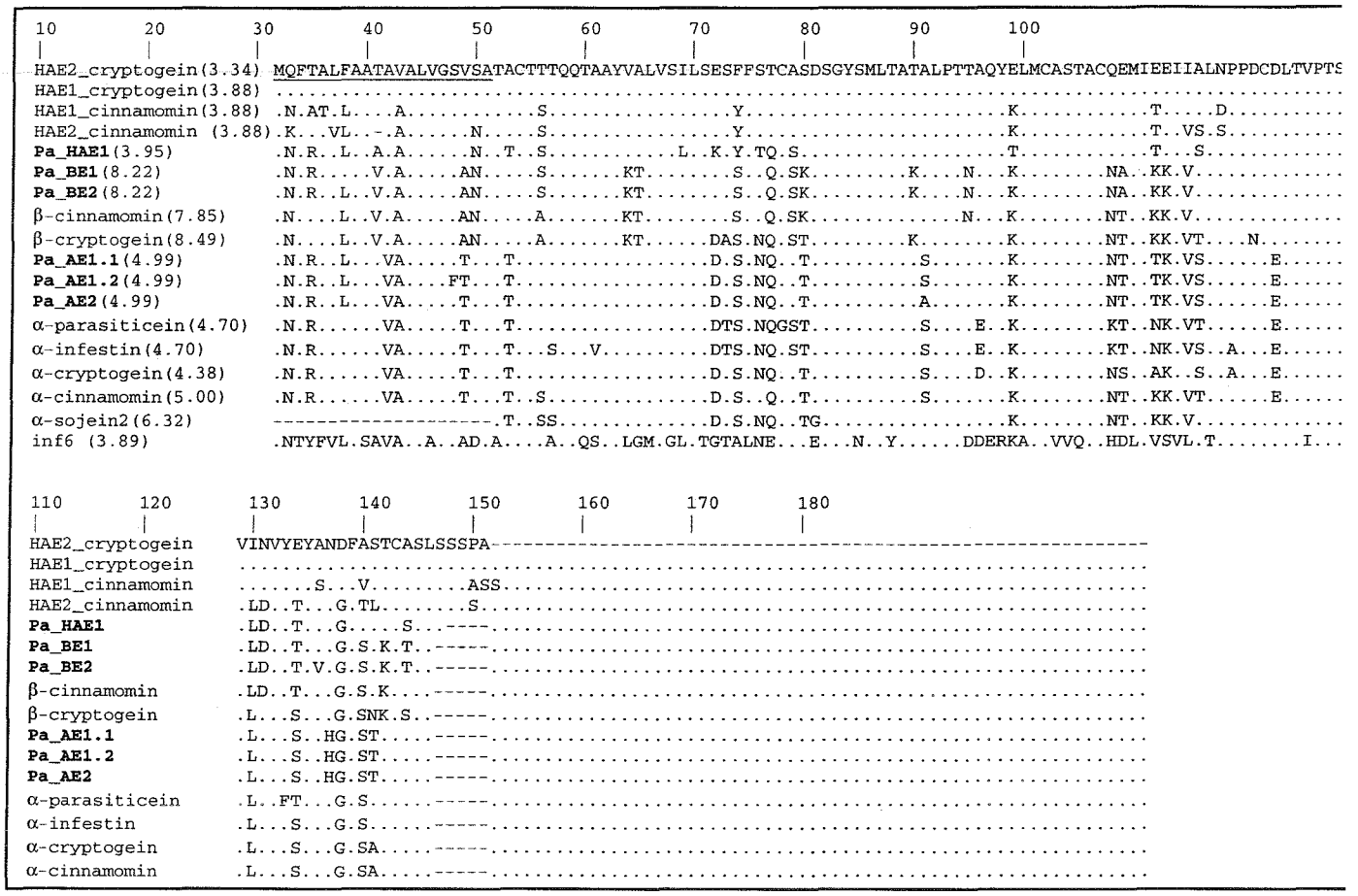

Figure 2: Multiple amino acid sequence alignment of the elicitins characterized in this study for *P. alni*, *P. cambivora* and *P. fragariae* (Pa_AE1.1, Pa_AE1.2, Pa_AE2, Pa_BE1, Pa_BE2 and Pa_HAE1) and well documented acidic (α-), basic (β-) and highly acidic (HAE-) elicitins retrieved from Genbank database (*P. cryptogea* Z34462, Z34459, Z34460, Z34461: Panabières *et al.*, 1995; *P. infestans* AY830090: Jiang *et al.*, 2005; *P. cinnamomi* AJ000071: Duclos et al., 1998; *P. sojae* AJ007859: Becker *et al.*, 2000; *P. parasitica* S67432: Colas *et al.*, 2001). The sequence corresponding to the peptide signal is underlined. Isoelectric point is indicated between parentheses, following sequence reference.

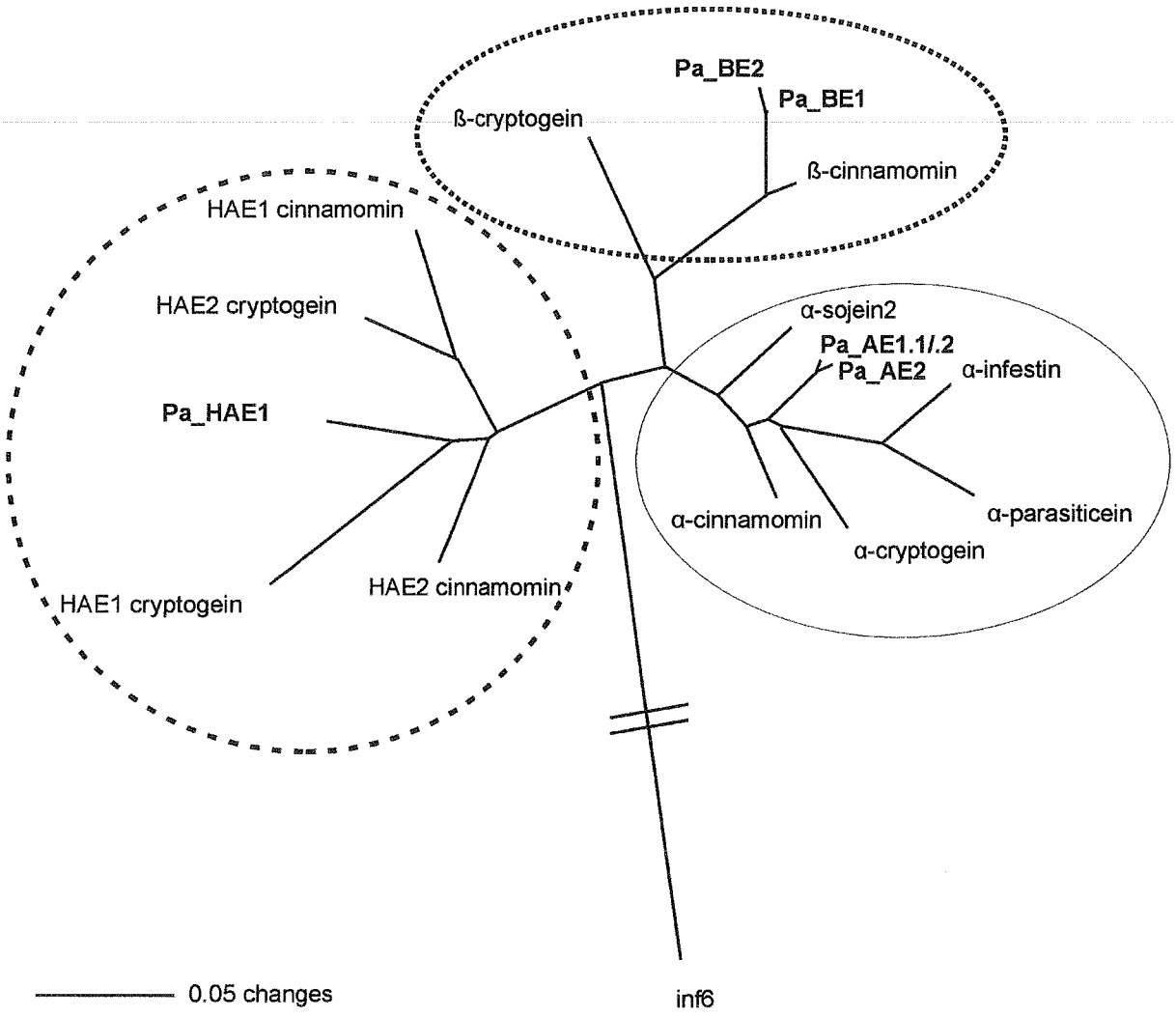

Figure 3: Phylogenetic relationships between elicitins, inferred from the sequence alignment of the mature polypeptides. The unrooted tree was constructed using the neighbour-joining method based on the multiple alignment of elicitin sequences listed in Figure 2 and using inf6 sequence as an outgroup. Pa_AE1.1/.2 and Pa_AE2 fall into the class I acidic elicitins group (full line), whereas Pa_BE1 and Pa_BE2 fall into class I basic elicitins (dotted line). Pa_HAE1 is closely related to Class II highly acidic elicitins (dashed line).

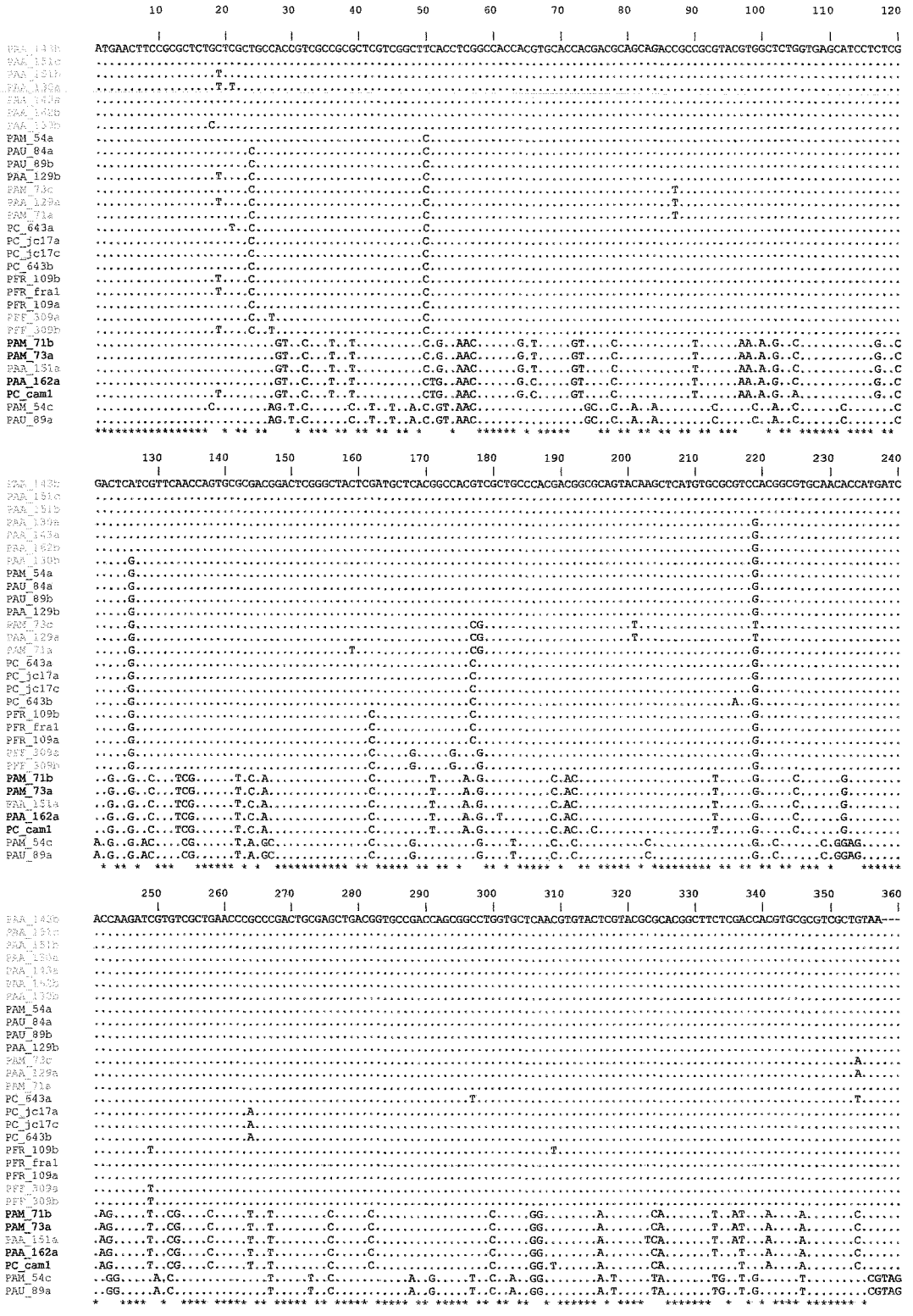

Figure 4: Sequence alignment of the coding region of the mRNAs, deduced from cDNA sequencing. Twenty-eight sequences obtained from our 15 isolate-panel are aligned along with 3'UTR mRNA sequences from *P. fragariae* var. *rubi* (*PFR_fra1*) and *P. cambivora* (*PC_cam1*) (F. Panabières, unpublished data). The code of each sequence is colour-labelled according to the protein encoded: Pa_AE1.1 (red), Pa_AE1.2 (pink), Pa_AE2 (orange), Pa_BE1 (dark green), Pa_BE2 (light green), Pa_HAE1 (blue).

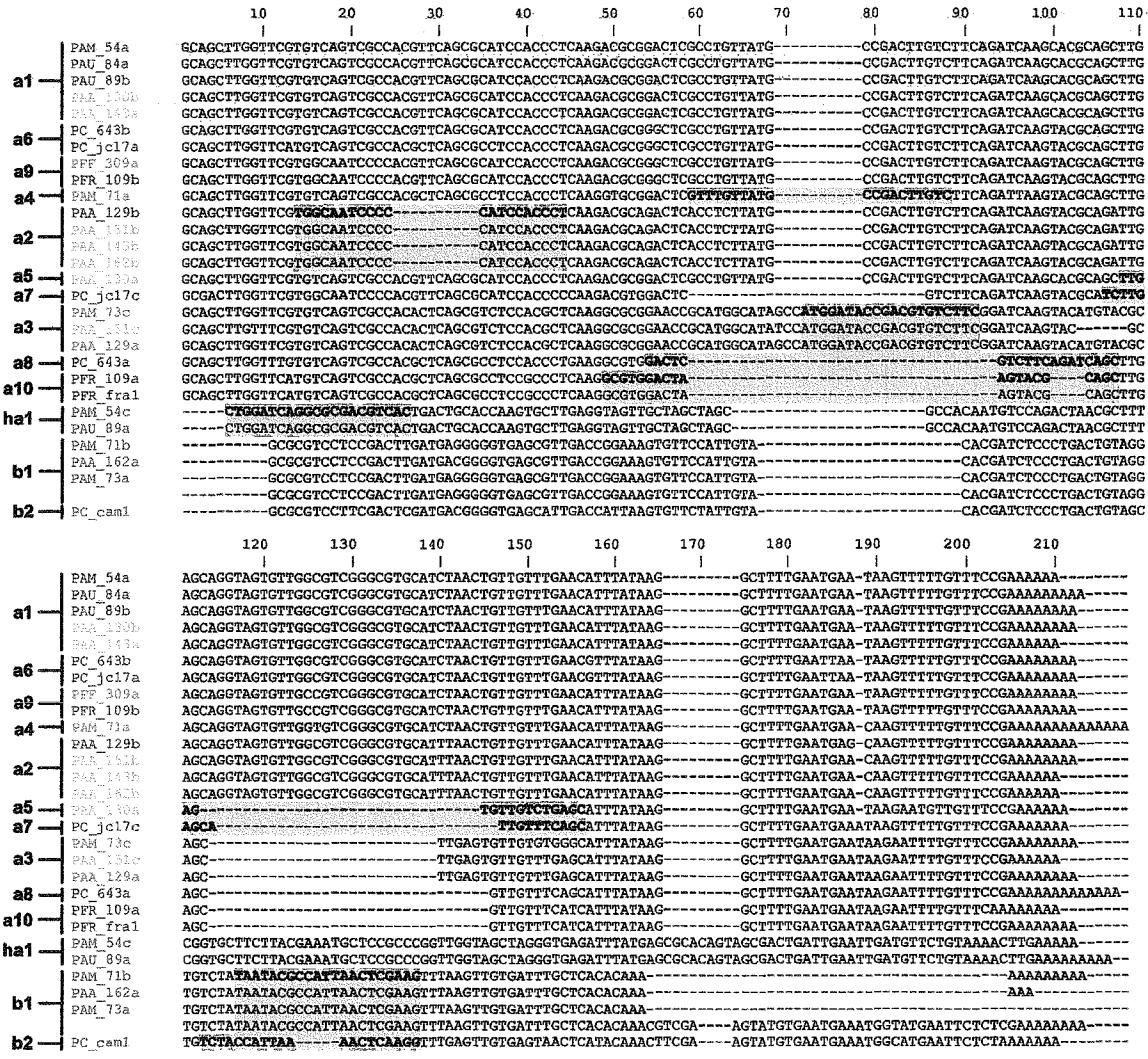

Figure 5: Sequence alignment of the region corresponding to the 3'UTR of the mRNAs, deduced from cDNA sequencing. Twenty-seven sequences obtained from our 15 isolate-panel are aligned along with 3'UTR mRNA sequences from *P. fragariae* var. *rubi* (*PFR_fra1*) and *P. cambivora* (*PC_cam1*) (F. Panabières, unpublished data). The code of each sequence is colour-labelled according to the protein encoded: Pa_AE1.1 (red), Pa_AE1.2 (pink), Pa_AE2 (orange), Pa_BE1 (dark green), Pa_BE2 (light green), Pa_HAE1 (blue).

3'UTR specific groups are indicated on the left-hand side and correspond to the clustering of identical or nearly identical sequences. For each group, shadowed sequence represents the polymorphic region from which a 3'UTR specific primer could be designed and the bold nucleotides correspond to the primer target. PFF309b 3'UTR sequence was so divergent that it could not be properly aligned. PFF309b was therefore not represented but a specific reverse primer could be designed (a11-R).

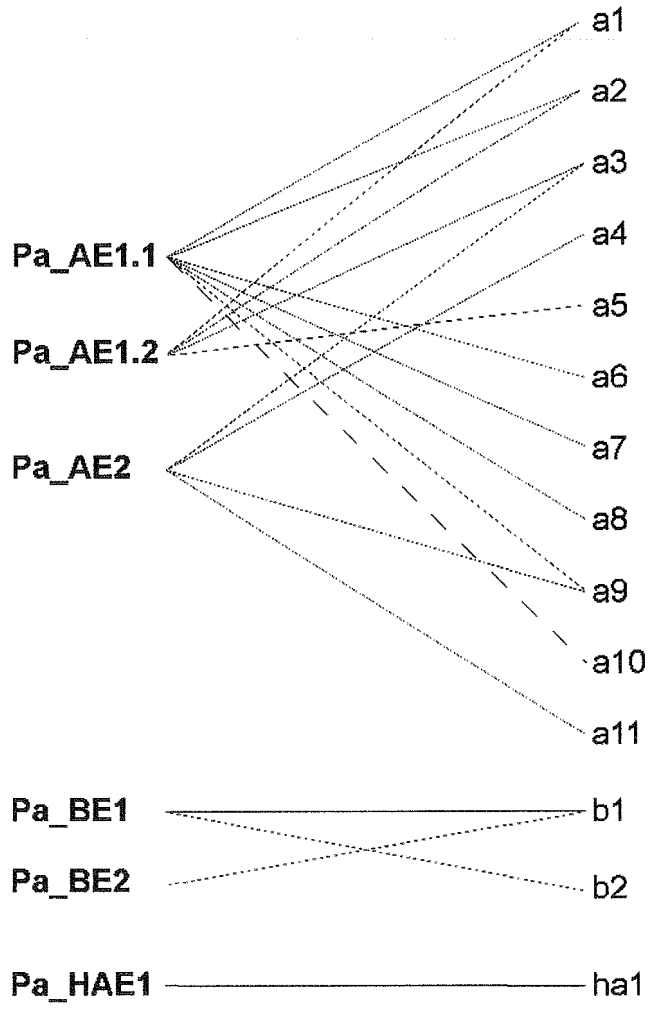

Figure 6: Schematic representation of the different associations between the different elicitin proteins (left) and the 3'UTR sequences (right) as inferred from elicitin-encoding genes sequencing for *P. alni*, *P. cambivora* and the two varieties of *P. fragariae*.

II.2. – Résultats obtenus et nouvelles hypothèses sur les origines de *Paa*, *Pam* et *Pau*.

II.2.1. – *Paa* semble avoir été généré par l'hybridation de *Pam* et *Pau*.

La première étude de caractérisation génétique de *Paa* (Publication 3) a montré que ce taxon possédait systématiquement trois allèles pour chacun des quatre gènes nucléaires étudiés, deux d'entre eux correspondant aux deux allèles observés chez *Pam* (allèles « PAM1 » et « PAM2 »), tandis que le troisième était identique à l'unique allèle mis en évidence chez *Pau* (allèle « PAU »). De plus, l'étude de l'ADN mitochondrial a démontré que les profils d'ADNmt de *Paa* étaient identiques soit à ceux de *Pam* ou à ceux de *Pau*.

En revanche, les allèles des gènes nucléaires ainsi que les profils mitochondriaux mis en évidence chez *P. cambivora* et chez *P. fragariae* différaient significativement de ceux observés chez *Paa*, *Pam* et *Pau*. Ces différences infirment l'hypothèse de Brasier et al. (1999) d'une implication directe de *P. cambivora* et *P. fragariae* dans le processus d'hybridation ayant conduit à la genèse de *P. alni*.

La combinaison des données nucléaires et mitochondriales a permis d'autre part d'infirmer l'hypothèse de Brasier et al. (1999) présentant *Pam* et *Pau* comme des ségrégants génétiques de *Paa*. Au contraire, il apparaît que *Paa* pourrait avoir été généré par l'hybridation entre *Pam* et *Pau*, ou éventuellement des taxons leur étant très proches. La distribution dichotomique des types mitochondriaux chez *Paa* (« type « M » ou type « U ») suggère enfin que l'hybridation a été sexuelle et non somatique, et s'est produite dans les deux directions, *Pam* et *Pau* ayant chacun joué les rôles oogonial et anthéridial.

Enfin, l'observation d'un seul allèle (« PAU ») pour chacun des 4 gènes nucléaires indépendants chez *Pau* montre que ce taxon, très proche de la diploïdie (2n+2, Brasier et al., 1999), est homozygote ou proche de l'homozygotie à chacun de ces gènes. En conséquence, la présence systématique de 2 allèles relativement divergents (« PAM1 » et « PAM2 ») chez *Pam* laisse supposer que le génome de ce taxon pourrait être constitué d'un assemblage de paires de chromosomes homéologues et donc être plutôt proche de la tétraploïdie. Ceci semble contradictoire avec les niveaux de ploïdie estimés entre 2n+4 et 2n+7 chez des isolats de *Pam* par Brasier et al. (1999) mais pourraient s'expliquer par *i)* des difficultés liées au comptage des chromosomes chez *Phytophthora* (Burnett, 2003 ; F. Panabières, comm. pers.) ou *ii)* un phénomène de diploïdisation de taxons initialement polyploïdes, comme observé chez de nombreuses plantes (Gross et Rieseberg, 2005). Dans ce cas de figure, *Pam* pourrait être un homoploïde, issu *i)* d'une ancienne hybridation interspécifique ou *ii)* d'une autopolyploïdisation. Enfin, *Paa* qui présente les trois allèles rencontrés chez *Pam* et *Pau* pourrait être par extension non pas quasi-tétraploïde (Brasier et al., 1999) mais hexaploïde.

Ces niveaux réels de ploïdie mériteraient donc une confirmation par le biais d'une autre technique que l'observation microscopique des chromosomes utilisée par Brasier et al. (1999). Les mesures relatives de quantité d'ADN chez les trois sous-espèces de *P. alni* avec *P. parasitica* comme espèce diploïde de

référence sont prévues, en se basant sur des données de florescence obtenues par la technique de cytométrie de flux (De Lucas et al., 1998 ; Si-Ammour, 2002 ; Ramsden et al., 2006).

Finalement, la présence systématique de trois allèles pour quatre gènes nucléaires indépendants permet de fournir une explication logique aux profils hétérodimériques inattendus (cinq bandes au lieu de trois chez un hétérozygote diploïde) au niveau du locus *Gpi* lors d'analyses isoensymatique chez *Paa* (Nagy et al., 2003 ; Brasier et al., 2004). De plus la présence systématique de deux allèles divergents chez *Pam* au lieu d'un seul chez *Pau* permet de mieux expliquer pourquoi *Pam* apparaissait phylogénétiquement plus proche de *Paa* que *Pau* sur le dendrogramme généré par AFLP (Figure 4 ; Brasier et al., 1999). En effet le « poids » du polymorphisme apporté par *Pam* à *Paa* est supérieur à celui de *Pau* et le nombre de bandes communes entre *Pam* et *Paa* devait nécessairement être supérieur à celui partagé par *Pau* et *Paa*.

II.2.2. - *P. alni*, *P. cambivora* et *P. fragariae* présentent des profils d'élicitines chevauchants.

La seconde approche de caractérisation génétique de *Paa* (Publication 4) était basée cette fois non plus sur des gènes nucléaires simple copie, mais sur une famille de gènes : les élicitines. L'étude a tout d'abord mis en évidence l'existence de profils de gènes d'élicitines complémentaires dans les trois taxons constituant *P. alni sensu lato*. *In fine*, le profil d'élicitines observé chez *Paa* représente l'exacte juxtaposition de ceux présentés par *Pam* et *Pau*. Ces informations confirment la probable implication de *Pam* et *Pau* dans la genèse de *Paa*, ou de taxons qui leurs sont très proches, comme suggéré par les résultats présentés dans la publication 3. De plus, l'existence de multiples gènes d'élicitines communs et spécifiques de *Paa*, *Pam*, *Pau*, *P. cambivora* et *P. fragariae* laisse supposer que la duplication de ces gènes se serait produite avant la radiation de ces espèces à partir de leur ancêtre commun. Enfin, l'association de mêmes séquences 3'UTR à des parties codantes différentes chez les trois espèces, phénomène qui n'avait jusqu'alors jamais été mis en évidence chez *Phytophthora*, traduit l'existence probable d'évènements de recombinaisons intra- ou interspécifiques.

Enfin, cette étude a démontré que le profil d'élicitines de *Pam* était plus riche que celui de celui de *Pau* ou de *P. fragariae* qui sont toutes deux des taxons diploïdes ou proche de la diploïdie. Les résultats de la Publication 3 ayant suggéré que *Pam* était de constitution allo(poly)ploïde (homoploïde et proche de la diploïdie ou tétraploïde), il apparaît logique que cette espèce présente un profil d'élicitines plus complexe que des espèces d'origine monophylétique. Par extension, le profil d'élicitines de *Paa* résultant de la juxtaposition de celui de *Pam* et de *Pau*, est encore plus complexe. A ce titre, nous avons aussi étudié l'expression de ces gènes, via l'analyse des ARNm par séquençage ou PCR 3'UTR-spécifiques sur les banques d'ARNm. Nous avons pu démontrer que chez *Paa* et *Pam*, qui apparaissent comme deux taxons allo(poly)ploïdes au vu des résultats de la Publication 3, les différents génomes qui co-existaient pouvaient être exprimés et que cette famille de gènes fortement

exprimée ne subissait pas (encore) de phénomènes de pseudogénisation, comme souvent observé chez les hybrides de plantes (Volkov et al., 1999).

II.3. - Valorisation du polymorphisme interspécifique des gènes étudiés par le développement de nouveaux outils de détection de *Phytophthora* spp..

La comparaison des séquences orthologues des quatres gènes nucléaires que nous avons utilisés dans la publication 3 (*ASF*-like, *GPA*1, *TRP*1, et *RAS-Ypt*) a montré qu'il existait un polymorphisme significatif entre les différentes espèces de *Phytophthora* étudiées, principalement localisé dans les régions introniques. Ces polymorphismes de nature interspecifique, voire intra-specifiques dans le cas des taxons allo(poly)ploïdes *Paa* et *Pam*, ont été exploités pour définir des amorces de PCR « allèle spécifiques ». Ces dernières ont par ailleurs confirmé qu'elles pouvaient aussi être spécifiques de l'espèce chez laquelle l'allèle en question était présent (Publication 3).

En reprenant cette stratégie de clonage/séquençage de chacun des quatre gènes chez un taxon donné de *Phytophthora* donné en utilisant les couples d'amorces dégénérées que nous avons définis, il est potentiellement possible de définir ensuite des amorces de PCR espèce-spécifique pour ce dernier dans les régions introniques.

Cette propriété a donc été mise en pratique pour définir des amorces de PCR spécifiques de *P. ramorum* et *P. fragariae sensu lato.* Ces deux espèces sont d'importance toute particulière en matière phytosanitaire puisqu'elles sont respectivement classées comme organisme réglementé pour *P. ramorum* (Anonyme, 2002) et de quarantaine pour *P. fragariae* (Anonyme, 2000). Une série d'amorces de PCR a été définie à partir des séquences orthologues de ces quatre gènes chez *P. ramorum* à partir de séquences tirées du projet de séquençage du génome (Joint Genome Institute ; (http://genome.jgi-psf.org/) et chez *P. fragariae* à partir des séquences obtenues par clonage et séquençage (Publication 3). Ces amorces ont été testées et pour chacune des deux espèces, deux couples d'amorces espèce-spécifiques ont été validées pour la détection de ces deux organismes *in planta.*

Ce travail annexe est résumé dans l'article suivant, sous presse dans la revue « European Journal of Plant Pathology » :

Publication 5 :

Ioos R., Laugustin L., Rose S., Schenck N., Husson C., and Frey P. (2006) Usefulness of single copy genes containing introns in *Phytophthora* for the development of detection tools for the regulated species *P. ramorum* and *P. fragariae. European Journal of Plant Pathology.* Sous presse.

Publication 5

Ioos R., Laugustin L., Rose S., Schenck N., Husson C., and Frey P. (2006) Usefulness of single copy genes containing introns in *Phytophthora* for the development of detection tools for the regulated species *P. ramorum* and *P. fragariae*. *European Journal of Plant Pathology*. Sous presse.

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Short Communication

Usefulness of single copy genes containing introns in *Phytophthora* for the development of detection tools for the regulated species *P. ramorum* and *P. fragariae*

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Abstract

Introns are generally highly polymorphic regions within genes and were proven to be of great interest for discriminating among phylogenetically-close *Phytophthora* species. *Phytophthora ramorum* and *P. fragariae* are considered as quarantine pathogens by the European Union and accurate detection tools are therefore necessary for their monitoring. From introns located in different single copy genes (*GPA*1, *RAS*-like, and *TRP*1), we developed a series of PCR primers specific to *P. ramorum* and *P. fragariae*. The specificity of these primers was successfully checked with a wide collection of *Phytophthora* isolates and a protocol was developed to detect both pathogens directly in infected plant tissues. These genes should be of particular interest for the development of additional species-specific detection tools within the *Phytophthora* genus.

Introduction

The genus *Phytophthora* includes major plant pathogens causing severe losses throughout the world. Some of them are ancient and well known devastating organisms, (e.g. *P. infestans* on potato crops), but this genus also harbours emergent and aggressive new species, such as *P. alni*, responsible for alder disease in Europe (Brasier et al., 2004) or *P. ramorum* causing Sudden Oak Death in North America (Rizzo et al., 2002). In addition, some species such as *P. ramorum* or *P. fragariae* var. *fragariae* (causing the root rot disease on strawberry) are regulated or quarantine registered pathogens for the European Union (Anon., 2000, 2002). Specific and sensitive detection tools are therefore needed for the monitoring of these particular species. Recently, a set of nuclear single copy genes containing intron(s) were studied for the genetic characterization of the hybrid species complex *Phytophthora alni* (Ioos et al., 2006). The four genes studied, namely *ASF*-like, *GPA*1, *RAS*-like, and *TRP*1, proved to be appropriate for the discrimination among the different subspecies of *P. alni*, and the phylogenetically close species *P. cambivora* and the two varieties of *P. fragariae* (var. *fragariae* and var. *rubi*). In order to isolate and clone these genes, Ioos et al. (2006) developed a set of four gene-specific degenerate primers. The design of the primers was based on sequence alignment conducted with original sequences of these genes deposited in GenBank, with orthologous sequences retrieved from the genome sequence project of *P. ramorum* and *P. sojae* (http://genome.jgi-psf.org/) and from the *P. infestans* EST database (http://www.pfgd.org/pfgd/filter.html).

In the present study, we successfully designed a set of *P. ramorum*-specific primers (Table 1) based

Table 1. Characteristics of the species-specific PCR primers for *P. ramorum* and *P. fragariae*

Species	Primer (forward/reverse)	Sequence (5'-3')	Size of the amplicon	Sensitivity (pg per 20 µl of PCR mixture)	Reference[a]
P. ramorum	GPA-PRAM-F[b]	TAAGGAACAAGGTACCAAAG	248 bp	0.5 pg	Scaffold_59 316212–318351
	GPA-PRAM-R[b]	CTCAGGAATTCACTCTCACG			
	TRP-PRAM-F[b]	GAGTAGAAACTTCGGGAATG	527 bp	0.5 pg	Scaffold_52 330699–333530
	TRP-PRAM-R[b]	GTTCGGCACATTAACGCAG			
P. fragariae var. *fragariae* (*Pff*)/*P. fragariae* var. *rubi* (*Pfr*)	RAS-PFR109h1-F[c]	TGTCGAGAGTGATTTATT	229 bp	1 pg (*Pff*)	DQ093986
	RAS-PFR109h1-R[c]	AATGGCAAGGCTAGTTACTA		1 pg (*Pfr*)	
	TRP-PFF309a9-F[c]	CTACCTCCCTAAGCTTATCA	403 bp	0.1 pg (*Pff*)	DQ094005
	TRP-PFF309a9-R[c]	ACGCAGCATCATAGAAAAT		0.5 pg (*Pfr*)	

[a]Refer to Genbank accession number for *P. fragariae* or localization of the orthologous sequence in the *P. ramorum* JGI sequencing project (Ioos et al., 2006).
[b]Primer designed and tested in this study.
[c]Primer designed in Ioos et al. (2006) and tested in this study.

on species-specific polymorphisms revealed by multiple alignments using all the orthologous sequences available for different *Phytophthora* species. Polymorphism was mainly located within the intronic regions of two of these genes (*GPA1* and *TRP1*). Similar species-specific polymorphisms have already been exploited by Ioos et al. (2006) in *RAS*-like and *TRP1* genes to develop a set of two *P. fragariae*-specific PCR primer pairs (Table 1). In this study, the *P. ramorum* and *P. fragariae*-specific PCR primers were assessed for their specificity and for their ability to detect both pathogens directly *in planta*.

Oomycete DNA was extracted from pure culture using a commercial plant DNA extraction kit (DNeasy plant mini kit®, Qiagen, Courtaboeuf, France) and as previously described (Ioos et al., 2005). Plant DNA was extracted as follows. Approximately 200 mg of fresh plant tissue (either *Fragaria* × *ananassa* or *Rubus idaeus* roots, or symptomatic tissues of *P. ramorum* potential hosts) were collected and first roughly cut using a sterile scalpel blade. Then the sample was transferred into a 2 ml micro-centrifuge tube and ground for 2 min with two 3 mm tungsten carbide beads at a frequency of 30 Hz with a mixermill grinder (Tissuelyser®, Qiagen). Genomic DNA was subsequently extracted using DNeasy plant mini kit® (Qiagen) following the manufacturer's instructions, except that after incubation with the lysis buffer, the microtubes were centrifuged for 4 min at 14,000*g* to pellet the cellular debris.

PCR was conducted using a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, California, USA) in 20 µl reaction mixtures consisting of 2 µl of template DNA (30–80 ng), 1× Taq polymerase buffer (Sigma-Aldrich, L'Isle d'Abeau, France), 2 mM $MgCl_2$, 0.6 µg μl^{-1} Bovine Serum Albumin (Sigma-Aldrich), 0.45 µM of each forward and reverse primer, 200 µM dNTPs, 0.5 unit of Taq DNA Polymerase (Sigma-Aldrich), and molecular biology grade water was added to 20 µl. The PCR conditions included an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C and elongation for 1 min at 72 °C, and a final extension step at 72 °C for 7 min.

The specificity of the four primer pairs was successfully checked with a wide collection of *Phytophthora* spp. and *Pythium* spp. (Table 2).

Table 2. Isolates of *Phytophthora* spp. and *Pythium* spp. tested in this study

Species	Isolate	Host	Geographical origin	PCR test GPA-PRAM-F/R	TRP-PRAM-F/R	RAS-PFR109h1-F/R	TRP-PFF 309a9-F/R	ITS6/ITS4[a]
P. ramorum (A1)	2N0983	*Rhododendron* sp. (Nursery)	France	+	+	–	–	+
P. ramorum (A1)	3N0003	*Viburnum* sp. (Nursery)	France	+	+	–	–	+
P. ramorum (A1)	F001	*Rhododendron* sp. (Nursery)	France	+	+	–	–	+
P. ramorum (A1)	RAM_Phot	*Photinia* sp. (Nursery)	Poland	+	+	–	–	+
P. ramorum (A1)	RAM_Cal	*Calluna* sp. (Nursery)	Poland	+	+	–	–	+
P. ramorum (A2)	2338	*Viburnum tinus* (Nursery)	Belgium	+	+	–	–	+
P. ramorum (A2)	05-1461-160	*Pieris japonica* (Nursery)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	05-1461-63	*Rhododendron* sp. (Nursery)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	9138	*Lithocarpus densiflorus* (Forest)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	9143	*Lithocarpus densiflorus* (Forest)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	4566	*Rhododendron macrophyllum* (Forest)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	9180	*Lithocarpus densiflorus* (Forest)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	04-189-B2	*Viburnum bodnantense* (Nursery)	Oregon (USA)	+	+	–	–	+
P. ramorum (A2)	04-79-B1	*Camelia* spp. (Nursery)	Oregon (USA)	+	+	–	–	+
P. ramorum (A1)	03-74-N11C	*Rhododendron* sp. (Nursery)	Oregon (USA)	+	+	–	–	+
P. ramorum (A1)	03-74-N11D	*Rhododendron* sp. (Nursery)	Oregon (USA)	+	+	–	–	+
P. fragariae var. *fragariae*	PFF1	*Fragaria x ananassa*	England	–	–	+	+	+
P. fragariae var. *fragariae*	CBS209.46	*Fragaria x ananassa*	England	–	–	+	+	+
P. fragariae var. *fragariae*	CBS309.62	*Fragaria x ananassa*	Scotland	–	–	+	+	+
P. fragariae var. *rubi*	PFRVR 59	*Rubus idaeus*	Great-Britain	–	–	+	+	+
P. fragariae var. *rubi*	PFR163-2	*Rubus idaeus*	France	–	–	+	+	+
P. fragariae var. *rubi*	PFR2	*Rubus idaeus*	Scotland	–	–	+	+	+
P. fragariae var. *rubi*	CBS967.95	*Rubus idaeus*	Scotland	–	–	+	+	+
P. fragariae var. *rubi*	CBS109.892	*Rubus idaeus*	Scotland	–	–	+	+	+
P. alni subsp. *alni*	PAA129	*Alnus glutinosa*	France	–	–	–	–	+
P. alni subsp. *uniformis*	PAU60	*Alnus glutinosa*	France	–	–	–	–	+
P. alni subsp. *multiformis*	PAM54	*Alnus glutinosa*	France	–	–	–	–	+
P. cactorum	CAC4810/TJ	unknown	France	–	–	–	–	+
P. cactorum	5N1449/T1	*Viola* spp.	France	–	–	–	–	+
P. cambivora	PCJC17	*Quercus* spp. soil	France	–	–	–	–	+
P. cambivora	4N1125/1	*Castanea sativa*	France	–	–	–	–	+
P. cinnamomi	DSFO2N0964	*Castanea sativa*	France	–	(+ >)[b]	–	–	+
P. cinnamomi	4N0741-O	*Castanea sativa*	France	–	(+ >)[b]	–	–	+
P. citricola	2AE5	*Quercus* sp. soil	France	–	–	–	–	+
P. citricola	4N697/1	*Syringa* spp.	France	–	–	–	–	+
P. citrophthora	2N1021	*Rosa* sp.	France	–	–	–	–	+
P. citrophthora	5N0373	*Pieris* sp.	France	–	–	–	–	+
P. cryptogea	990675	*Actinidia sinensis*	France	–	–	–	–	+
P. cryptogea	CH 2555/00	*Chamaecyparis lawsonia*	Poland	–	–	–	–	+

Table 2. continued

Species	Isolate	Host	Geographical origin	PCR test GPA-PRAM-F/R	TRP-PRAM-F/R	RAS-PFR109h1-F/R	TRP-PFF 309a9-F/R	ITS6/ITS4[a]
P. erythroseptica	960713	*Polygonum oberti*	France	–	–	–	–	+
P. europaea	AL5	*Quercus* sp. soil	France	–	–	–	–	+
P. gonapodyides	Gonap 4	*Quercus* sp. soil	France	–	–	–	–	+
P. gonapodyides	5N0391	*Rhododendron* sp.	France	–	–	–	–	+
P. humicola	3N1245-j	*Alnus glutinosa* soil	France	–	–	–	–	+
P. inundata	9500802	*Alnus glutinosa* soil	France	–	–	–	–	+
P. lateralis	98093.1-SPV	*Chamaecyparis* sp.	France	–	–	–	–	+
P. megasperma	3N1245-m	*Alnus glutinosa* soil	France	–	–	–	–	+
P. megasperma	9900557	*Prunus* sp.	France	–	–	–	–	+
P. nicotianae	960579	*Nicotiana tabacum*	France	–	–	–	–	+
P. taxon forestsoil	8CARPPOC1	*Quercus* sp. soil	France	–	–	–	–	+
P. palmivora	970423	*Hedera* sp.	France	–	–	–	–	+
P. parasitica	970029	*Lycopersicon esculentum*	France	–	–	–	–	+
P. taxonPgchlamydo	Haye,3,1	*Quercus* sp. soil	France	–	–	–	–	+
P. pseudosyringae	EW5	*Quercus* sp. soil	France	–	–	–	–	+
P. psychrophila	FF20	*Quercus* sp. soil	France	–	–	–	–	+
P. quercina	FNA	*Quercus* sp. soil	France	–	–	–	–	+
P. sojae	443	*Glycine max*	Unknown	–	–	–	–	+
P. syringae	2JZ2	*Quercus* sp. soil	France	–	–	–	–	+
P. syringae	4N0247/5	*Rhododendron* sp.	France	–	–	–	–	+
Pythium aphanidermatum	Ctsa	unknown	France	–	–	–	–	+
Pythium sylvaticum	0675/a	unknown	France	–	–	–	–	+
Pythium intermedium	02/84/1	unknown	France	–	–	–	–	+
Pythium irregulare	02/57/1	unknown	France	–	–	–	–	+
Pythium ultimum	433/3	unknown	France	–	–	–	–	+
Pythium spp	5N0691	*Photinia* sp.	France	–	–	–	–	+
Pythium spp.	3N1345-11	*Alnus glutinosa* soil	France	–	–	–	–	+

[a]ITS6 and ITS4 primers efficiently target *Phytophthora* and *Pythium* spp. ITS regions (Cooke et al., 2000).
[b]yielded a faint band of ca. 650 bp.

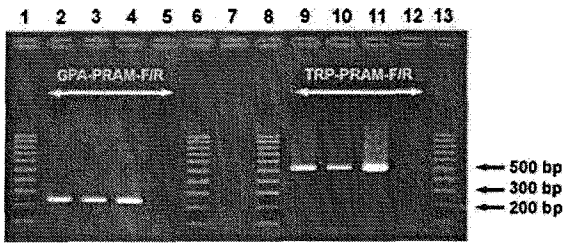

Figure 1. PCR products obtained with the two *P. ramorum* primer pairs developed in this study: GPA-PRAM-F/R and TRP-PRAM-F/R, respectively. Lanes 1, 6, 8 and 13: 100 bp DNA ladder (Sigma-Aldrich); lanes 2 and 9: DNA extracted from a naturally *P. ramorum*-infected *Rhododendron* spp. leaf; Lanes 3 and 10: DNA extracted from a naturally *P. ramorum*-infected *Viburnum* spp. leaf, Lanes 4 and 11: *P. ramorum* isolate 2N0983 DNA extract (positive control); Lanes 5 and 12: negative control (water). Lane 7 was not used.

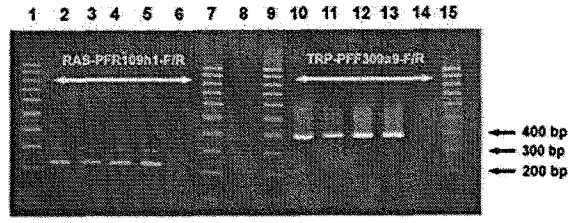

Figure 2. PCR products obtained with the two *P. fragariae* primer pairs tested in this study: RAS-PFR109h1-F/R and TRP-PFF309a9-F/R, respectively. Lanes 1, 7, 9 and 15: 100 bp DNA ladder (Sigma-Aldrich); lanes 2 and 10: DNA extracted from naturally *P. fragariae* var. *fragariae*-infected strawberry root sample PV35; Lanes 3 and 11: DNA extracted from naturally *P. fragariae* var. *fragariae*-infected strawberry root sample PV62; Lanes 4 and 12: *P. fragariae* var. *rubi* isolate CBS109.892 DNA extract (positive control); Lanes 5 and 13: *P. fragariae* var. *fragariae* isolate CBS309.62 DNA extract (positive control); Lanes 6 and 14: negative control (water). Lane 8 was not used. Using the RAS-PFR109h1F/R primer pair, a shadow aspecific band of ca 300 bp was systematically co-amplified with the DNA target but did not interfere with the sensitivity of the test.

Both *P. ramorum*-specific primer pairs yielded a positive signal of the expected size only when tested with *P. ramorum* isolates, regardless of their natural host, geographical origin and mating type (A1 or A2). Likewise, only the two varieties of *P. fragariae* yielded a positive signal with the two *P. fragariae*-specific primer pairs. However, *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi*, occurring on strawberry and on *Rubus* spp., respectively, could not be distinguished with these PCR tests.

The sensitivity threshold for each primer pair was determined experimentally using pure oomycete DNA (Table 1). Several molecular detection tools have already been developed for *P. fragariae* detection and many of them were extensively compared by Bonants et al. (2004). In this respect, the two *P. fragariae* PCR tests described here seem to be equally or even more sensitive than other published single-round PCR tests, except for those using Molecular Beacons™ which were able to detect down to 0.1 pg of *P. fragariae* DNA (Bonants et al., 2004). Likewise, the two *P. ramorum* primer PCR tests described here appear more sensitive than previously published *P. ramorum*-specific single-round PCR tests (Kroon et al., 2004), despite being less sensitive than the real-time PCR-based assay described by Tomlinson et al. (2005) or the nested PCR protocols developed to detect this pathogen (e.g. Martin et al., 2004; Hayden et al., 2004).

Our species-specific primer pairs were also successfully used to detect *P. ramorum* or *P. fragariae* in naturally infected plant tissues (Figures 1 and 2). In addition, during a 2-year survey of *P. ramorum*, the French Plant Protection Laboratory (LNPV-UMAF) carried out a comparative assessment of one of the *P. ramorum* PCR tests described here (TRP-PRAM-F/R) vs. isolation on the *Phytophthora* selective medium PARBHY (Robin et al., 1998). Total of 448 plant samples were simultaneously analysed using both methods. The results showed that for 86.7% of the samples, both techniques yielded identical results, whereas for 12.1% of the samples the PCR test detected the presence of *P. ramorum* while the isolation technique failed to do so. The 12.1% positive samples were further successfully confirmed using the alternative *P. ramorum*-specific primer pair GPA-PRAM-F/R. In the remnant 1.2%, PCR failed to detect *P. ramorum*, while it was successfully isolated on the selective medium. For these samples, the *P. ramorum* isolates recovered reacted positively with the TRP-PRAM-F/R PCR test. Therefore, this suggests that for these samples the biomass of *P. ramorum* was probably heterogeneously present between the respective PCR or isolation sub-samples.

The PCR primer pairs developed in this study should be of great interest for quarantine survey purposes. In addition, the use of at least two independent PCR tests for both pathogens may

strengthen the diagnosis. Such confirmation tests targeting independent loci might prevent the occurrence of 'false-positive' samples (Garbelotto, 2003).

Finally, the intronic regions from which these PCR primers were designed could also be potentially used for the development of detection tools for other economically important *Phytophthora* species.

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References

Anon. (2000) Council directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the community of organisms harmful to plants or plant products and against their spread within the community. OJ No L169, 2000.7.10, 1–112.

Anon. (2002) Commission decision (2002/757/EC) of 19 September 2002 on provisional emergency phytosanitary measures to prevent the introduction into and the spread within the Community of *Phytophthora ramorum* Werres, De Cock & Man in 't Veld sp. nov. OJ No L252, 2002-09-20, 3739.

Bonants PJM, vanGent-Pelzer MPE, Hooftman R, Cooke DEL, Guy DC and Duncan JM (2004) A combination of baiting and different PCR formats, including measurement of real-time quantitative fluorescence, for the detection of *Phytophthora fragariae* in strawberry plants. European Journal of Plant Pathology 110: 689–702.

Brasier CM, Kirk SA, Delcan J, Cooke DEL, Jung T and Man in't Veld WA (2004) *Phytophthora alni* sp. nov. and its variants: Designation of emerging heteroploid hybrid pathogens spreading on Alnus trees. Mycological Research 108: 1172–1184.

Cooke DEL, Drenth A, Duncan JM, Wagels G and Brasier CM (2000) A molecular phylogeny of Phytophthora and related oomycetes. Fungal Genetics and Biology 30: 17–32.

Garbelotto M (2003) Molecular diagnostics of *Phytophthora ramorum*, causal agent of Sudden Oak Death. Sudden Oak Death Online Symposium. www.apsnet.org/online/SOD.

Hayden KJ, Rizzo D, Tse J and Garbelotto M (2004) Detection and quantification of *Phytophthora ramorum* from California forests using a real-time polymerase chain reaction assay. Phytopathology 94: 1075–1083.

Ioos R, Husson C, Andrieux A and Frey P (2005) SCAR-based PCR primers to detect the hybrid pathogen *Phytophthora alni* and its subspecies causing alder disease in Europe. European Journal of Plant Pathology 112: 323–335.

Ioos R, Andrieux A, Marçais B and Frey P (2006) Genetic characterization of the natural hybrid species *Phytophthora alni* as inferred from nuclear and mitochondrial DNA analyses. Fungal Genetics and Biology 43: 511–529.

Kroon LPNM, Verstappen ECP, Kox LFF, Flier WG and Bonants PJM (2004) A rapid diagnostic test to distinguish between American and European populations of *Phytophthora ramorum*. Phytopathology 94: 613–620.

Martin FN, Tooley PW and Blomquist C (2004) Molecular detection of *Phytophthora ramorum*, the causal agent of Sudden Oak Death in California, and two additional species commonly recovered from diseased plant material. Phytopathology 94: 621–631.

Rizzo DM, Garbelotto M, Davidson JM, Slaughter GW and Koike ST (2002) Phytophthora ramorum as the cause of extensive mortality in Quercus spp. and *Lithocarpus densiflorus* in California. Plant Disease 86: 205–214.

Robin C, Desprez-Loustau ML, Capron G and Delatour C (1998) First record of *Phytophthora cinnamomi* on cork and holm oak in France and evidence of pathogenicity. Annals of Forest Science 55: 869–883.

Tomlinson JA, Boonham N, Hughes KJD, Griffin RL and Barker I (2005) On-site extraction and Real-time PCR for detection of *Phytophthora ramorum* in the field. Applied and Environmental Microbiology 11: 6702–6710.

Chapitre III

Développement de marqueurs microsatellites chez le taxon hybride *Phytophthora alni* subsp. *alni*.

III.1. - Présentation de la stratégie scientifique.

Les résultats présentés dans le chapitre 2 tendent à démontrer que *Paa* a été généré par l'hybridation de *Pam* et *Pau* ou de taxons qui leurs sont très proches. Les marqueurs microsatellites, qui ont la propriété d'être codominants, devraient permettre de confirmer la constitution génétique de *Paa*, c'est-à-dire une juxtaposition de génomes de type *Pam* et de type *Pau*. De plus, l'étude de la variabilité des allèles observés devrait permettre une première estimation de la variabilité génétique au sein d'une collection limitée mais représentative de la population européenne des différents taxons constituant *P. alni sensu lato*. Cette dernière partie de la thèse a consisté à isoler des marqueurs microsatellites à partir d'un isolat de *Phytophthora alni* subsp. *alni* en utilisant un protocole de criblage par hybridation avec des sondes de motifs microsatellites marquées à la digoxygénine. L'ADN total de l'isolat de *Paa* utilisé a tout d'abord été enrichi en motifs microsatellites via l'utilisation de sondes de motifs microsatellites greffées sur billes magnétiques.
Les séquences présentant des motifs microsatellites exploitables devaient ensuite permettre de définir des couples d'amorces de PCR spécifiques de chaque locus. Les produits de PCR locus-spécifiques obtenus avec la collection de *P. alni* devaient ensuite être caractérisés par génotypage (marquage des amorces avec différents fluorochromes et analyse par électrophorèse capillaire).

Le développement et les résultats fournis par l'utilisation de ces marqueurs microsatellites chez *P. alni* subsp. *alni* sont présentés dans un article accepté pour publication dans la revue « Molecular Ecology Notes ».

Publication 6 :

Ioos R., Barrès B., Andrieux A., and Frey P. (2006) Characterization of microsatellite markers in the interspecific hybrid *Phytophthora alni* subsp. *alni* and cross-amplification with related taxa. *Molecular Ecology Notes*. Sous presse.

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PRIMER NOTE

Characterization of microsatellite markers in the interspecific hybrid *Phytophthora alni* ssp. *alni*, and cross-amplification with related taxa

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Abstract

***Phytophthora alni* ssp. *alni* is an interspecific hybrid oomycete causing a large-scale decay of alders throughout Europe. In this study we developed a set of 10 microsatellite markers that shows promise for population studies and for studying hybridization events between the parental species of the hybrid. Moreover, the genotype and the ploidy of the different subspecies of *P. alni* might be inferred from the quantitative ratio of amplified genome-specific alleles. Nine primer pairs cross amplified with the related species *Phytophthora cambivora* and *Phytophthora fragariae* and yielded distinct alleles.**

Keywords: genotyping, oomycete, polyploidy

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Phytophthora alni ssp. *alni* (*Paa*) is a recently described interspecific hybrid oomycete causing a lethal disease of alder throughout Europe (Brasier *et al.* 2004). Two other taxa close to *Paa* are isolated from diseased alders or from soil beneath alders: *Phytophthora alni* ssp. *uniformis* (*Pau*) and *Phytophthora alni* ssp. *multiformis* (*Pam*). All three taxa are phylogenetically very close to *Phytophthora cambivora* (*Pc*) and *Phytophthora fragariae* (*Pf*) (Brasier *et al.* 1999). Previously hypothesized to originate from genetic breakdown of *Paa* (Brasier *et al.* 1999), *Pam* and *Pau* were actually suggested to represent distinct species and to have generated *Paa* through interspecific hybridization (Ioos *et al.* 2006). In addition, Brasier *et al.* (1999) reported that *Paa* was a near tetraploid species (4n + 2) in respect with the typical diploid (2n) status of the *Phytophthora* genus, and that *Pam* and *Pau* were aneuploid species with 2n + 4 to 2n + 7, and 2n + 2 chromosomes, respectively. However, Ioos *et al.* (2006) demonstrated that three divergent alleles for four unlinked single copy nuclear genes were observed within the *Paa* genome. Two of them matched the two alleles also found in *Pam* and the third one corresponded to the single allele found in *Pau*. As these results contrasted with the ploidy levels reported, and as little is known about the genetic polymorphism within *Paa* and the two other taxa, we isolated and characterized microsatellite loci in *Paa*. These codominant markers are especially useful to address questions relating to origin and genetic diversity of fungi (Weising *et al.* 1995).

Microsatellite markers were isolated from *Paa* using enrichment libraries. DNA was extracted from *Paa* isolate PAA130 using DNeasy Plant Mini Kit (QIAGEN). Approximately 500 ng μL^{-1} DNA were recovered and used for the construction of five enrichment libraries using biotinylated oligoprobes [$(AC)_{13}$, $(AG)_{13}$, $(ACG)_6$, $(AAC)_6$ and $(AAG)_6$] and streptavidin-coated magnetic beads, according to Dutech *et al.* (2000). The libraries were cloned using a TOPO TA Cloning kit (Invitrogen). For each of the five enrichments, 288 clones were screened by colony blot following a protocol developed by Estoup *et al.* (1993). A total of 172 positive clones were subsequently size-screened by polymerase chain reaction (PCR) using vector's primers and 43 clones with insert between 400 and 1000 bp were retained for sequencing. Eighteen primer pairs could be designed from the sequences, either manually or using PRIMER 3 software (Rozen & Skaletski 2000). The primer pairs were tested by PCR with a panel of 6 *Paa*, 4 *Pam*, 8 *Pau*, 2 *Pc* and 2 *Pf* isolates, from various geographical origins in Europe and displaying different mitotypes according to Ioos *et al.* (2006) (Table 1). PCR conditions were optimized for each primer pair. PCR was conducted in a 20-µL volume containing 15–20 ng of

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Table 1 List of the isolates used in the study. Mitotypes of the isolates determined as described in Ioos *et al.* (2006)

Species	Isolate	Mitotype	Host	Geographical origin	Year isolated
Phytophthora alni ssp. *alni*	PAA129	U	*Alnus glutinosa*	SW France	2003
	PAA130*	M	*Alnus glutinosa*	NE France	2003
	PAA143	M″	*Alnus glutinosa*	Poland	2002
	PAA151	U	*Alnus glutinosa*	NE France	2004
	PAA162	U	*Alnus glutinosa*	Germany	2004
	PAAa2′	U	*Alnus glutinosa*	NE France	2005
	PAAe3	M	*Alnus glutinosa*	NE France	2005
	PAA151	U	*Alnus glutinosa*	NE France	2004
	PAA70	M	*Alnus glutinosa*	the Netherlands	Unknown
	PAAa2	M	*Alnus glutinosa*	NE France	2005
	PAA38	M	*Alnus glutinosa*	N France	2002
	PAA74	U	*Alnus glutinosa*	Scotland	2000
	PAA75	M	*Alnus viridis*	Scotland	2000
	PAA81	U	*Alnus glutinosa*	England	1997
	PAA82	M	*Alnus glutinosa*	England	1996
	PAAe1	M	*Alnus glutinosa*	NE France	2005
	PAA85	M	*Alnus glutinosa*	England	Unknown
	PAA86	M	*Alnus glutinosa*	Belgium	1999
	PAA88	M	*Alnus glutinosa*	Belgium	2001
	PAA91	M′	*Alnus glutinosa*	Hungary	2001
	PAA112	M	*Alnus glutinosa*	NW France	2003
	PAA114	M	*Alnus glutinosa*	NE France	2002
	PAA134	M	*Alnus glutinosa*	Germany	2000
	PAA141	U′	*Alnus glutinosa*	Austria	Unknown
	PAA144	M″	*Alnus glutinosa*	Poland	2003
	PAA178	M″	*Alnus glutinosa*	Poland	2003
	PAA185	M	*Alnus glutinosa*	NE France	2004
	PAA194	M	*Alnus glutinosa*	NW France	2005
	PAAd6′	U	*Alnus glutinosa*	NW France	2005
	PAAa10	M	*Alnus glutinosa*	N France	2005
	PAAc10	M	*Alnus glutinosa*	N France	2005
	PAAd2′	M′	*Alnus glutinosa*	NE France	2005
	PAAe2′	M	*Alnus glutinosa*	NE France	2005
	PAAa11′	M	*Alnus glutinosa*	Germany	2005
Phytophthora alni ssp. *multiformis*	PAM54	M	*Alnus glutinosa*	NW France	2000
	PAM71	M	*Alnus glutinosa* soil	the Netherlands	1995
	PAM90	M	*Alnus glutinosa* soil	the Netherlands	1995
	PAM186	M′	*Alnus glutinosa*	Belgium	2001
	PAM73	nd	*Alnus glutinosa*	England	1996
Phytophthora alni ssp. *uniformis*	PAU60	U″	*Alnus glutinosa*	NE France	1999
	PAU84	U′	*Alnus glutinosa*	Sweden	1999
	PAU87	U	*Alnus glutinosa*	Belgium	2001
	PAU89	U	*Alnus cordata*	Italy	2000
	PAU142	U	*Alnus glutinosa*	Slovenia	2000
	PAU188	U	*Alnus incana*	Belgium	2001
	PAUb3	U″	*Alnus glutinosa*	NE France	2005
	PAUc6′	U	*Alnus glutinosa*	NE France	2005
	PAU187	U	*Alnus glutinosa*	Belgium	2001
	PAU96	U	*Alnus glutinosa*	Hungary	1999
	PAU97	U	*Alnus glutinosa* soil	Hungary	1999
	PAU98	U	*Alnus glutinosa* soil	Hungary	1999
	PAUb3	U′	*Alnus glutinosa*	NE France	2005
Phytophthora cambivora	PCJC17	C2	*Quercus* sp. soil	NE France	1999
	PC643	C1	*Castanea sativa* soil	SW France	2000
	PC463	C2	*Castanea sativa*	SW France	1994
	PCga1	C2	*Quercus* sp. soil	NE France	1999

Table 1 *Continued*

Species	Isolate	Mitotype	Host	Geographical origin	Year isolated
	PC99428	C2	*Castanea sativa*	France	1999
	PCst3r1	C2	*Quercus petraea*	SW France	1999
	PC627	C2	*Castanea sativa*	Italy	1999
	PC1a21	C2	*Quercus* sp. soil	NW France	1999
	PC4n444	C2	*Castanea sativa*	NW France	2004
	PC4n1125	C2	*Castanea sativa*	NW France	2004
	PC051422	C2	*Castanea sativa*	NW France	2005
Phytophthora fragariae var. *fragariae*	PFF309	FF	*Fragaria* × *ananassa*	Great Britain	1962
	PFFCSL	FF	*Fragaria* × *ananassa*	Unknown	Unknown
	PFF209	FF	*Fragaria* × *ananassa*	England	1946
Phytophthora fragariae var. *rubi*	PFR109	FR	*Rubus* sp.	Great Britain	1991
	PFR163	FR	*Rubus* sp.	France	Unknown
	PFR59	FR	*Rubus* sp.	Scotland	Unknown
	PFRCSL	FR	*Rubus* sp.	England	Unknown
	PFR96795	FR	*Rubus* sp.	Scotland	1985
Phytophthora cactorum	CAC4810	nd	Unknown	France	Unknown
Phytophthora cinnamomi	DSF970060	nd	*Quercus suber*	France	1999
Phytophthora citricola	AUL045	nd	*Alnus glutinosa*	France	1999
Phytophthora citrophthora	2 N1021	nd	*Rosa* sp.	France	2002
Phytophthora cryptogea	990675	nd	*Actinidia sinensis*	France	1999
Phytophthora erythroseptica	960713	nd	*Polygonum oberti*	France	1999
Phytophthora europaea	AL5	nd	*Quercus* sp. soil	France	1998
Phytophthora gonapodyides	Gonap4	nd	*Quercus* sp. soil	France	1998
Phytophthora humicola	3 N1245j	nd	*Alnus glutinosa* soil	France	2003
Phytophthora inundata	9500802	nd	*Alnus glutinosa* soil	France	1998
Phytophthora lateralis	98093–1 PV	nd	*Chamaecyparis* sp.	France	1998
Phytophthora megasperma	BK1	nd	*Quercus* sp. soil	France	1998
Phytophthora nicotianae	960579	nd	*Nicotiana tabacum*	France	1996
Phytophthora taxon forest soil	8CARPPOC1	nd	*Quercus* sp. soil	France	1998
Phytophthora palmivora	970423	nd	*Hedera* sp.	France	1997
Phytophthora parasitica	970029	nd	*Lycopersicon* sp.	France	1997
Phytophthora taxon Pgchlamydo	Haye,3,1	nd	*Quercus* sp. soil	France	1998
Phytophthora pseudosyringae	EW5	nd	*Quercus* sp. soil	France	1998
Phytophthora psychrophila	FF20	nd	*Quercus* sp. soil	France	1998
Phytophthora quercina	FNA	nd	*Quercus* sp. soil	France	1999
Phytophthora ramorum	2 N083	nd	*Rhododendron* sp.	France	2002
Phytophthora sojae	443	nd	*Glycine max*	Unknown	Unknown
Phytophthora syringae	2JZ2	nd	*Quercus* sp. soil	France	1999

*, isolate used for microsatellite isolation; nd, not determined.

template DNA, 2 μL of 10× polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, 0.5 U of *Taq* polymerase (Sigma) and water was added to 20 μL. Touchdown PCR conditions were first five cycles at 94 °C for 30 s, 66–62 °C (see initial annealing temperatures, Table 2) for 30 s and 72 °c for 1 min; followed by 8 cycles at 94 °C for 30 s, 66–55 °C minus 0.5 °C per cycle for 30 s, and 72 °C for 1 min; 22 cycles at 94 °C for 30 s, 62–51 °C for 30 s, and 72 °C for 1 min; and a final elongation step at 65 °C for 30 min. All retained forward primers were labelled to allow size and dye multiplexing. Allele sizes were determined on a CEQ 8000 Genetic Analysis System (Beckman Coulter). Eight out of the 18 primer pairs tested yielded no polymorphism or unclear pattern and were therefore discarded. The remaining 10 primer pairs were tested by PCR on a larger collection of *Paa, Pau, Pam, P. cambivora* and *P. fragariae* isolates as well as with 23 other *Phytophthora* spp. (Table 1).

Except PA12-F/R that were specific to *Paa* and *Pam* but gave no size polymorphism, all the primers retained yielded an amplicon with *Paa, Pau, Pam, Pc* and *Pf*, confirming their close kinship. In *Paa*, the number of alleles for the nine polymorphic loci ranged from two to five indicating a low level of allelic variation, consistent with this hybrid species being of recent origin (Brasier *et al.* 2004; Ioos *et al.* 2006). In addition, all the alleles observed in *Paa* were those encountered either in *Pam* or in *Pau* (Table 2), strengthening the

Table 2 Primer sequences and characteristics for the nine polymorphic microsatellite loci isolated from *Phytophthora alni* ssp. *alni*. All reverse PCR primers were fluorescently labelled

Locus	Repeat motif	GenBank Accession no.	Primer sequence (5′–3′)	Size (bp)	Annealing T°C*	Total number of different alleles/no. of alleles per individual (allele sizes)						
						P. alni ssp. *alni*	*P. alni* ssp. *multiformis*	*P. alni* ssp. *uniformis*	*P. cambivora*	*P. fragariae* var *fragariae*	*P. fragariae* var *rubi*	other species
PA3	$(CAA)GA(CAA)_5$	DQ665899	F: CTTGGATAGAGCCGTCGTTC R: TCCTACTGTTGGGAGCAAGG	201	62 °C	3/2† (198, 212, 216)	2/1–2† (198, 216)	2/1–2 (198, 212)	4/3 (210, 211, 213, 215)	1/1 (217)	1/1 (217)	—
PA6	$(CAA)_{11}TAA(CAA)$	DQ665900	F: AACACCGCGTTGAAGACG R: GTAGCCACCGCACATGAATC	303	66 °C	3/3† (278, 284, 287)	1/1 (278)	3/3 (278, 284, 287)	2/2 (278, 281)	2/1–2 (275, 288)	2/2 (275, 288)	—
PA8	$(ACA)_5T(ACA)$	DQ665901	F: GGTCAGCCAAGAGCAAAGAG R: CTGTGAGCTGCAAGAAGCAG	376	66 °C	5/3–5† (356, 359, 361, 367, 368)	3/2–3† (356, 359, 368)	2/2 (361, 367)	2/1–2 (367, 369)	2/2 (364, 367)	1/1 (367)	*P. europaea* (368)
PA11	$(CAA)_5CAG(CAA)$	DQ665902	F: TGCAAACAGTGCGTCTCTTC R: CCTAGATCCAGCGACAGCTC	226	62 °C	2/2† (226, 232)	2/1–2† (226, 232)	1/1 (226)	2/1–2 (224, 225)	1/1 (226)	1/1 (225)	—
PA14	$(CT)_7(\ldots)(TTTC)_4$	DQ665904	F: TGGCAAACAGACACGAAGTC R: GAAACCCAGTCATCCGAGAG	173	62 °C	3/3† (173, 175, 177)	2/2† (173, 175)	1/1 (177)	4/2 (174, 177, 179, 181)	2/2 (168, 174)	2/2 (175, 177)	—
PA17	$(GTC)_4(\ldots)(GC)_4$	DQ665905	F: AGCGACAATGCAGGAAGC R: CTGTCTGGGCATTCATGTCG	317	62 °C	2/2 (313, 317)	2/2 (313, 317)	1/1 (313)	2/1–2 (309, 313)	2/2 (308, 312)	1/1 (315)	*P. europaea* (385)
PA23	$(GAA)_7GGA(GAA)_3$	DQ665906	F: GGAGATAGCCACGAGACACC R: CAAGCATCGCTGTAAACGAC	155	62 °C	2/2† (133, 148)	2/2 (133, 148)	1/1 (148)	1/1 (148)	1/1 (148)	1/1 (148)	—
PA30	$(AC)_8$	DQ665907	F: TAGGGGACTTAAACCCCA R: GTTGGCGGACTAGAGATTT	127	66 °C	4/4† (120, 122, 123, 124)	3/3† (120, 122, 124)	1/1 (123)	2/2 (118, 122)	2/2 (120, 122)	2/2 (120, 122)	—
PA31	$(CAA)_8$	DQ665908	F: GCTTCTCACTGCACAGCAAC R: AGGGTATTGGAGCCTGATGC	195	62 °C	2/1–2† (183, 192)	1/1 (192)	2/2 (183, 192)	4/3–4 (174, 180, 186, 192)	1/1 (150)	1/1 (150)	—

*, initial annealing temperature for touchdown PCR; †, loci with alleles consistently showing different peak areas.

hypothesis that *Pau* and *Pam* may actually be the progenitors of *Paa*. Due to the uncertain nature of ploidy in these different taxa, expected heterozygosities and linkage disequilibria were not computed. However, depending on the locus, up to three or five different alleles were simultaneously observed in individual *Pam* and *Paa* isolates, respectively. In addition, genotyping of the PCR products obtained with the primer pairs PA3, PA6, PA8, PA11, PA14, PA23, PA30 and PA31 showed that for individual *Paa* and *Pam* isolates, the respective quantities of the different alleles (based on peak areas) were not always equal. The occurrence of unbalanced peak ratios suggests these species are polyploid (Christiansen 2006). The genotype amplification patterns for these loci will be particularly useful to unravel the genotypes of *Paa* and *Pam*.

References

Brasier CM, Cooke DEL, Duncan JM (1999) Origin of a new *Phytophthora* pathogen through interspecific hybridization. *Proceedings of the National Academy of Sciences, USA*, **96**, 5878–5883.

Brasier CM, Kirk SA, Delcan J *et al.* (2004) *Phytophthora alni* sp. nov. & its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. *Mycological Research*, **108**, 1172–1184.

Christiansen DG (2006) A microsatellite-based method for genotyping diploid and triploid water frog of the *Rana esculenta* hybrid complex. *Molecular Ecology Notes*, **5**, 190–193.

Dutech C, Amsellem L, Billotte N, Jarne P (2000) Characterization of $(GA)_n$ microsatellite loci using an enrichment protocol in the neotropical tree species *Vouacapoua americana*. *Molecular Ecology*, **9**, 1433–1435.

Estoup A, Solignac M, Harry M, Cornuet JM (1993) Characterization of $(GT)_n$ and $(CT)_n$ microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*. *Nucleic Acids Research*, **21**, 1427–1431.

Ioos R, Andrieux A, Marçais B, Frey P (2006) Genetic characterization of the natural hybrid species *Phytophthora alni* as inferred from nuclear and mitochondrial DNA analyses. *Fungal Genetics and Biology*, **43**, 511–529.

Rozen S, Skaletski H (2000) PRIMER3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey.

Weising K, Nybom H, Wolff K, Meyer W (1995) *DNA Fingerprinting in Plants and Fungi*. CRC press, Boca Raton, Florida.

III.2. - Utilité des marqueurs microsatellites développés.

III.2.1. - Les amorces microsatellites développées sont majoritairement trans-spécifiques au sein du groupe *P. alni* / *P. cambivora* / *P. fragariae.*

Une série de 9 marqueurs microsatellites polymorphes chez *Paa* a pu être développée. Ces derniers ont permis par ailleurs l'amplification des mêmes loci chez *Pam* et *Pau*, comme attendu, mais aussi chez *P. cambivora* et *P. fragariae*. En revanche, les allèles observés chez *P. cambivora* et *P. fragariae* étaient souvent distincts de ceux observés chez *Paa*, *Pam* et *Pau*. Ceci confirme bien la proximité génétique entre les trois espèces, mais infirme à nouveau la possibilité d'une implication directe de *P. cambivora* et *P. fragariae* dans la genèse de *P. alni* subsp. *alni*. Comme lors de l'étude des quatre gènes nucléaires (Publication 3), les allèles identifiés chez *Paa* étaient exclusivement composés de ceux présents soit chez *Pam* ou chez *Pau*. De même, les génotypes observés chez *Paa* correspondaient à l'addition des génotypes observés chez *Pam* et chez *Pau*, preuve supplémentaire de l'implication de ces deux taxons dans la genèse de *Paa*. Enfin, pour certains loci, différents génotypes de *Paa* ont pu être observés, chacun correspondant à l'addition de deux génotypes particuliers parmi ceux observés chez les différents *Pam* et les différents *Pau*, respectivement. Ceci semble indiquer qu'il aurait pu y avoir plusieurs évènements d'hybridations entre ces deux dernières entités, comme le laisser déjà supposer la distribution dichotomique de l'ADN mitochondrial chez les différents *Paa* que nous avons étudiés dans le cadre de la publication 3.

III.2.2. - Observation d'un faible niveau de polymorphisme.

Globalement, les marqueurs microsatellites que nous avons pu isoler sont peu polymorphes au sein du groupe *Paa*, *Pam* et *Pau*. En revanche, les génotypes obtenus avec la plupart d'entre eux permettent de discriminer *Pau* de *Pam*. Chez *Paa*, le polymorphisme observé correspond à la somme des polymorphismes mis en évidence chez les isolats de *Pam* et de *Pau.* Au total, de 2 à 5 allèles ont été observés selon le locus microsatellite. Ce faible niveau de polymorphisme est toutefois en accord avec le faible nombre d'allèles observé chez d'autres espèces de *Phytophthora* : 2 à 4 allèles par locus chez *P. ramorum* (Prospero et al., 2004), 1 à 4 allèles par locus chez *P. cinnamomi* (Dobrowolski et al., 2002), 2 à 9 allèles par locus chez *P. infestans* (Lees et al., 2006).

Néanmoins, le faible niveau de polymorphisme que nous avons observé pour les marqueurs que nous avons isolés peut avoir deux explications pouvant d'ailleurs se compléter : *i)* tous les loci microsatellites de *P. alni sensu lato* sont faiblement polymorphes ce qui est une conséquence conjuguée de la création récente de l'hybride, et surtout de l'absence apparente de reproduction sexuée chez *Paa*, *Pam* et *Pau* ou *ii)* certains loci microsatellites de *P. alni* sont plus polymorphes mais les techniques d'enrichissement et de criblage que nous avons utilisées ne nous ont pas permis de sélectionner suffisamment de marqueurs, ce qui aurait augmenté la probabilité d'isoler des loci plus polymorphes. Cette dernière hypothèse a par ailleurs déjà été vérifiée dans le cas de *P. ramorum*

puisque Ivors et al. (2006) ont développé des marqueurs significativement plus polymorphes (2 à 9 allèles par locus) que Prospero et al. (2004) en recherchant, non plus par des techniques de criblage chimique, mais de façon exhaustive et *in silico* des loci microsatellites chez *P. ramorum* à partir de la séquence complète de son génome.

Cependant, *Paa*, *Pau* et *Pam* étant des espèces homothalliques, il ne reste au demeurant pas surprenant d'observer un niveau de polymorphisme équivalent ou un peu plus faible que chez les espèces citées plus haut, toutes hétérothalliques.

Enfin, le polymorphisme des différents loci microsatellites qui ont été isolés n'a été estimé que sur un faible nombre d'isolats des taxons *Pam* et *Pau.* Malgré leur apparente rareté dans la nature (Brasier et al., 2004 ; R. Ioos, observations personnelles), une certaine variabilité génétique a pu être mise en évidence avec ces outils, et il serait intéressant de pouvoir collecter de nouveaux isolats.

III.2.3. - Génotypes complexes, ratios d'amplifications alléliques et polyploïdie.

Le génotypage des produits de PCR générés avec les couples d'amorces que nous avons définis a montré que pour quatre loci parmi les dix utilisés, plus de deux allèles pouvaient être observés pour un même isolat chez *Paa*. Par exemple au locus PA8, jusqu'à 5 allèles différents ont pu être observés simultanément chez des isolats de *Paa*. De même, à deux loci particuliers (PA8 et PA30) jusqu'à trois allèles ont pu être observés chez des isolats de *Pam*, tandis que 3 allèles ont été systématiquement observés chez *Pau* au locus PA6. L'existence de ces génotypes multi-alléliques peut s'expliquer de deux façons : *i)* polysomie voire polyploïdie du taxon en question et/ou *ii)* duplication de certains loci microsatellites dans le génome.

Bien que ces deux explications ne soient pas exclusives l'une de l'autre, l'observation des hauteurs relatives des pics correspondant aux différents allèles sur les profils de génotypage permet de favoriser une interprétation rigoureuse du profil multi-allélique observé, locus par locus. En effet, Christiansen (2005) et Christiansen et al. (2005) ont démontré que l'observation et la mesure des ratios entre les logarithmes des hauteurs des différents pics détectés (correspondant chacun à un allèle), permettait de définir le génotype d'espèces polyploïdes et de ploïdie connue. Cette démonstration part du principe que des séquences similaires présentant des séquences cibles identiques pour les amorces (i.e. les différentes séquences alléliques d'un locus donné) sont amplifiées avec le même rendement. Ainsi à titre d'exemple, un ratio « log [hauteur pic allèle « A » / hauteur pic allèle « B »] » égal à 0.5 (*i.e.* log (3/1)) correspond donc théoriquement à un génotype « hétérozygote complexe » 'A/A/A/B' chez un taxon tétraploïde (on parlera aussi d'hétérozygotie homéologue). En revanche, lorsque deux pics « A » et « B » présentent la même hauteur, ils sont présents en même quantité initiale dans le génome, et l'on a alors affaire à un génotype « hétérozygote simple » du type 'A/B' pour un taxon diploïde ou 'A/A/B/B' pour un taxon tétraploïde.

Au final, tous les isolats de *Pau* ont présenté des profils simples, homozygotes ou hétérozygotes. Notable exception, le locus PA6, pour lequel tous les isolats de *Pau* présentent simultanément 3 allèles

a très probablement été dupliqué car les trois pics présentent la même hauteur (Figure 5). Cependant, une trisomie ponctuelle de ce taxon *Pau* à ce locus PA6 pourrait être une explication alternative, puisque ce phénomène a déjà été observé chez *P. cinnamomi* (Dobrowolski et al., 2002) et *P. infestans* (Lees et al., 2006).

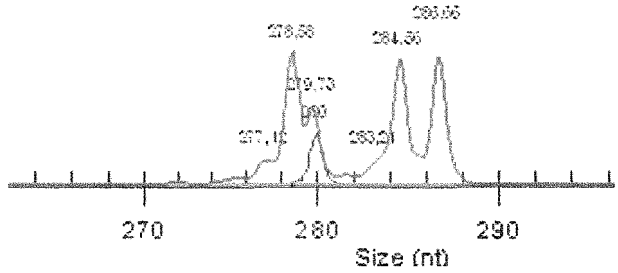
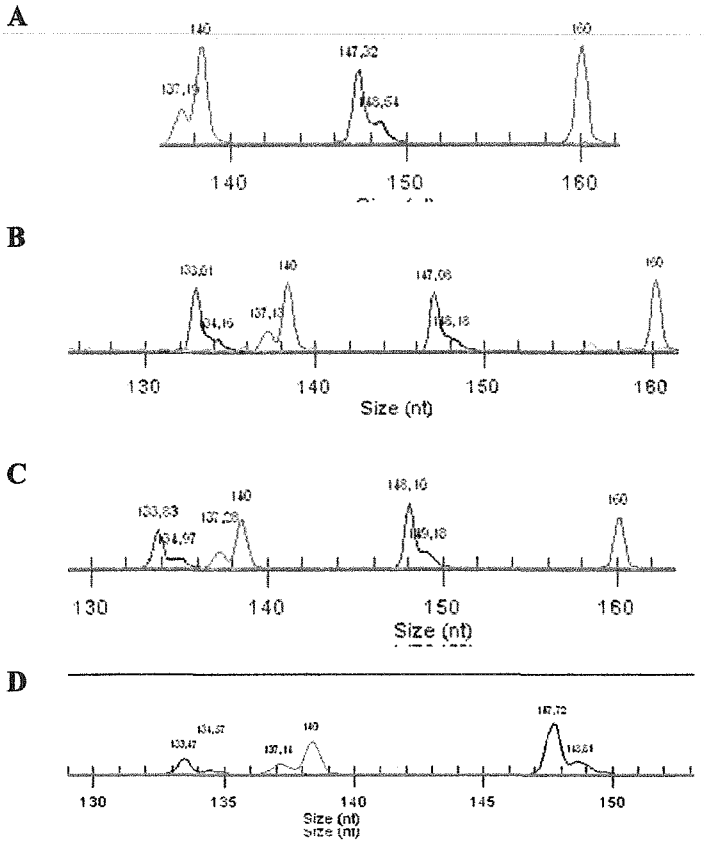


Figure 5 : Chromatogramme obtenu suite au génotypage par électrophorèse capillaire de produits de PCR générés en utilisant le couple d'amorces microsatellites PA6 (marquage 'bleu'). Trois pics sont observés chez *Pau*, mais présentent des hauteurs équivalentes. On peut donc en conclure que ce locus *i)* est trisomique, ou *ii)* a fait l'objet d'une duplication.

Comme *Pau* ne présente que un à deux allèles pour tous les autres loci, il est raisonnable de postuler que ce taxon est bien proche de la diploïdie.

En revanche, pour un certain nombre de loci chez *Paa* et chez *Pam*, le phénomène de différence de hauteur de pics alléliques est systématiquement observé, ce qui confirmerait bien la nature polyploïde de ces deux espèces. Néanmoins, il a été impossible en l'état actuel des résultats de réaliser des calculs précis en matière de ratios, et donc d'en déduire des génotypes précis, puisque la ploïdie exacte de ces différents taxons n'est pas connue avec exactitude. A titre d'exemple, les figures 6 et 7 présentent l'interprétation de différents génotypes obtenus à partir d'isolats des trois taxons et de mélanges artificiels. Il est notamment possible de reconstituer artificiellement un génotype « hétérozygote complexe » de *Paa*, en mélangeant de l'ADN de *Pam* et de *Pau*.



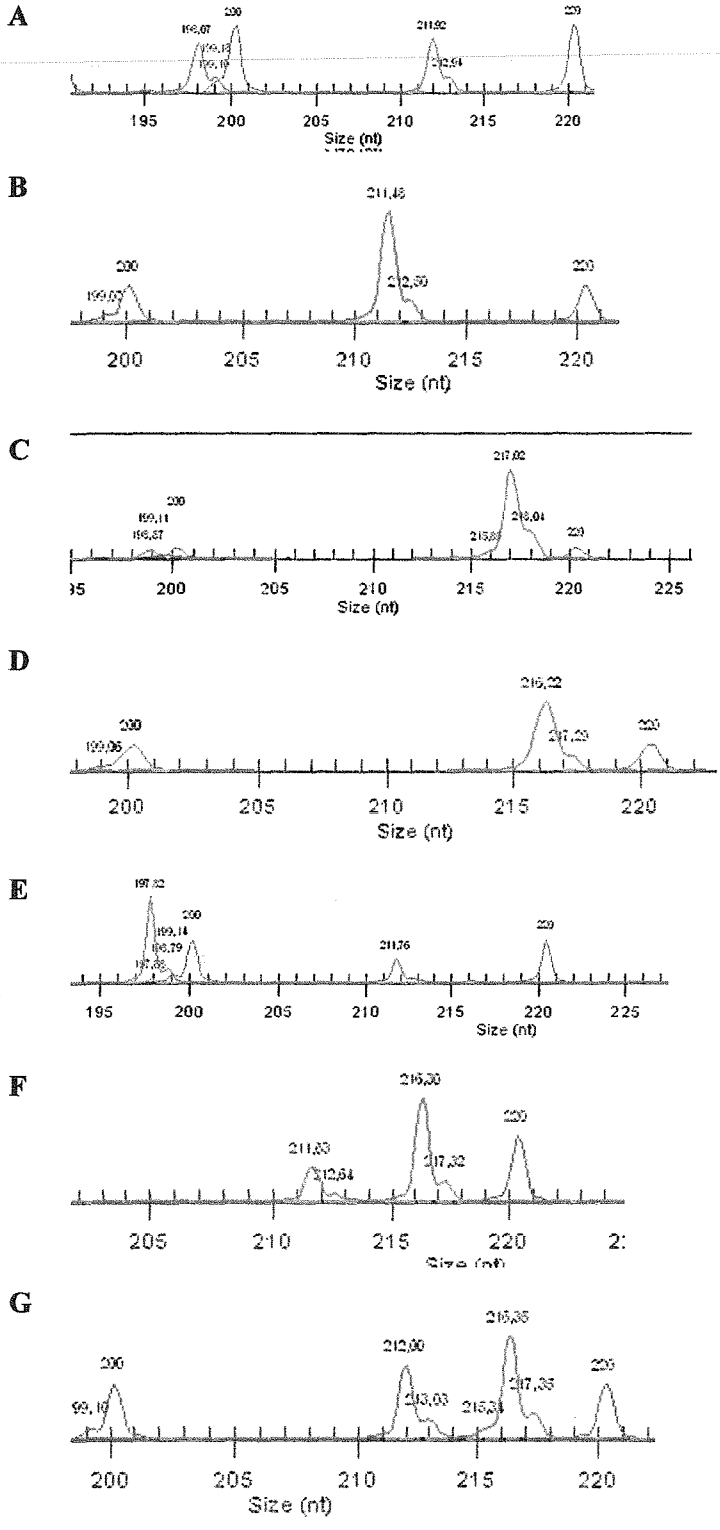
Pau (isolat PAU60)
Un seul allèle est détecté, noté 148. On a donc affaire à un génotype homozygote '**148/148**'

Pam (isolat PAM54)
Deux allèles sont détectés, notés 133 et 148. Les pics présentent la même hauteur. On a donc affaire à un génotype « hétérozygote simple » '$(133)_{n\ fois}/(148)_{n\ fois}$', puisqu'on ne connaît pas la ploïdie exacte.

Paa (isolat PAA88)
Deux allèles sont détectés, notés 133 et 148. Les pics présentent des hauteurs différentes. On a donc affaire à un génotype « hétérozygote complexe » '$(133)_{x\ fois}/(148)_{y\ fois}$', puisqu'on ne connaît pas la ploïdie exacte.

Mélange artificiel *Pam*+*Pau* (isolats PAM54 et PAU60)
Deux allèles sont détectés, notés 133 et 148. Les pics présentent des hauteurs différentes. On a donc recréé artificiellement un génotype « hétérozygote » complexe '$(133)_{n\ fois}/(148)_{n+2\ fois}$', similaire à celui d'un *Paa*.

Figure 6 : Chromatogrammes obtenus suite au génotypage par électrophorèse capillaire de produits de PCR générés en utilisant le couple d'amorces microsatellites PA23 (marquage 'noir'). Un seul génotype est observé chez *Pau* (A), et un seul autre génotype est observé chez *Pam* (B). Un génotype « hétérozygote complexe » est observé chez *Paa* (C). En génotypant le produit de PCR obtenu avec PA23-F/R sur un mélange expérimental de quantités équivalentes d'ADN de *Pam* et de *Pau*, un génotype de type « hétérozygote complexe » ou de *Paa* similaire au cas C peut être créé artificiellement. (D).



Pau (isolat PAU84)
Deux allèles sont détectés, notés 198 et 212. Les pics présentent la même hauteur. On a donc affaire à un génotype « hétérozygote simple » '**198/212**'

Pau (isolat PAU60)
Un seul allèle est détecté, noté 212. On a donc affaire à un génotype homozygote '**212/212**'

Pam (isolat PAM90)
Deux allèles sont détectés, notés 198 et 216. Les pics présentent des hauteurs différentes. On a donc affaire à un génotype « hétérozygote complexe » '$(198)_{x\ fois}/(216)_{y\ fois}$', puisqu'on ne connaît pas la ploïdie exacte.

Pam (isolat PAM54)
Un seul allèle est détecté, noté 216. On a donc affaire à un génotype « homozygote » '$216/(216)_{n\ fois}$', puisqu'on ne connaît pas la ploïdie exacte.

Paa (isolat PAA88)
Deux allèles sont détectés, notés 198 et 212. Les pics présentent des hauteurs différentes. On a donc affaire à un génotype « hétérozygote complexe » '$(198)_{p\ fois}/(212)_{q\ fois}$', puisqu'on ne connaît pas la ploïdie exacte.

Paa (isolat PAA178)
Deux allèles sont détectés, notés 212 et 216. Les pics présentent des hauteurs différentes. On a donc affaire à un génotype « hétérozygote complexe » '$(212)_{m\ fois}/(216)_{n\ fois}$', puisqu'on ne connaît pas la ploïdie exacte.

Mélange artificiel *Pam+Pau* (isolats PAM54 et PAU60)
Deux allèles sont détectés, notés 212 et 216. Les pics présentent des hauteurs différentes. On a donc recréé artificiellement un génotype « hétérozygote complexe » '$(212)_2/(216)_{n+1\ fois}$', similaire à celui d'un *Paa*.

Figure 7 : Chromatogrammes obtenus suite au génotypage par électrophorèse capillaire de produits de PCR générés en utilisant le couple d'amorces microsatellites PA3 (marquage 'bleu'). Deux génotypes sont observés chez *Pau* (A ou B), et deux autres génotypes sont observés chez *Pam*, dont un « hétérozygote complexe » (C ou D). Deux génotypes « hétérozygote complexe » sont observés chez *Paa* (E ou F). En génotypant le produit de PCR obtenu avec PA6-F/R sur un mélange expérimental de quantités équivalentes d'ADN de *Pam* et de *Pau*, un génotype de type « hétérozygote complexe » de *Paa* similaire au cas F peut être créé artificiellement (G).

Chapitre IV

L'hybridation interspécifique dans le genre *Phytophthora*.

Publication 7

Ioos R., Man in't Veld W.A., and Frey P. An appraisal of interspecific hybridization in the genus *Phytophthora*. En préparation.

An appraisal of interspecific hybridization in the genus *Phytophthora*.

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Abstract

Interspecific hybridization in the genus *Phytophthora* was for long restricted to laboratory experiments. However, the recent descriptions of a series of new natural hybrid taxa within this genus demonstrate that this phenomenon should not be underestimated nor neglected, as many of the *Phytophthora* species are severe plant pathogens. In this review, we first describe the different features of both artificially and naturally generated hybrids reported in the literature and assess the cytological, genetical, and ecological consequences of interspecific hybridization within this genus. In a second part, we highlight the requirements and barriers that determine the chances of hybridization between different *Phytophthora* taxa. Last, we propose a series of complimentary tools or strategies reported in the literature that can be used to assess the hybrid status in *Phytophthora* and to decipher its parenthood. Owing to the probable morphological similarity with their parents, natural hybrid *Phytophthora* may remain unnoticed unless they exhibit striking new properties unless molecular tools are used for their characterization.

Key words: reticulation, emerging pathogen, natural hybridization.

Introduction

The genus *Phytophthora* encompasses more than fifty morphological species, much of them being severe plant pathogens (Brasier, 1992). Some species of the genus *Phytophthora* are ancient and well known devastating organisms, (e.g. *P. infestans* causing potato late blight), but other appeared recently as emergent and aggressive taxa, such as *P. alni*, responsible for alder disease in Europe (Brasier et al., 2004) or *P. ramorum* causing Sudden Oak Death in North America (Rizzo et al., 2002).

The major sources of genetic variation in the genus *Phytophthora* encompass mutations in both nuclear and mtDNA (substitutions and insertion/deletion), mitotic recombination caused by crossing-overs during mitosis, and the introgression of DNA material following interspecific hybridization (Goodwin, 1997). Despite natural hybridization is recognized as very common in the plant and animal Kingdoms, until recently there had been much less attention paid to this phenomenon in Fungi and by extension to Stramenopiles, including oomycetes (Olson and Stenlid, 2002; Shardl and Craven, 2003). Yet, a number of natural interspecific hybrids belonging to the genus *Phytophthora* have been recently described (Man in't Veld et al, 1998; Brasier et al., 1999; Man in't Veld et al., 2006) suggesting this type of gene flow was not as uncommon as previously imagined (Goodwin, 1997).

Sensu Brasier and Hansen (1992), species of *Phytophthora* are "groups of populations that share a common lineage and have maintained genetic similarity in morphology, physiology, and ecological behaviour". Initially based on the biological species concept, species boundaries in *Phytophthora* has become much clearer since the advent of modern molecular tools such as isozyme analysis (Oudemans and Coffey, 1991), analysis of portions of ribosomal DNA, such as ITS (Internal Transcribed Spacer) (Cooke et al., 2000), analysis of mitochondrial genes (Martin and Tooley, 2003), or the analysis of combination of both nuclear and mitochondrial genes (Kroon et al., 2004). In this respect, the term "hybrid" will be used herein as the outcome of a cross between different species of *Phytophthora*, regardless of the "age" of the hybrid (new F1, later generations up to established hybrid species) and with the assumption that the parental species are distinct taxa.

The purpose of this paper is to review the literature on reports of both artificial and natural interspecific hybridization within the genus *Phytophthora*. We will first discuss about the various outcomes of interspecific hybridization and the characteristics of the hybrids. Second, the requirements and the barriers determining the chance of interspecific crossing within this genus will be assessed. Last, we will emphasize the existing possibilities to support the hybrid status and will highlight the practice of dealing with the reconstruction of the hybrid origin.

1. Characteristics of the *Phytophthora* interspecific hybrids.

1.1. Artificial and natural hybrids

Outcrossing is a regular reproduction system for heterothallic *Phytophthora* species in which offspring may be either outcome of selfing or of intraspecific hybridization (Goodwin, 1997). However,

natural intraspecific hybridization has also been observed between different strains of the same homothallic species (e.g. Whisson et al, 1994). In other respects, the possibility of interspecific hybridization was first assessed *in vitro* before the first cases of natural hybridization were reported (Table 1).

1.1.1 Laboratory-made hybrids

In a wide assessment of mating behavior within the genus *Phytophthora*, Savage et al. (1968) paired several heterothallic species of *Phytophthora* and reported the observation of gametangial fusion and subsequent oospore formation between opposite mating types of *P. infestans* and *P. capsici*. Boccas (1973) hypothesized later that two heterothallic species, namely *P. parasitica* and *P. cinnamomi*, could hybridize by sexual crossing. However, re-examination of the possibility of true interspecific hybridization between these species by Boccas and Zentmyer (1976) using analysis of protein pattern demonstrated that induced autofertilizations were probably responsible for the range of progeny observed. Among the all the F1 oospores obtained by further attempts to cross *P. capsici* and *P. palmivora,* a single isolate appeared to be a product of interspecific hybridization as inferred from its singular protein pattern (Boccas, 1981). Later, sexual interspecific crosses between two heterothallic species, namely *P. infestans* and *P. mirabilis* were successfully achieved and proven to have occurred in both directions (Goodwin and Fry, 1994).

More recent studies resorted to chemical or physical process to achieve intraspecific crosses. Using a polyethylene glycol (PEG)-mediated zoospore fusion protocol, Érsek et al. (1995) artificially generated hybrids of the unrelated *P. nicotianae* and *P. capsici,* whereas Érsek et al. (1998) and Bakonyi et al. (2002) could fuse *P. nicotianae* with a more phylogenetically closer species, namely *P. infestans*. With the same protocol, Ersek et al. (1997) could also produce a tri-parental species hybrid with fused zoospores from the heterothallic species *P. capsici, P. parasitica* and *P. citrophthora*. The possibility of outbreeding was also recently demonstrated between two homothallic species, *P. sojae* and *P. vignae*, both pathogenic on soybean (May et al., 2003). Last, Gu and Ko (2000a, 2001) created for the first time artificial interspecific hybrids between *P. parasitica* and *P. capsici* through the independent transplantation of nuclei or mitochondria.

1.1.2. Putative natural hybrid species

Sansome et al. (1991) observed polyploidy in some isolates of *Phytophthora meadii* pathogenic to Rubber trees in Sri Lanka and suggested for the first time the possibility of occurrence of a natural hybrid species. However, allopolyploidy remains hypothetical for this species and still need confirmation studies (See section 'Exploration of the hybrid status in *Phytophthora*'). The first cases of natural hybridizations between different species of *Phytophthora* were extensively documented by Man in't Veld et al. (1998) and Bonants et al. (2000) and all probably involved the two polyphageous species *P. nicotianae* and *P. cactorum*. The different hybrids were first recovered from plants grown in hydroponic systems in the Netherlands (*Spathiphyllum*, *Primula*, *Lavandula*, *Cyclamen* and *Lewisia* spp.), but were later also found on Loquat trees in Taiwan (Man in't Veld, 2001).

Based on ambiguous nuclear DNA sequences, especially additivity within the sequence of β-tubulin (β-*tub*) and Translation Elongation Factor 1α (*tef*1α) genes, Kroon et al. (2004) suggested that *P. andina,* a new species akin to *P. infestans* (Adler et al., 2004), could actually be a hybrid taxon with the latter as a parent species. However, additivity in *tef*1α was further proven to be a consequence of gene duplication (W. Man in't Veld & H. Meijer, unpublished data). Last, a few isolates of *Phytophthora* collected in citrus orchards from Corsica displayed at the same time morphological characteristics of *P. parasitica* and *P. citricola*, respectively, but with their protein patterns very close to *P. citrophthora*. These abnormal features led Ricci et al. (1990) to suggest that these taxa could actually be hybrid species. Besides, gene flow between *P. citricola, P. nicotianae* and *P. citrophthora* had been previously hypothesized by Erselius and De Vallavieille (1984) to explain the similar electrophoretic protein profiles observed among isolates also recovered from citrus located in Corsica.

The sterile taxon previously described as *Phytophthora* 'O' group by Brasier et al. (1993), was further ranked to the species level and named *Phytophthora inundata* (Brasier et al., 2003). This species was previously supposed to be of hybrid origin based of a particular protein-banding pattern (Brasier et al., 1993), but after deeper investigations, heteroploidy was only inferred for a few isolates of *P. inundata* (Brasier et al., 2003).

In contrast with the natural hybrids reported above that only caused limited damages, the spread of a recently emerged *Phytophthora* hybrid, *P. alni* subsp. *alni* (*Paa*) (Brasier et al., 2004), has reached a pandemical level by attacking and killing hundreds of thousands of alder trees throughout Europe since the beginning of the 1990' (Gibbs et al., 2003).

Last, a series of new hybrid taxa morphologically resembling to *P. cactorum* were isolated from *Rhododendron* sp., *Allium* spp., *Idesia* sp. and *Penstemon* sp. in the Netherlands in 2005 (Man in't Veld et al., 2006). These hybrids probably involve once again *P. cactorum* and a recently described species *P. hedraiandra* (De Cock & Lévesque, 2004).

1.1.3. Less documented species as potential hybrid derivatives.

Several taxa that are still poorly characterized and different separate groups within some taxa as inferred from DNA or protein investigations were suggested to originate from past reticulation events. These include *P. citrophthora* which different isolates are morphologically highly variable and often sterile and *P. capsici* (Brasier and Hansen, 1992). The observation of unstable F1 progeny among *P. botryosa* intraspecific crossings, with some of them showing affinities with *P. palmivora* (Chee, 1973) was interpreted by Brasier and Hansen (1992) as a consequence of a past reticulation event. Studying the *P. cryptogea* / *P. dreschsleri* complex through isozyme and DNA polymorphisms, Mills et al. (1991) resolved up to nine groups within this complex, and observed a few isolates (Group "K") equally akin to both species that could possibly originate from hybridization between the two taxa. Brasier et al. (1993) suggested that isolates belonging to group "K" and designated as *P.* taxon Pgchlamydo, as well as other isolates previously assigned to *P. gonapodyides* and designated *P.* taxon salixsoil, could indeed be of hybrid origin, but further studies conducted with ITS sequencing failed to confirm clearly these hypotheses (Brasier et al., 2003).

Furthermore, the striking mtDNA polymorphisms observed between different isolates of *P. citricola* comparing to the close species *P. cactorum* (Förster and Coffey, 1991) could be a result of past somatic hybridizations between them or with other *Phytophthora* providing opportunities to generate mtDNA recombinants (Brasier and Hansen, 1992).

Last, based on ITS (Internal Transcribed Spacers) polymorphisms observed for individual isolates of *P. cambivora*, it was suggested that this species might have been involved in reticulation events (Cooke and Duncan, 1997; Brasier et al., 1999). Recent studies showed the presence of multiple alleles for several nuclear genes for individual isolates and strengthen the hypothesis *P. cambivora* could have been introgressed (Ioos et al., 2006a).

1.2. Mating types.

Artificial pairing of two heterothallic *Phytophthora* species often leads to the production of oospores. The necessary contact between two heterothallic species could be favorable to interspecific crossing. However, two kinds of oospores may be produced during such crosses: the first would arise from self-fertilization of each parental isolate and the second would result from pairing of gametangial structures of the two parents (Haasis and Nelson, 1963; Brasier, 1972; Brasier and Sansome, 1975; Boccas, 1981). Nevertheless, in most of the cases, the resulting oospores proved to be products of induced self-fertilizations, rather than true interspecific hybrids (Brasier, 1972; Boccas and Zentmyer, 1976; Boccas, 1981; Ann and Ko, 1988; Chang and Ko, 1993). However, exceptions are reported in the literature and outcrossing two heterothallic species could generate a few true interspecific hybrids. Boccas (1981) and Vorobèva and Gridnev (1981) obtained interspecific hybrid progeny when crossing *P. capsici* and *P. palmivora*, and *P. infestans* and *P. capsici*, respectively. *P. infestans* x *P. mirabilis* hybrids were also generated using strains of opposite mating types (Goodwin and Fry, 1994) and segregated into both mating types following Mendelian's expectations. By contrast, Ersek et al. (1997) circumvented the mating type requirements to cross the heterothallic species *P. nicotianae* and *P. capsici* by using a zoospore-based somatic fusion, involving two strains of both A2 mating type.

On the other hand, interspecific cross between two homothallic species would not be expected since self-fertilization is the rule for this type of breeding. However, May et al. (2003) investigated 800 oospores produced by cultural contact between the homothallic species *P. sojae* and *P. vignae* and found that two of them appeared as interspecific hybrids.

Furthermore, the different natural hybrids or putative hybrids encountered so far represent either possible hybridizations between a heterothallic species (*P. nicotianae*) and a homothallic species (*P. cactorum*) (Man in't Veld et al., 1998; Bonants et al., 2000) or between two homothallic species: *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis* (Ioos et al., 2006a); *P. cactorum* and *P. hedraiandra* (Man in't Veld et al., 2006). These examples suggest that both homothallic and heterothallic species may be candidate for hybridization despite the need for mating type compatibility would make it less likely for the latter. All the natural *Phytophthora* hybrids described so far appear to be homothallic in their behavior.

1.3. Modification of host range and aggressiveness.

Hybrid vigor, also called heterosis, is a frequently encountered feature in F1 hybrids resulting from crosses between plants. This enhanced vigor comparing to the parental species may be attributed to the results of the interaction of several genetic factors, such as the accumulation of dominant genes, overdominance, or epistasis (Stoskopf et al., 1993).

In the genus *Phytophthora,* significantly increased growth vigor was observed in the artificial nuclear hybrids between *P. parasitica* and *P. capsici* engineered by Gu and Ko (2001). In this study, the nuclear-transplanted hybrids grew 20-40 % faster than the better wild parent. However, Gu and Ko (2001) also observed that the hybrid growth vigor was repressed after both mitochondria and nucleus were transplanted concomitantly in the receptor cell, thus mitigating the potential hybrid vigor in somatic hybrids. The hybrid vigor in these artificial nuclear hybrids was therefore attributed to nuclear interactions.

In other instances, the artificially generated hybrid *P. nicotianae* x *P. capsici* exhibited a cumulative host range of both parental species (Érsek et al., 1995). *P. nicotianae* is able to attack radish and tomato but is not aggressive on lemon, whereas *P. capsici* induces severe lesions on lemon and tomato but is not pathogenic to radish. Among the somatic hybrid progeny, two *P. nicotianae* x *P. capsici* isolates were able to induce lesions when inoculated on the three host plants. However, the symptoms observed were significantly less severe than in the cases of inoculation with the parental species on the same host plant, indicating a loss of aggressiveness accompanied this expanded host range in the hybrid progeny (Érsek et al., 1995). A last type of *P. nicotianae* x *P. capsici* hybrid was only able to infect tomato and thus differed from its parents and the other hybrid by its reduced host range. In contrast with these results, some strains of other *P. nicotianae* x *P. infestans* hybrids generated *via* zoospore-fusion did not exhibit any host range expansion. Moreover, a complete loss of pathogenicity was observed towards potato, the common host of both parents (Bakonyi et al., 2002). Furthermore, the tri-parental species hybrids generated by Ersek et al. (1997) could not reproduce symptoms of either parental species on their different respective hosts and exhibited reduced fitness, suggesting that the co-existence of distinct genomes and the potential genetic rearrangements could be deleterious to the hybrid descent. Likewise, most of the interspecific *P. infestans* x *P. mirabilis* F1 progeny completely failed to produce symptoms on the hosts respectively attacked by the parents, i.e. potato and tomato (Goodwin and Fry, 1994). Only a single interspecific isolate proved to be aggressive towards potato and was suggested to have arisen via non meiotic disjunction of the chromosome(s) holding the pathogenicity and host specificity gene(s) during the interspecific crossing process. The F1 progeny between *P. sojae* and *P. vignae*, despite pathogenic on the parent's hosts, also showed a considerable reduction of aggressiveness (May et al., 2003). Since the parental species are both homothallic, the hybrid progeny should possess the full complementary set of genes from both parents and therefore, a possible different number of chromosome inherited from both parents was suggested to explain this reduced aggressiveness.

Artificially generated hybrids seem to be prone to decreasing aggressiveness, which contrasts with the features reported for the natural hybrids. Some of the natural hybrids *P. cactorum* x *P. nicotianae*

seemed equally aggressive towards their hosts than their counterpart parental species (Man in't Veld et al., 1998), but later Bonants et al. (2000) reported additional hybrids possibly involving the same parental species that were pathogenic on *Cyclamen* sp. As the latter was not within the known host range of both putative parental species, Bonants et al. (2000) hypothesized that these new natural hybrids displayed an extended host range. Man in't Veld (2001) further reported the occurrence of other *P. cactorum* x *P. nicotianae* hybrids on loquat trees, despite *P. cactorum* had never been described on that host previously. The recently discovered hybrid taxa *P. cactorum* x *P. hedraiandra* were isolated from *Allium*, *Idesia* and *Penstemon* whereas the putative parental species have never been reported on these hosts (Man in't Veld et al., 2006). However, since the pathogenicity of the putative parental species was never assessed on these new hosts by in vitro experiments (Man in't Veld, pers. comm.), the acquisition of a new pathogenicity for these hybrid taxa involving *P. cactorum* is not fully demonstrated. Last, Brasier et al. (1999) hypothesized that the putative parental species of the hybrid *Phytophthora alni* subsp. *alni* (*Paa*) were *P. cambivora* and a taxon close to *P. fragariae*. As both species are unable to cause significant symptoms on alder (Brasier and Kirk, 2001), this hybrid would have acquired new host specificity through the hybridization process, and would have been an example of the "superpathogen" imagined by Brasier (2001). However, recent investigations rather demonstrated that the two putative parents of *Paa* could possibly be two other subspecies of *P. alni*: *P. alni* subsp. *uniformis* (*Pau*) and *P. alni* subsp *multiformis* (*Pam*), both able to induce necrosis on alder. In this respect, the hybrid *Paa* would be significantly more aggressive towards alder than its direct progenitors (Brasier and Kirk, 2001).

1.4. Ploidy levels.

Diploidy in the Oomycetes is now universally accepted and extensively documented since the beginning or the 1980' (Brasier, 1992). However, in particular taxa such as hybrid *Phytophthora*, different ploidy levels may be observed. The zoospore progeny of the artificial *P. capsici* x *P. parasitica* hybrids described by Gu and Ko (2000a) was of normal size and uninucleate, which suggested that the initial and instable tetraploid hybrid protoplasts were gradually transformed into diploid organisms during zoosporogenesis. This inter-specific experiment exactly mirrored previous results observed with *P. parasitica* nucleus-transferred heterokaryon that regained a diploid status after mitotic recombination and loss of chromosome during zoosporogenesis (Gu and Ko, 1998).

In other respects, cytological examinations led Sansome et al. (1991) to consider some isolates of *P. meadii* as tetraploid (4n), diploid (2n) or even sometimes consisting of a mix of 2n and 4n nuclei in individual oogonial cells. The polyploid status for isolates of a same species was suggested to be a consequence of the co-existence of distinct genomes (allopolyploidy), which disturbed meiosis. Consequently, whereas aneuploid or diploid isolates failed to produce normal oospheres because of difficulties for chromosome pairing during meiosis leading to gametic abortion, chromosomal doubling in the tetraploid isolates enabled normal meiosis and oosphere production.

Despite the ploidy was not directly assessed for the natural hybrids *P. cactorum* x *P. nicotianae*, Bonants et al. (2000) interpreted the increased number of resolvable fragments in the hybrids' AFLP

patterns comparing to the presumed parental species as a direct consequence of polyploidy. Using light microscopy and acetoorcein staining during gametangial meiosis, Brasier et al. (1999) could estimate the number of chromosomes in the hybrid *Phytophthora alni* subsp. *alni*, previously named "standard type". In comparison to *P. cambivora* and *P. fragariae*, that are typical diploid species (Brasier et al., 1999), the standard type of alder *Phytophthora* was suggested to be near-tetraploid as inferred from chromosomal counts. As a mirror to the situation of *P. meadii*, Brasier et al. (1999, 2004) also proposed that the species *P. alni* was actually a hybrid swarm that comprised subspecies of different ploidy: *P. alni* subsp *uniformis*, close to diploidy (2n+2) and *P. alni* subsp. *multiformis*, gathering different aneuploid entities (2n+4 to 2n+7). However, further studies revealed that the respective ploidy levels of *Paa* and *Pam* were probably underestimated and should be reinvestigated with more accurate techniques (Ioos et al., 2006a,b).

1.5. Phylogenetic relatedness of the parental species.

Phylogenetically close species are assumed to derive from a common ancestor. In return, it may be interesting to check whether the possibility of hybridization between two distinct taxa is dependant on the phylogenetical relatedness.

The *P. parasitica*-nuclear transplanted *P. capsici* hybrids created by Gu and Ko (2000a) were shown instable in culture and readily segregated into parental types. These results proved that the assembling of unrelated taxa nuclei may not be genetically stable, and suggested the occurrence of natural hybridization between unrelated species of *Phytophthora* was unlikely. This mirrored the results previously obtained by Boccas (1973) and Boccas and Zentmyer (1976) who failed to generated interspecific hybrid by crossing the two unrelated species *P. parasitica* and *P. cinnamomi.*

Nevertheless, the *P. nicotianae* x *P. capsici* somatic hybrids artificially generated by Érsek et al. (1995) remained stable after subculturing or inoculation/re-isolations steps and successfully asexually reproduced via zoosporogenesis, despite the parental species are not phylogenetically related since placed in divergent clades (Cooke et al., 2000). However, *P. nicotianae* and *P. capsici* share common host plants such as *Capsicum annuum*, *Cucumis* spp., *Lycopersicon esculentum*, *Macadamia* sp., or *Piper nigrum* (Erwin and Ribeiro, 1996) and co occur in common geographical areas (Satour and Butler, 1967). On the contrary, two closely related species, such as *P. capsici* and *P. palmivora*, *P. infestans* and *P. mirabilis or P. vignae and P. sojae*, could be crossed and the hybrid progeny further proved to remain stable in culture (Boccas, 1981; Goodwin and Fry, 1994; May et al., 2003).

Of particular interest is the close relationship between the parental species of most of the natural *Phytophthora* hybrid described so far. All the well-documented natural hybrids seem to have been generated by hybridization between phylogenetically close taxa, which is the general rule in other biological Kingdoms (Mallet, 2005). The natural hybrids *P. cactorum* x *P. nicotianae* all result from the hybridization between two distinct but phylogenetically close species: *P. cactorum* and *P. nicotianae* (subclades "1a" and "1d", respectively, *sensu* Kroon et al. (2004)) and also share common hosts. Likewise, the ITS sequences of *P. cactorum* and *P. hedraiandra* share 99% identity showing the hybrid taxa probably involving these species was also generated by crossing of closely related

species. Last, *P. alni* subsp. *alni* may have also been generated by sexual hybridizations between two sibling species: *Pau* and *Pam* or *Pau-* and *Pam*-like taxa (Ioos et al. 2006a).

A single report contrasts with this propensity, two phylogenetically unrelated species, namely *P. botryosa* and *P. heveae*, (Cooke et al., 2000), were first proposed as parental species for the presumed hybrid species *P. meadii* (Sansome et al., 1991) based on the ploidy level and the fact they also occur on rubber. On the other hand, Kroon et al. (2004) later demonstrated that two other unrelated species, *P. colocasiae* and *P. hibernalis,* were more probable candidates, respectively inferred from nuclear and mitochondrial sequence data.

1.6. Mitochondrial inheritance.

Unlike nuclear genes, which are inherited from both maternal and paternal parents, mitochondrial genes seem to be exclusively transmitted via the maternal line during sexual crossing in *Phytophthora* (Förster and Coffey, 1990; Whittaker et al., 1994). This was verified following natural (Man in' Veld et al., 1998; Ioos et al., 2006a) and artificial (Goodwin and Fry, 1994) hybridization. However, despite the paternal mitochondria are excluded from the female gametangium during sexual fertilization, there should be no restriction when hybridization occurs through somatic fusion of both parental types. In this respect, both mitochondrial types may be inherited and co-exist in the cytoplasm of the somatic hybrid, and hypothetic mtDNA recombination events cannot be excluded (Gu and Ko, 2000b). Many of these recombinations could remain unnoticed following mtDNA restriction patterns analyses and would probably require extensive sequencing data to be demonstrated.

During a sexual interspecific hybridization event, both parents could potentially act as the antheridial strain. However, examples reported in the literature tend to indicate that despite hybridization occurred in both directions, one of them seems to be favored, leading to an unequal transmission of the mitochondrial types. All the *P. nicotianae* x *P. cactorum* strains studied by Man in't Veld (1998) displayed a *P. nicotianae* mtDNA pattern. In addition, more than 82 % of the hybrid progeny generated by *P. mirabilis* x *P. infestans* crossings inherited of the mitochondrial DNA from *P. infestans* (Goodwin and Fry, 1994), while most the European hybrid *P. alni* subsp. *alni* studied so far displayed a *P. alni* subsp. *multiformis* pattern (Nagy et al., 2003; Ioos et al., 2006a; Ioos R. personal observation). In these cases of interspecific hybridizations, both homothallic (*P. alni* subsp. *multiformis and P. alni* subsp. *uniformis*) or heterothallic (*P. infestans*, *P. nicotianae*) proved to behave as a "strong female' (Galindo and Gallegly, 1960). Whether this skewed repartition of the mtDNA is influenced by a pre-zygotic determinism or a poorer fitness of the progeny generated by one of the combination remains unknown.

Interestingly, Kroon et al. (2004) showed that despite the putative hybrid species *P. meadii* was highly similar to *P. colocasiae* in regard with nuclear DNA sequences and *nadh*1 mitochondrial gene, it shared an almost identical *cox*1 mitochondrial sequence with *P. hibernalis*. This would first suggest that *P. hibernalis* could be a parental species of the hybrid and second that mitochondrial DNA would have also been uniparentally inherited from one of the parent during a sexual hybridization event.

The results obtained with artificial experiments are less univocal. Laboratory-made interspecific mitochondrial hybrids of *P. parasitica* x *P. capsici* were proven to be stable in culture, unlike their nuclear hybrid counterparts (Gu and Ko, 2001a). Most of the mitochondrial patterns observed in the zoospore progeny of the mitochondrial hybrid could be explained by different ratios of distribution for the different mitochondrial types. Nevertheless, a few unexpected phenotypes were encountered following first and second asexual reproductions, and were interpreted as possible consequences of fusions between different mitochondrial types (Gu and Ko, 2001a).

1.7. Autapomorphic characters of the hybrids.

Interspecific hybridization may enhance one or both of the parental species characteristic(s) through heterosis but could also generate one or more autapomorphic features, i.e. "new" characteristics, apparently unexpressed in any of the parental species (numerous example throughout plants, animals, insects or fungi are reviewed by Arnold, 2004a,b). For instance, new combinations of avirulence seem to have been generated by mitotic gene conversion following intraspecific hybridization in some strains of *P. sojae* (Chamnanpunt et al., 2001). Another striking autapomorphic feature could be the pathogenicity towards a host plant unaffected by both parents. Such a phenomenon was previously attributed to the hybrid *Phytophthora* aggressive to alder trees since none of the previous hypothesized parental species, i.e. *P. cambivora* and *P. fragariae* (Brasier, 2000), was pathogenic to this tree. However, Ioos et al. (2006a) demonstrated that the involvement of these species was unlikely and that the two other *P. alni* subspecies, namely *Pau* and *Pam*, both pathogenic to alder (Brasier and Kirk, 2001, De Merlier et al., 2005) could actually be the progenitors of the hybrid.

2. Factors affecting the chances of hybridization in *Phytophthora.*

Genetic exchange between different species of *Phytophthora* through hybridization may be naturally precluded by a series of pre-zygotic and post-zygotic barriers, including genetic as well as ecological or geographical limitations. However, numerous examples in the literature demonstrate that these restrictions may be by-passed by various genetical mechanisms, or simply canceled by man's activities.

2.1. Genetic compatibility.

The results reported following attempts of artificial interspecific crosses showed that both pre- and postzygotic genetic compatibility are strongly negatively correlated with genetic distance, and therefore with time separation of *Phytophthora* taxa. Although scarce, the putative natural *Phytophthora* hybrids certainly obeyed the same law.

The likelihood of natural hybridization via anastamosis or zoospore fusion were judged as unlikely (Brasier 1992) and first attempts to artificially fuse protoplasts from the closely related *P. sojae* and *P. medicaginis* failed (Hansen and Maxwell, 1991). However, the generation of artificial hybrids generated by zoospore fusion was successfully achieved between two or more phylogenetically unrelated species of *Phytophthora*, but was dependant on the use of chemical treatment to inhibit cell walls development or encystment and favor protoplast fusion (Ersek et al., 1995, 1997). Zoospores are released from sporangia in a wall less state, and the development of culturing systems such as hydroponic systems of water recirculation systems of nurseries where several different species of *Phytophthora* are frequently encountered (Mac Donald et al., 1994; Themann et al., 2002) might favor the meeting and the potential fusion of zoospores from alien species. In this view, the apparent increase of hybrid descriptions in greenhouse recirculation systems in Netherlands could be a consequence of such somatic hybridization (Bonants et al., 2000). The first description of natural *Phytophthora* hybrids on diseased *Spathiphyllum* and *Primula* grown in hydroponic systems is as such meaningful since the two putative parental species *P. cactorum* and *P. nicotianae* are phylogenetically closely related (Cooke et al., 2000) and may have diverged from a common ancestor. The limited divergence between the two genomes and therefore homology between the respective homoeologues chromosomes may have enabled their successful coexistence in a common cell. However, all the natural hybrids reported up to now were more probably generated through sexual crossings, involving phylogenetically close taxa.

2.2. Frequency of contact

Obviously, host specificity provides a prezygotive reproducing barrier for most of the species of *Phytophthora* that have a narrow host range. In return, polyphageous species will not be affected by this reproductive isolating mechanism as their respective host range may overlap, thus enabling contact between them.

Ersek et al. (1995) artificially fused zoospores from *P. capsici* and *P. nicotianae* and demonstrated the somatic hybrids were stable, though phylogenetically distant. Despite natural *P. capisici* x *P. nicotianae* hybrids have never been reported, these two species sharing common host plants might occur in the same field (Satour and Butler, 1967) or even on the same single plant, and interspecific cross could be imagined (English et al., 1999). *Phytophthora* zoospores are very abundant in a water recirculation system, which provides additional opportunities for zoospores of different species to meet and fuse. Nevertheless, the natural hybrids encountered so far are presumed to originate from sexual crossings (Man in't Veld et al., 1998, 2006; Bonants et al., 2000; Ioos et al., 2006). Sexual crossing requires meeting of hyphae in a common substrate, either during the pathogenic stage (on a common host plant) or during the saprotrophic stage (in soil or plant debris). For all the natural *Phytophthora* hybrids described so far, the presumed parental species may have met during the saprotrophic stage or in a common substrate in nurseries but also share common host plants: alder trees for *Pam* and *Pau* (Brasier et al., 2004; Ioos et al., 2006), *Spathiphyllum* and *Primula* for *P. cactorum* and *P.*

nicotianae (Man in't Veld, et al., 1998) or rhododendron sp. for *P. cactorum* and *P. hedraiandra* (Man in't Veld et al., 2006).

Several alien species of *Phytophthora*, not affected by phytosanitary restrictions, may be introduced by human activity and brought into physical contact with resident species of *Phytophthora*. Since a resident and an introduced species of *Phytophthora* will probably lack an efficient reproductive isolating mechanism, hybridization between two allopatric progenitor strains would not be precluded by prezygotic barriers. This was one of the hypotheses proposed by Sansome et al. (1991) to explain that the putative hybrid species *P. meadii* was only found on rubber, a man's introduced plant, in a limited area of the world, i.e. southern India and Sri Lanka. In this view, remote sibling taxa that have diverged from a common ancestor, through a combination of geographical and /or climatic isolation, and host specialization could be artificially brought into physical contact by commercial trade (Brasier, 2001).

2.3. Postzygotic barriers.

As previously mentioned, the F1 progeny generated by the hybridization between unrelated *Phytophthora* species may be unable to thrive due to genetic instability (Gu and Ko, 2000a). Incompatibility between the genomes of divergent taxa could therefore lead to another type of postzygotic isolation process since the hybrid would exhibit a reduced fitness. However, if this transient and/or less fit F1 progeny is able to backcross with one or both parental species, the horizontal gene transfers may enable the parental the introgressed parental species to acquire a novel host range of an increased virulence (Olson and Stenlid, 2002). Other examples suggest that somatic hybrid may develop normally, and despite less aggressive than the respective parental species, laboratory made *P. nicotianae* x *P. capsici* hybrids remain stable for more than two years (English et al., 1999) and could still produce zoospores from infected plant tissues (Ersek et al., 1995).

Nevertheless, despite the formation of oospores following interspecific crosses is well documented, (Haasis and Nelson, 1963; Savage et al.1968; Boccas and Zentmyer, 1976; Boccas, 1981; Erselius and Shaw, 1982; Goodwin and Fry, 1994; May et al., 2003), only a few of these interspecific mating gave rise to viable and germinating oospores (Boccas, 1981). This loss of such a surviving structure may greatly affect the probability of such a hybrid to survive from season to season. However, this kind of post-zygotic barrier in the resultant hybrid may be by-passed by a vegetative dispersion strategy, such as observed for *P. cactorum* x *P. nicotianae* (Bonants et al., 2000) or *P. alni* subsp. *alni* (Delcan and Brasier, 2001). Finally, hybridization could yield a progeny with a new genetic combination providing a fitness advantage that enables the F1 crosses to thrive. Hence, the hybrid *Paa* is highly aggressive of *Alnus*, and significantly more aggressive that its putative parents. By extent, host-specialization enabling the hybrid to be aggressive on a new host would automatically provide the hybrid an opportunity to maintain in this new niche, and this specialized virulence system will sooner or later lead to speciation (Brasier and Hansen, 1992). Finally, once hybridization has occurred, the resulting genotypes may be fixed instantly by a vegetative mode of reproduction that restricts recombination or may evolve more slowly if selection works to sieve out the best adapted

genotypes among an array of recombinants. Hybridity may be stabilized and circumvent the postzygotic barriers by several mechanisms going from cytological controls such as polyploidy or permanent translocation heterozygosity, to an exclusive clonal growth and dispersal (Ellstrand and Schienrenbeck, 2000)

3. Exploration of the hybrid status in *Phytophthora.*

Artificially created hybrids in *Phytophthora* are usually screened out using a set of dominant mutational characters initially present in the parental species, such as fungicide resistance (Érsek et al., 1995; Érsek et al., 1997; Gu and Ko, 2000a; Bakonyi et al., 2002). In case of a suspected natural hybrid, such tools are obviously not available to unravel the parental species. However, numerous complementary characters can be used to establish their credentials as hybrid taxa.

3.1. Host range and aggressiveness.

A potentially expanded host range may be the consequence of a hybridization event, as reported both for natural (Man in't Veld et al., 1998, 2006) as well as for artificial (Ersek, 1995) interspecific crosses. The sudden apparition of a new host for a well-known species of *Phytophthora* might be indicative of a genetical modification of the pathogen, and a reticulation event and/or subsequent gene flow via introgression might not be ruled out as possibilities. On the other hand, the loss of aggressiveness was also described as a possible consequence of interspecific hybridization (Goodwin and Fry, 1994; Bakonyi et al., 2002). However, a reduced host range observed for a particular taxon could be indicative of hybridization as well as of induced autofertilization, as previously demonstrated by Boccas and Zentmyer (1976) by studying the *P. parasitica* x *P. cinnamomi* hybrid progeny. Boccas (1981) further hypothesized that this reciprocal self-fertilization between compatible strains of different species may result in phenotypic variation of the progeny if the parental strains are heterozygous. As a whole, an abnormal host range or a modified aggressiveness may indicate a genetic modification but additional features should be assessed to support a reticulation event.

3.2. Morphological and cultural traits.

English et al., (1999) assessed the morphological characters from the set of four *P. nicotianae* x *P. capsici* artificial hybrids generated by Ersek et al. (1995) and observed that the colony morphology of the species hybrids was intermediate to those of the respective parents. Bakonyi et al. (2002) observed a maximal growth temperature for the *P. nicotianae* x *P. infestans* hybrids intermediate to those of the parent species but the other cultural of morphological traits were highly variable. Ersek et al. (1997) first hypothesized the occurrence of several artificial triparental species hybrid based on morphological traits and drug resistance pattern. However, only one out of these putative hybrids contained detectable amount of different parental species, showing that more

evidence than intermediate morphology must be provided to support a hybrid status. By extension, this is even more necessary when the putative hybrid is a previously unrecognized taxon. The hybrid nature of *P. meadii,* and of the isolates of *P. cactorum* x *P. nicotianae* described by Sansome et al. (1991) and Man in't Veld (1998), respectively, could not be inferred from any morphological characters, nor the parental species unambiguously determined.

Finally, relying only morphological characters to state about the hybrid status of a taxon would not be very rigorous, since *Phytophthora* species frequently exhibit overlapping morphological features (Brasier, 1991; Hansen, 1991) and a hypothetical hybrid between two very similar species might possibly be lumped with one or other parent species (e.g. Man in't Veld et al., 1998, 2006).

3.3. Cytological evidences.

The observation of abnormally–sized nuclei in zoospore progeny may be the consequence of changes in ploidy of the taxon. Ersek et al. (1995) reported significantly enlarged zoospores within the progeny of their *P. nicotianae* x *P. capsici* somatic hybrids and hypothesized that the occurrence of binucleate or single enlarged nuclei were indicative of the addition of the two parental genomes. Similarly, Sansome et al. (1991) associated significantly larger oogonia with the tetraploid isolates of *P. meadii* in comparison with diploid ones, and this was presumed to be a consequence of the occurrence of a double set of chromosomes in the polyploid isolates.

However, polyploidy may not be systematically a consequence of a hybridization event. For instance, some isolates of *P. infestans* (Sansome, 1977; Tooley and Therrien, 1987, Shaw and Shattock, 1991), or of the different biological species encompassed by the *P. megasperma* group (Sansome and Brasier, 1974; Hansen et al., 1986), are presumably tetra-, hexaploid or even aneuploid and in this case a consequence of autopolyploidy, i.e. self-duplication of the whole genome, which is a very common phenomenon in the plant Kingdom (Otto and Whitton, 2000). Also, triploidy may also be one of the aberrant outcomes of intraspecific mating (Carter et al., 1999). Furthermore, hybrids may have a homoploid structure, i.e. they may have regained a diploid level while retaining a set of chromosome of both parental species. The hybrid status of such homoploid species will therefore remain cryptic if only based on ploidy level. This could be the case with *P. alni* subsp. *multiformis* pathogenic on alder, in which two divergent alleles are systematically observed for four physically unlinked nuclear genes (Ioos et al., 2006a) despite this (sub)species is reported to be close to diploidy (Brasier et al., 2004).

Last, most of the ploidy data rely upon microscopic examination of chromosomal structure, following staining protocols such as described by Sansome and Brasier (1974) or Tooley and Therrien (1987). Unfortunately, with these techniques an accurate chromosome number is sometimes difficult to determine (Sansome et al., 1991; Burnett, 2003). New technologies, based upon DNA fluorescent labeling, such as cytophotometry (Toley and Therrien, 1987) or flow cytometry (Si-Ammour, 2002) should provide more reliable relative intracellular amounts of DNA in putative polyploid *Phytophthora.*

3.4. Protein and isozyme patterns.

The resolution of protein patterns was one of the pioneer techniques used to distinguish *Phytophthora* species (Gill and Zentmyer, 1978) and Boccas (1981) suggested the occurrence of a single hybrid oospore resulting from multiple *P. capsici* x *P. palmivora* crossings based on a protein pattern quantitatively and qualitatively different from the parents.

Isozyme patterns are of special interest because of their ability to distinguish true hybrid organism from physical mixture of two or more species when using ad hoc dimeric enzymes (Goodwin et al., 1994). For instance, protein and isozyme patterns were combined by Ricci et al. (1990) to draw up the hypothesis of genetic exchange between *P. citrophthora* and *P. citricola* or *P. parasitica* for a few isolates of *Phytophthora* collected from citrus. Furthermore, the elicitin genes pattern observed for this putative *P. parasitica* x *P. citrophthora* hybrid was the exact juxtaposition of the patterns observed for the parental species (F. Panabières, pers. comm.). Goodwin and Fry (1994) identified recombinant *Gpi* genotypes in the artificially generated crosses between *P. infestans* and *P. mirabilis*, showing that the hybrids inherited one allele from each of the parental species. Likewise, isozyme investigations conducted by Man in't Veld et al. (1998, 2006) reinforced the possibility of a hybrid status for *P. cactorum* x *P. nicotianae* and *P. cactorum* x *P. hedraiandra* isolates, as inferred from the occurrence of a heterodimeric isozyme for two loci and one locus, respectively. This demonstrated that both hybrid taxa were heterozygous at these loci, and possessed the respective single allele observed for each putative parental species. However, the results were not sufficient to identify the parental species with certainty since other species may share identical isozyme products. *P. cambivora* was finally rejected as a parental species for the hybrid *Paa* (Ioos et al., 2006a, b, c) despite this species share identical alleles with *Paa* for several isozyme loci (W. Man in't Veld, unpublished data). Nevertheless, the different isozyme alleles resolved for *Paa* are also those observed for the two putative parental species *Pam* and *Pau* (Brasier et al., 2004).

Furthermore, caution is required for the interpretation of isozyme banding pattern since the occurrence of a heterodimeric band does not necessarily reflect a hybrid status, and the heterozygous state may be fixed, such as in *P. infestans* (Fry et al., 1992), or may also explained by gene duplication or null allelism (Richardson et al., 1986). In addition, isozyme analysis may not always lead to a clear and readily interpretable pattern. For instance, Bakonyi et al. (2002) analysed the isozyme pattern of artificial *P. infestans* x *P. nicotianae* hybrids and consistently observed a unique phenotype with the dimeric isocitrate dehydrogenase corresponding to *P. nicotianae*. Furthermore, a single putative hybrid isolate displayed another distinct pattern, which could neither be interpreted as a parental pattern nor as a recombination of this dimeric enzyme.

3.5. Molecular markers.

3.5.1. Additivity in ITS sequence.

In *Peronosporales* as in most Eukaryotic organisms, ribosomal genes are repeated in tandem in the genome and up to 100-200 copies are present within a single nucleus (White et al., 1990). Of particular interest are the Internal Transcribed spacers (ITS) tandemly repeated in these regions, since they were proven to be useful for species delineation within the genus *Phytophthora* (Cooke and

Ducan, 1997; Cooke et al. 2000). Since these repeated ITS should be identical within a genome, the presence of different sequences of ITS within a single genome would indicate that this taxon was subjected to gene flow, either by hybridization of further introgression. Hence, Brasier et al. (1999) and Man in't Veld et al. (2006) observed a combination of different ITS types in individual isolates of the natural hybrids *Paa* and *P. hedraiandra* x *P. cactorum*, respectively. Additivity, i.e. the occurrence of two bases at a few dimorphic sites within the ITS region, was first suggested based on the observation of overlapping peaks in the electropherograms of sequenced PCR products, and further confirmed by PCR-RFLP assays, targeting the polymorphic sites.

However, traces of past reticulation could be erased in 'old' hybrid *Phytophthora* taxa by a process called bidirectional concerted evolution that leads to homogenization of the ITS sequences in eukaryotic organisms (e.g. Dover and Tauz, 1986), possibly reverting to one parent type (Wendel et al., 1995). One parental ITS may also first be eliminated before the other remaining ITS underwent rearrangement (Volkov et al., 1999) or the number of copies of one parental type may over dominate the other type, making it hardly detectable (Rauscher et al., 2002). With these scenarii, the final ITS sequence of a hybrid taxon will eventually be different from both parental types thus blurring each parental contribution to the hybrid. Other possibilities are that the divergent ITS may undergo various levels of recombination leading to chimerical ITS (Baldwin et al., 1995), or that the divergent copies gathered in the polyploidy genome are maintained, evolving independently without recombination (Baldwin et al., 1995; Aguilar and Feliner, 2003). In the latter case, reconstructing the hybrid history is obviously much easier.

Last, as two phylogenetically close but distinct *Phytophthora* species may share identical ITS sequence, a hybrid between these two species would remain undetected from the ITS point of view (Cooke et al., 2000), thus demonstrating here again the need for additional molecular markers.

3.5.2. Genomic fingerprinting.

Genomic fingerprinting accurately reflects the phylogenetic relationships between hybrid and their parental species. Man in't Veld et al. (1995) demonstrated that the Randomly Amplified Polymorphic DNA (RAPD) patterns of the natural hybrids encountered on *Primula* and *Spathiphyllum* almost exclusively consisted of the sum of the bands resolved with their respective parents *P. cactorum* and *P. nicotianae*. The observation of the addition of the parental RAPD patterns in the artificial crosses *P. vignae* x *P. sojae* was also used as an evidence to demonstrate their hybrid status by May et al. (2003). These results were in line with those previously reported by Brasier et al. (1999) and Bonants et al. (2000) who showed that the Amplified Fragment Length Polymorphism (AFLP) fingerprinting generated with the standard type of alder *Phytophthora* and the *P. cactorum x P. nicotianae* hybrid, respectively, were almost entirely a composite of the bands resolved with the putative parental species, i.e. *Pam* and *Pau* on the one hand and *P. cactorum* and *P. nicotianae* on the other hand.

3.5.3. Observation of the parental alleles.

Ersek et al. (1995) confirmed the hybrid status of the progeny of the artificial *P. nicotianae* x *P. parasitica* somatic hybrid by demonstrating the co-occurrence of both parental species DNA with southern blots and species-specific PCR tests. Likewise, Bakonyi et al. (2002) inferred the presence of *P. infestans* DNA in the laboratory made *P. nicotianae* x *P. infestans* by positive *P. infestans* specific, ITS-based PCR tests. The occurrence of both putative parental species ITS sequences in the natural hybrids studied by Bonants et al. (2000) was also confirmed using species-specific ITS-based PCR assays. In addition, restriction Fragment Length Polymorphism (RFLP) of these PCR products revealed that among the hybrid progeny, some isolates probably possessed more copies of *P. nicotianae* than *P. cactorum* ribosomal gene. Last, Goodwin and fry (1994) demonstrated unambiguously that the progeny of the crossings between the two heterothallic species *P. infestans* and *P. mirabilis* were not generated through self fertilization but following hybridization by analyses of the segregation ratios for alleles at several loci revealed by southern-blotting assays using a fingerprinting DNA probe.

The examination of natural putative hybrids requires much attention, as by definition the parental species are not ascertained. Based on the combination of nuclear and mitochondrial sequence data, Kroon et al. (2004) could re-examine the origin of the putative hybrid *P. meadii*. This demonstrated the usefulness of study combining data bi- and uni-parentally inherited to decipher the origin of a hybrid taxon. New insights into the probable origin of *P. alni* subsp. *alni* were provided by the study of the allelic distribution of four unlinked single copy nuclear genes (Ioos et al., 2006a). Indeed, single copy DNA may be highly homologous to the sequences present in the genome of hybrid progenitors (Okamuro and Goldberg, 1985). Fort each gene, *Paa* was proven to possess at the same time three alleles which were those also found in *Pam* and *Pau*, but differed significantly from those exhibited by *P. cambivora* and *P. fragariae sensu lato*, previously suggested to represent the hybrid progenitors (Brasier et al., 1999). However, these results were not sufficient to support that *Paa* originated from the hybridization of *Pam* and *Pau*, because *Pam* and *Pau* could also represent genetical breakdowns of *Paa*, inheriting part of its alleles. Uniparentally inherited DNA such as mitochondrial DNA is therefore of much interest and should help to unravel the relationship between a hybrid and its putative parental counterparts. Last, it should be kept in mind that the presence of multiple alleles for a gene potentially present at single copy in the genome may not be only attributed to reticulation events since episodic and ancient gene duplication may also generate paralogues, such as those observed in *P. cambivora* (Ioos et al., 2006a).

In other respects, microsatellites are codominant markers that are especially useful to address questions relating to origin and genetic diversity of fungi (Weising et al., 1995) and are now famous tools to conduct population genetic studies. Despite they may cross-react with closely related taxa, microsatellite markers are mostly intrinsically species-specific and should therefore enable the development of species-specific markers. The co-dominancy of these markers should enable the amplification of the respective parental species alleles, with the assumption they are phylogenetically close. Hence, the characterization of microsatellite loci showed that the natural hybrid *Paa* exhibited

both the alleles from the putative parental species *Pam* and *Pau* and that the genotype amplification pattern at several loci could additionally reflect the ploidy level in hybrid species (Ioos et al., 2006b).

3.5.4. Mitochondrial genes.

The co-existence of two or more mitochondrial types within a single taxon could be a consequence of a somatic hybridization event. However, Gu and Ko (2000b) demonstrated that fusion and / or displacement of mitochondrial type could occur following artificial transplantation of alien mitochondria in a *Phytophthora* receptor cell. Kroon et al. (2004) hypothesized that the abnormal mutations patterns resolved during its phylogenetics studies based on mitochondrial gene sequences could be explained by "leakage" of paternal mtDNA, followed by recombination between both mitochondrial types. Therefore, somatic and at a lesser extent, sexual hybridization could eventually lead to a new mitochondrial type, different from both parental species.

However, in the cases of natural hybridization reported up to now, only a single mitochondrial type has been inherited in the hybrid progeny. All the *P. cactorum* x *P. nicotianae* hybrids examined by Man in't Veld et al. (1998) displayed the same mtDNA restriction pattern typical of *P. nicotianae,* and in this view, the latter species would have acted as the oogonial mate during the sexual hybridization. Likewise, Ioos et al. (2006a) and Man in't Veld et al. (2006) resolved two different mitotypes among the different hybrid taxa *P. alni* subsp. *alni* and *P. cactorum* x *P. hedraiandra*, respectively, corresponding to either of the putative parental species. This dichotomous repartition of the mitochondrial types also proved that at least two sexual hybridization events occurred during which each parental species acted as a maternal strain.

3.6. Combining data to trace back the parental species.

Deciphering the origin of a hybrid taxon in the genus *Phytophthora*, as well as for any other biological group, remains a delicate exercise. Putative kinship should be supported by several and independent criterions. Morphological, cytological, nuclear and mitochondrial data should be combined in order to unravel the relationship between a natural species hybrid and its putative parents (e.g. Man in't Veld et al., 1998; Bonants et al., 2000; Ioos et al., 2006a, b). However, the ideal evidence would be the artificial generation of the *Phytophthora* hybrid by *in vitro* crossing(s) between the putative parental species. Such crossing studies should be needed to support any hybrid hypothesis (Vriesendorp and Bakker, 2005). This artificially generated hybrid should share identical features with the putative natural hybrid and complete Koch's postulate. To the last of our knowledge, such a demonstration has never been successfully conducted with any of the natural hybrid reported. In this view, the parental species should remain quoted as 'presumed' parents until evidence of successful artificial crossing has been provided. Indeed, despite a large set of data would indicate the involvement of one more species in the genesis of a natural hybrid, it may also be hypothesized that one or more previously unknown phylogenetically and morphologically close species are involved. In this respect, the observation of Goodwin and Fry (1994): "Claims of interspecific hybridization should be confirmed with

unambiguous genetic markers" could also fit to the demonstration of the identity of the parental species that also requires the use of a multiloci analysis, combining bi-parentally and uni-parentally inherited DNA. However, the retrospective analysis of the hybridization events can represent somewhat a challenge if the genetic modifications remained very discrete or blurred by subsequent genetic rearrangements (Brasier, 1995). For instance, intraspecific hybrids of *P. sojae* displayed considerable genetic rearrangement produced by mitotic gene conversion during their vegetative growth (Chamnanpunt et al., 2001), and it may be hypothesized that interspecific hybrid might undergo such rearrangement making the deciphering of each parental contribution sometimes even more difficult.

4. Conclusion and prospective.

Hybridization in the fungal kingdom may sometimes lead to obvious beneficial interactions (Tsai et al., 1994). However, the rise of natural fungal hybrids (Brasier, 2000) appears more often as a threat to the ecosystems. In particular, the natural hybrids occurring within the genus *Phytophthora* pose serious problems owing to the fact that most of the pre-existing species are already aggressive plant pathogens.

Boccas (1981) concluded from his experiments that the likelihood of genetic exchange between the closely related species he tempted to cross was weak, and based on his results stated that "interspecific hybridization was unlikely to be an important source of variability in the genus *Phytophthora*". If one considers the amount of example of successful artificial hybridizations reported up to now, the potential for gene flow between species of *Phytophthora* seems on the contrary not insignificant. This is however mitigated by the fact that only a few cases of natural hybridization are reported. Under the biological species concept, which seems appropriate for *Phytophthora* (Brasier and Hansen, 1992), the species are internally compatible and externally reproductively isolated and hybridization should remain scarce by definition. In this respect, it might be hypothesized that other cryptic natural hybrids and backcross hybrids may exist, although undetected because of the lack of striking differences with existing and well defined taxa and/or have gone unrecognized in morphologically uniform subgroups (Burnett, 2003), or because, *Phytophthora* taxa occurring in natural habitats have been yet poorly investigated in comparison with agricultural pathogens (Brasier and Hansen, 1992). In contrast with other Kingdoms where intraspecific hybridization outcomes may be conspicuous owing to intermediate or striking phenotypic differences (Mallet, 2005), natural *Phytophthora* hybrids may only be encountered *i)* if they exhibit markedly new properties, such as exacerbated aggressiveness or a new host range, or *ii)* during survey studies using modern molecular tools (e.g. Man in't Veld et al., 1998, 2006).

Greatly improved genetic data show clearly that horizontal gene transfer, hybridization, and introgression between species are ongoing and regular, if not always common process in nature (Arnold, 2004a,b). Hence, in spite of the difficulties to address the putative hybrid status of a taxon and the awkwardness to trace back the parental species, it seems very likely that interspecific hybridization

is actually also a significant mean to generate variation in the genus *Phytophthora* that will deserve particular attention in the near future.

Interspecific hybridization is one of the routes leading to sympatric speciation (Kohn, 2005), but the commonest result of hybridization remains more likely introgression, i.e. the transfer or genetic material between hybridizing taxa through backcrosses (Anderson, 1948, 1949). We have seen than in the genus *Phytophthora*, a hybrid may encompass different taxa, from established hybrid species or still evolving recent hybrid, to taxa with different amount of introgression and change in ploidy level. As a mirror to the assumptions of Anderson and Stebbins (1954) for plants, transfer of part or whole genetic material from a *Phytophthora* species to another may produce new evolutionary lineages that possess new phenotypes and ecological amplitude.

All the artificial attempts to hybridize different taxa of *Phytophthora* reported in this review demonstrate the usefulness of investigating the behavior of the different progenies because they may anticipate some of the consequences of such an event, if naturally occurring. Some authors emphasized the beneficial potential applications of interspecific hybridization, such as the production of superior strains for usage in industry (Gu and Ko, 2001). However, an expanded host range or an exacerbated aggressiveness obviously represent a great threat to our environment and agriculture and all the successful attempts for artificial interspecific hybridization show that the actual quarantine regulations would not be protective and relevant to prevent the meeting of two allopatric *Phytophthora* species. These regulations would neither be efficient to prevent the spread of hybrid *Phytophthora* taxa if the diagnosis techniques do not evolve toward the use of relevant molecular markers, since morphological features or ITS-based detection assays could confound the hybrid with the parental species.

It should be kept in mind that human activity could be a powerful agent for bringing together cross-compatible species of *Phytophthora*, including agricultural as well as non-agricultural taxa, which had been previously isolated by ecology or geography. In addition, it is now well recognized that manhood activity is also responsible for climate change and pollution that likely represent additional stress sources for plants. The latter might therefore be more susceptible to these new *Phytophthora* genotypes generated by interspecific hybridization, especially the slower-reproducing elements of natural ecosystems such as trees and shrubs (Brasier, 2001). However, it is now accepted that numerous examples of natural interspecific hybridizations throughout the plant, animal or insect kingdoms were not connected to any environmental disturbances (Mallet, 2005) and it may therefore be speculated that hybridization within the *Phytophthora* genus may also occur "naturally".

References

Adler N.E., Erselius L.J., Chacón M.G., Flier W.G., Ordoñez M.E., Kroon L.P.N.M., Forbes G.A. (2004) Genetic diversity of *Phytophthora infestans sensu lato* in Ecuador provides new insight into the origin of this important plant pathogen. Phytopathology 94: 154-162.

Aguilar J.F., Feliner G.N. (2003) Additive polymorphisms and reticulation in an ITS phylogeny of thrifts (*Armeria*, Plumbaginaceae). Molecular Phylogenetics and Evolution 28: 430-447.

Anderson E. (1948) Hybridization of the habitat. Evolution 2: 1-9.

Anderson E. (1949) Introgressive hybridization. John Wiley and sons, New York.

Anderson E. and Stebbins G.L. (1954) Hybridization as an evolytionnary stimulus. Evolution 8: 378-388.

Ann P.J. and Ko W.H. (1988) Hormonal heterothallism in *Phytophthora parasitica*: a novel mode of sexual reproduction? Journal of General Microbiology 134: 2985-2992.

Arnold M.L. (2004a) Natural hybridization and the evolution of domesticated, pest and disease organisms. Molecular Ecology 13: 997-1007.

Arnold M.L. (2004b) Transfer and origin of adaptation through natural hybridization: were Anderson and Stebbins right ? The Plant Cell 16: 562-570.

Bakonyi J., Láday M., and Érsek T. (2002) Characterization of parental traits in somatic fusion progeny of *Phytophthora infestans* and *Phytophthora nicotianae*. Acta Phytopathologica Entomologica Hungarica 37: 33-46.

Baldwin B.G., Sanderson M.J, Porter J.M., Wojciechowski M.F., Campbell C.S., Donohoghue M.J. (1995) The ITS region of nuclear ribosomal DNA- a valuable source of evidence of angiosperm phylogeny. Annals of the Missouri Botanical Garden 82 : 247-277.

Boccas B. (1973) Observation d'un cas d'hybridation interspécifique entre le *Phytophthora parasitica* Dast. et le *Phytophthora cinnamomi* Rands. Cahiers de l'ORSTOM 20: 63-69.

Boccas B.R. (1981) Interspecific crosses between closely related heterothallic *Phytophthora* species. Phytopathology 71: 60-65.

Boccas B.R., and Zentmayer G.A. (1976) Genetical studies with interspecific crosses between *Phytophthora cinnamomi* and *Phytophthora parasitica*. Phytopathology 66: 477-484.

Bonants P.J.M., Hagenaar-de Weerdt M., Man in't Veld W.A., and Baayen R. (2000) Molecular characterization of natural hybrids of *Phytophthora nicotianae* and *P. cactorum*. Phytopathology 90, 867-874.

Brasier C.M. (1972) Observations on the sexual mechanism in *P. palmivora* and related species. Transactions of the British Mycology Society 58: 237-251.

Brasier C.M. (1991) Current questions in *Phytophthora* systematics: the role of the population approach. In: *Phytophthora* (J.A. Lucas, R.C. Shattock, D.S. Shaw & L.R. Cooke, eds.): Cambridge University Press, Cambridge, UK. 104-128.

Brasier C.M. (1992) Evolutionary Biology of *Phytophthora*. Part I: Genetic system, sexuality and the generation of variation. Annual Review of Phytopathology 30: 153-171.

1 Brasier C.M. (1995) Episodic selection as a force in fungal microevolution, with special reference to
2 clonal speciation and hybrid introgression. Can. J. Bot. 73: 1213-1221.
3 Brasier C.M. (2000) The rise of the hybrid fungi. *Nature* 405: 134-135.
4 Brasier C.M. (2001) Rapid evolution of introduced plant pathogens via interspecific hybridization.
5 Bioscience 51: 123-133.
6 Brasier C.M., Cooke D.E.L. and Duncan J.M. (1999) Origin of a new *Phytophthora* pathogen through
7 interspecific hybridization. Proceedings of the National Academy of Sciences of the USA 96: 5878-
8 5883.
9 Brasier C.M., Cooke D.E.L., Duncan J.M. and Hansen E.M. (2003) Multiple taxa from trees and
10 riparian ecosystems in *Phytophthora gonapodyides* - *P. megasperma* ITS clade 6, which tend to be
11 high-temperature tolerant and either inbreeding or sterile. Mycological Research 107: 277-290.
12 Brasier C.M., Hamm P.B. and Hansen E.M. (1993) Cultural characters protein patterns and unusual
13 mating behaviour of *Phytophthora gonapodyides* isolates from Britain and North America. Mycological
14 Research 97: 1287-1298.
15 Brasier C.M., and Hansen E.M. (1992) Evolutionary Biology of *Phytophthora*. Part II: Phylogeny,
16 speciation, and population structure. Annual Review of Phytopathology 30: 173-200.
17 Brasier C.M. and Kirk S.A. (2001) Comparative aggressiveness of standard and hybrid alder
18 Phytophthoras, *P. cambivora* and other *Phytophthora* species on bark of *Alnus*, *Quercus* and other
19 woody hosts. Plant Pathology 50: 218-229.
20 Brasier C.M., Kirk S.A., Delcan J., Cooke D.E.L., Jung T., and Man in't Veld (2004). *Phytophthora alni*
21 sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus*
22 trees. Mycological Researcy 108: 1172-1184.
23 Brasier C.M., Sanchez-Hernandez E., and Kirk S.A. (2003) *Phytophthora inundata* sp. nov., a part
24 heterothallic pathogen of trees and shrubs in wet or flooded soils. Mycological Research 107: 477-
25 484.
26 Brasier C.M. and Sansome E. R. (1975) Diploidy and gametangial meiosis in *Phytophthora*
27 cinnamomi, P. infestans, P. dreschleri. Transactions of the British Mycological Society 65: 49-65.
28 Burnett J.H. (2003) Fungal populations and species, Oxford University Press, UK. 368 pp.
29 Carter D.A., Buck K.W., Archer S.A., Van der Lee T., Shaw D.S. (1999) The detection of nonhybrid,
30 trisomic ans triploid offspring in sexual progeny of a mating of *Phytophthora infestans*. Fungal Gentics
31 and Biology 26: 198-208.
32 Chang T.T., and Ko W.H. (1993) Evidence for absence of hybridization in crosses between
33 *Phytophthora infestans* and *Phytophthora parasitica*. Mycological Research 97: 675-678.
34 Chamnanpunt J., Shan W.-X. and Tyler B.M. (2001) High frequency mitotic gene conversion in genetic
35 hybrids of the oomycete *Phytophthora sojae*. Proceedings of the National Academy of Sciences of the
36 USA 98: 14530-14535
37 Chee K.H. (1973) Phenotypic differences among single oospores culture of *P. palmivora* and *P.*
38 *botryosa* from *Hevea brasiliensis*. Mycopathologia and Mycologia Applicata. 50: 275-292.
39 Cooke D.E.L., Drenth A., Duncan J.M., Wagels G., and Brasier C.M. (2000) A molecular phylogeny of
40 *Phytophthora* and related Oomycetes. Fungal Genetics and Biology 30, 17-32.

1 Cooke D.E.L. and Ducan J.M. (1997) Phylogenetic analysis of *Phytophthora* species based on ITS1
2 and ITS2 seqduence of ribosomal DNA. Mycological Research101: 667-677.
3 de Cock A. W. A. M. and C. A. Lévesque (2004). New species of *Pythium* and *Phytophthora*. Studies
4 in Mycology 50: 481-487.
5 De Merlier D., Chandelier A., DeBruxelles N., Noldus L., Laurent F., Dufays E., Classens H. and
6 Cavelier M. (2005) Characterization of alder *Phytophthora* isolates from Wallonia and development of
7 SCAR primers for their specific detection. Journal of Phytopathology 153: 99-107.
8 Dover G.A., Tauz D. (1986) Conservation and divergence in multigene families: alternation to
9 selection and drift. Philosophical Transactions of the Royal Society B: Biological Sciences 312: 275-
10 289.
11 Ellstrand N.C. and Schienrenbeck K.A. (2000) Hybridization as a stimulus for the evolution of
12 invasiveness in plants. Proceedings of the National Academy of Sciences of the USA 97: 7043-7050.
13 English J.T., Laday M., Bakonyi J., Schoelz J.E., and Ersek T. (1999) Phenotypic and molecular
14 characterization of species hybrids derived from induced fusion of zoospores of *Phytophthora capsici*
15 and *Phytophthora nicotianae*. Mycological Research 103: 1003-1008.
16 Érsek T., Bakonyi J., and English J.T. (1998) [Morphology and pathogenicity of interspecific progenies
17 obtained via zoospore fusion of *Phytophthora* spp.. Novenyvedelem 34: 529-537.
18 Érsek T., English J., and Schoelz J.E. (1995) Creation of species hybrids of *Phytophthora* with
19 modified host ranges by zoospore fusion. Phytopathology 85: 1343-1347.
20 Érsek T., English J., and Schoelz J.E. (1997) Triparental species hybrids from fused zoospores of
21 *Phytophthora*. Czech Mycologia 50: 13-20.
22 Erselius L.J. and De Vallavieille C. (1984) Variation in protein profiles of *Phytophthora*: comparison of
23 six species. Transactions of the British Mycological Society 83: 463-472.
24 Erselius L.J., and Shaw D. (1982) Protein and enzyme differences between *Phytophthora parasitica*
25 and *P. megakarya*: evidence for self-fertilization in pairings of the two species. Transactions of the
26 British Mycological Society 78: 227-238.
27 Erwin D.C. and Ribeiro D.C. (1996) *Phytophthora* diseases worldwide. APS Press, Americal
28 Phytopathological Society, St Paul.
29 Förster H. and Coffey M.D. (1990) Mating behavior of *Phytophthora parasitica*: evidence for sexual
30 recombination in oospores using DNA restriction fragment length polymorphism as genetic markers.
31 Experimental Mycology 14: 351-359.
32 Förster H. and Coffey M.D. (1991) Approaches to the taxonomy of *Phytophthora* using polymorphisms
33 in mitochondrial and nuclear DNA. . In: *Phytophthora* (J.A. Lucas, R.C. Shattock, D.S. Shaw & L.R.
34 Cooke, eds.) Cambridge University Press, Cambridge, UK 164-183.
35 Fry W.E., Goodwin S.B., Matuszak J.M., Spielman L.J., Milgroom M.G. and Drenth A. (1992)
36 Population genetics and intercontinental migrations of *Phytophthora infestans*. Annual Review of
37 Phytopathology 30: 107-129.
38 Gallindo J. and Gallegly M.E. (1960) The nature of sexuality in *Phytophthora infestans*.
39 Phytopathology 50: 123-128.

1 Gibbs, J., Van Dijk, C., Webber, J., 2003. *Phytophthora* disease of alder in Europe. Forestry
2 Commission Bulletin 126, Edinburgh, 82 pp
3 Gill H.S., and Zentmyer G.A. (1978) Identification of *Phytophthora* species by disc electrophoresis.
4 Phytopathology 68: 163-167.
5 Goodwin S.B. (1997) The population genetics of *Phytophthora*. Phytopathology 87: 462-473.
6 Goodwin S.B., and Fry W.E. (1994) Genetic analyses of interspecific hybrids between *Phytophthora*
7 *infestans* and *Phytophthora mirabilis*. Experimental Mycology 18: 20-32.
8 Goodwin S.B., Webster R.K., and Allard R.W. (1994) Evidence for mutation and migration as sources
9 of genetic variation in populations of *Rhynchosporium secalis*. Phytopathology 84: 1047-1053.
10 Gu Y.-H. and Ko W.-H. (1998) Occurrence of parasexual cycle following transfer of isolated nuclei into
11 protoplast of *Phytophthora* parasitica. Current Genetics 34: 120-123.
12 Gu Y.-H. and Ko W.-H. (2000a) Segregation following interspecific transfer of isolated nuclei between
13 Phytophthora *parasitica* and *P. capsici*. Canadian Journal of Botany *46: 410-416.*
14 Gu Y.-H. and Ko W.-H. (2000b) Transplantation and subsequent behaviour of mitochondria in cells of
15 *Phytophthora*. Canadian Journal of Microbiology 46: 225-230.
16 Gu Y.-H. and Ko W.-H. (2001) Creation of hybrid vigor through nuclear transplantation in
17 *Phytophthora*. Canadian Journal of Microbiology 47: 662-666.
18 Haasis F.A., and Nelson R.R. (1963) Studies on the biological relationships of species of
19 *Phytophthora* as measured by oospore formation in intra and interspecific crosses. Plant Disease
20 Reporter 47: 705-709.
21 Hansen E.M. (1991) Variation in the species of the *Phytophthora megasperma* complex. In:
22 *Phytophthora* (J.A. Lucas, R.C. Shattock, D.S. Shaw & L.R. Cooke, eds.): Cambridge University
23 Press, Cambridge, UK. 148-163..
24 Hansen E.M., Brasier C.M., Shaw D.S., Hamm P.B. (1986) The taxonomic structure of *Phytophthora*
25 *megasperma*: evidence for emerging biological species groups. Transactions of the British
26 Mycological Society 87: 557-573.
27 Hansen E.M., Maxwell D.P. (1991) Species of the *Phytophthora megasperma* complex. Mycologia 83:
28 376-381.
29 Ioos R., Andrieux A., Marçais B., and Frey P. (2006a) Genetic characterization of the natural hybrid
30 species *Phytophthora alni* as inferred from nuclear and mitochondrial DNA analyses. Fungal Genetics
31 and Biology 43: 511-529.
32 Ioos R., Barrès B., Andrieux A., Frey P. (2006b) Characterization of microsatellite markers in the
33 hybrid *Phytophthora alni* subsp. *alni*, and cross-amplification with related subspecies and species.
34 Molecular Ecology Notes. *In press.*
35 Ioos R., Panabières F., Industri B., Andrieux A., and Frey P. (2006c) Overlapping elicitin genes
36 patterns resolved within the hybrid oomycete *Phytophthora alni* and the related species *P. cambivora*
37 and *P. fragariae. Submitted.*
38 Kohn L.M. (2005) Mechanisms of Fungal speciation. Annual Review of Phytopathology 43: 279-308.

1 Kroon L.P.N.M., Bakker F.T., van den Bosch G.B.M., Bonants P.J.M., and Flier W.G. (2004)
2 Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences.
3 Fungal Genetics and Biology 41, 766-782.
4 Mac Donald J.D., Ali-Shtayeh M.S., Kabashima J. and Stites J. (1994) Occurrence of *Phytophthora*
5 species in recirculated nursery irrigation effluents. Plant Disease 78: 607-611.
6 Mallet J. (2005) Hybridization as an invasion of the genome. Trends in Ecology and Evolution 20: 229-
7 237.
8 Man in't Veld W.A. (2001) First report of natural hybrids of *Phytophthora nicotianae* and *P. cactorum*
9 on loquat in Taiwan. Plant Disease 85: 98.
10 Man in't Veld W.A., de Cock A.W.A.M., Summerbell R.C. (2006) Natural hybrids of resident and
11 introduced *Phytophthora* species proliferating on new hosts. European Journal of Plant Pathology. In
12 press.
13 Man in't Veld W.A., Veenbaas-Rijk W.J., Ilieva E., De Cock A.W.A.M., Bonants P.J.M. and Pieters R.
14 (1998). Natural hybrids of *Phytophthora nicotianae* and *P. cactorum* demonstrated by isozyme
15 analysis and random amplified polymorphic DNA. Phytopathology 88, 922-929.
16 Martin F.N. and Tooley P.W. (2003) Phylogenetic relationships among *Phytophthora* species inferred
17 from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. Mycologia 95:
18 269-284.
19 May K.J., Drenth A., and Irwin J.A.G. (2003) Interspecific hybrids between the homothallic
20 *Phytophthora sojae* and *Phytophthora vignae.* Australasian Plant Pathology 32: 353-359.
21 Mills S.D., Förster H., Coffey M.D. (1991) Taxonomic structure of *Phytophthora cryptogea* and
22 *Phytophthora drechsleri* based on isozyme and mitochondrial DNA analyses. Mycological Research.
23 95: 31-48.
24 Nagy Z.A., Bakonyi J., Ersek T. (2003) Standard and Swedish variant types of the hybrid alder
25 *Phytophthora* attacking alder in Hungary. Pest Management Science 59: 484-492.
26 Okamuro J.K. and Goldberg R.B. (1985) Tobacco single copy DNA is highly homologous to
27 sequences present in the genome of its diploid progenitor. Molecular Genomic and Genetic 198: 290-
28 298.
29 Olson A. and Stenlid J. (2002) Pathogenic fungal species hybrid infecting plants. Microbes and
30 infection 4: 1353-1359.
31 Otto S.P., Whitton J. (2000) Polyploid incidence and evolution. Annual Review of Genetic 34: 401-427.
32 Oudemans P and Coffey M.D. (1991) Isozyme comparison within and among worldwide sources of
33 three morphologically distinct species of *Phytophthora.* Mycological Reseach 95: 19-30.
34 Rauscher J.T. Doyle J.J., Brown A.H.D. (2002) Internal transcribed spacer repeat-specific primers and
35 the analysis of hybridization in the *Glycine tomentella* (Leguminosae) polyploidy complex. Molecular
36 Ecology 11: 2691-2702.
37 Ricci P., Pope-De Vallavieille C., Panabières F., Marais A., and Augé G. (1990) Caractères comparés
38 des espèces de *Phytophthora* pathogènes des agrumes. EPPO Bulletin 20: 19-28.
39 Richardson B.J., Baverstock P.R., and Admas M. (1986) Electrophoresis. P 23, in: Allozyme
40 electrophoresis. Academic Press, New York.

Rizzo D.M., Garbelotto M., Davidson J.M., Slaughter G.W., Koike S.T. (2002) *Phytophthora ramorum* as the cause of extensive mortality in *Quercus* spp. and *Lithocarpus densiflorus* in California. Plant Disease 86: 205-214.

Si-Ammour A. (2002) Molecular analysis of the arabidopsis-*Phytophthora* pathosystem. PhD Thesis, University of Fribourg, Switzerland.

Sansome E.R. (1977) Polyploidy and induced gametangial formation in the British isolates of *Phytophthora* infestans. Journal of General Microbiology 99: 311-316.

Sansome E. and Brasier C.M. (1974) Polyploidy associated with varietal differentiation in the *megasperma* complex of *Phytophthora*. Transaction of the British Mycological Society 63: 461-467.

Sansome E., Brasier C.M., and Hamm P.B. (1991) *Phytophthora meadii* may be a species hybrid. Mycological Research 95: 273-277.

Satour M.M., and Butler E.E. (1967) A root and crown rot of tomato caused by *Phytophthora capsici* and *P. parasitica*. Phytopathology 57: 510-515.

Savage E.J., Clayton C.W., Hunter J.H., Brenneman J.A., Laviola C., and Gallegly M.E. (1968) Homothalism, heterothallism, and interspecific hybridization in the genus *Phytophthora*. Phytopathology 58: 1004-1021.

Shardl C.L. and Craven K.D. (2002) Interspecific hybridization in plant-associated fungi and oomycetes: a review. Molecular Ecology 12: 2861-2873.

Shaw D.C., and Shattock R.C. (1991) Genetics of *Phytophthora infestans*: the mendelian approach. In: *Phytophthora* (J.A. Lucas, R.C. Shattock, D.S. Shaw, and L.R. Cooke, Eds.) pp. 218-230. Cambridge Univ. Press, Cambridge, UK.

Stoskopf N.C., Tomes D.T., and Christie B.R. (1993) Plant breeding: theory and practice. Westview press, Boulder, Colo.

Themann K., Werres S., Lüttmann R.D, Diener H.-A. (2002) Observation of *Phytophthora* spp. in recirculation systems in commercial hardy ornamental nursery stock. European Journal of Plant Pathology 108: 337-343.

Tooley P.W., Therrien C.D. (1987) Cytophotometric determination of the nuclear DNA content of 23 Mexican and 18 non-Mexican isolates of *Phytophthora infestans*. Experimental Mycology 11: 19-26.

Tsai H.F., Liu J.S., Staben C., Christensen M.J., Latch. G.C.M., Siegel M.R., and Schardl C.L. (1994) Evolutionary diversification of fungal endophytes of tall fescue grass by hybridization with *Epichloë* species. Proceedings of the National Academy of Sciences of the USA 91: 2542-2546.

Volkov R.A., Borisjuk N.V., Panchuk I.I., Schweizer D., and Hemleben V.(1999). Elimination and rearrangement of parental rDNA in the allotetraploid *Nicotiana tabacum*. Molecular Biology and Evolution 16, 311-320

Vorobèva Y.V., and Gridnev V.V. (1981) Crossability of various heterothallic species of *Phytophthora* fungi. Genetika 17: 992-995.

Vriesendorp B. and Bakker F.T. (2005) Reconstructing patterns of reticulate evolution in angiosperms: what can we do ? Taxon 54: 593-604.

Weising K., Nybom H., Wolff K., Meyer W. (1995) *DNA fingerprinting in plants and Fungi*. CRC presse, USA.

1 Wendel J.F., Schnabel A., Seelanan T. (1995) Bidirectional interlocus concerted evolutin following
2 allopolyploid speciation in cotton (*Gossypium*) Proceedings of the National Academy of Sciences of
3 the USA 92: 280-284.
4 Whisson S.C., Drenth A., MacLean D.J., and Irwin J.A.G. (1994) Evidence of routcrossing in
5 *Phytophthora* sojae and linkage of a DNA marker to two avirulence gnes. Current Genetics 27: 77-82.
6 White T.J., Bruns T., Lee S., Taylor J. (1990). Amplification and direct sequencing of fungal ribosomal
7 RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ eds. *PCR protocols:*
8 *a guide to method and applications.* Academic Press, NewYork. 315-322.
9 Whittaker S.L., Assinder S. J., and Shaw D.S. (1994). Inheritance of mitochondrial DNA in
10 *Phytophthora infestans.* Mycological Research 98, 569-575.

Table 1 : List and characteristics of hybrid or putative hybrid lineages described throughout the genus *Phytophthora*.

Derived taxon or parent taxa (reference)	Phylogenetical relatedness of the parental species	Host range / parents	Aggressiveness / parents,	Habit of the hybrid lineage	ploidy	Reproduction system (parent A x parent B / hybrid taxon)	Sexual or vegetative cross	mtDNA inheritance	Evidence of hybrid status / Markers used	Stability / hybrid vigor
Artificial hybrids										
P. infestans x *P. capsici* (Savage et al., 1968)	Different phylogenetical clades[a]	*n.i.*	*n.i.*	n/a	*n.i.*	He* x He / *n.i.*	Sexual cross between opposite mating types	*n.i.*	Observation of mature oospores produced by fusion of gametangia from each species	*n.i.*
P. palmivora x *P. capsici* (Boccas, 1981)	Different phylogenetical clades[a]	Host range of *P. capsici*	*n.i.*	n/a	*n.i.*	He x He / *n.i.*	Sexual cross between opposite mating types	*n.i.*	Protein pattern	*n.i.*
P. infestans x *P. mirabilis* (Goodwin and Fry, 1994)	Common phylogenetical clade[a]	Reduced	Reduced	n/a	*n.i.*	He x He / *n.i.*	Sexual cross between opposite mating types	*P. infestans* (82% of the progeny) or *P. mirabilis* (18%)	Isozyme patterns, Fingerprinting with a polymorphic hybridization probe	Stable
P. nicotianae x *P. capsici* (Ersek et al., 1995; English et al., 1998	Different phylogenetical clades[a]	Pathogenic to all host plants from each parent or reduced host range	Reduced	n/a	*n.i.*	He (A2) x He (A2) / He	Somatic fusion between zoospores	*n.i.*	Morphology, Allele-specific PCR, Hybridization with species-specific probe, RAPD	Stable
P. nicotianae x *P. infestans* (Ersek et al., 1998)	Common phylogenetical clade[a]	Pathogenic to all host plants from each parent or to the hosts of one or the other parent	*n.i.*	n/a	*n.i.*	He (A2) x He (A2) / *n.i.*	Somatic fusion between zoospores	*n.i.*	Expression of drug resistance	*n.i.*
P. nicotianae x *P. infestans* (Bakonyi et al., 2002)	Common phylogenetical clade[a]	Host range of *P. nicotianae*	Reduced	n/a	*n.i.*	He (A2) x He (A2) / *n.i.*	Somatic fusion between zoospores	*n.i.*	Allele-specific PCR, Hybridization with species-specific probe	*n.i.*
P. nicotianae x *P. capsici* x *P. citrophthora* (Ersek et al., 1997)	*P. nicotianae* belongs to a distinct phylogenetical clade[a]	Loss of pathogenicity to the parent's hosts	Complete loss of aggressiveness	n/a	*n.i.*	He (A2) x He (A2) x He (A2) / *n.i.*	Somatic fusion between zoospores	*n.i.*	Expression of drug resistance, Allele-specific PCR, Hybridization with species-specific probe	Instable
P. sojae x *P. vignae* (May et al., 2003)	Common phylogenetical clade[a]	Host range of *P. sojae* + *P. vignae*	Reduced	n/a	*n.i.*	Ho** x Ho	Sexual cross between two strains of different species	*n.i.*	RAPD + AFLP patterns	No germinating oospore
P. parasitica x *P. capsici* (Gu and Ko, 2000a)	Different phylogenetical clades[a]	*n.i.*	*n.i.*	n/a	transient tetraploidy and diploidization	He x He / *n.i.*	Nuclear transplantation	n/a	Cultural features, Drug resistance	Stable

P. parasitica x *P. capsici* (Gu and Ko, 2001)	Different phylogenetical clades[a]	*n.i.*	Increased growth rate, increased sporulation	n/a	*n.i.*	He x He / *n.i.*	Nuclear transplantation	n/a	Drug resistance	Stable
Natural hybrids										
P. meadii (Sansome et al., 1991) *i) P. botryosa* x *P. heveae*? Less probable involvement of *P. palmivora* and *P. capsici* (Sansome et al., 1991) *ii) P. colocasiae* x *P. hibernalis*? (Kroon et al., 2004)	*i)* Different phylogenetical clades[a] *ii)* Different phylogenetical clades[c]	*i)* Rubber and Cocoa are common hosts between both putative parents and hybrid[d] *ii)* Mainly host range of *P. colocasiae*	No increased aggressiveness observed	Mainly located in south India and Sri-Lanka, and mainly confined to rubber trees.	2n or 4n or mix of 2n and 4n	*i)* He x Ho / He[a] *ii)* He x Ho / He[c]	Probably sexual cross but needs further demonstration[bc]	Possibly inherited from *P. hibernalis* but needs further demonstration[c]	Polyploid nuclei, Incomplete meiosis in diploid isolates[b]	Thriving on rubber trees, but diploid isolates unable to produce viable oospores[b]
P. nicotianae x *P. cactorum* (Man in't Veld et al., 1998; Bonants et al., 2000)	Common phylogenetical clade[ac]	Probable expanded host range (*Cyclamen* sp.)	*n.i.*	Several ornamental species in hydroponic systems in the Netherlands Loquat Trees in Taiwan	polyploidy suggested as inferred from AFLP patterns[f]	He x Ho / Ho	mtDNA characterization suggests sexual crossing	Exclusively inherited from *P. nicotianae*	Isozyme analysis[e], RAPD[e] and AFLP[f] patterns, Southern blots with species-specific probes[e], mtDNA RFLP[e], ITS-RFLP[f], Species-specific PCR[f]	Stable
P. alni subsp. *alni* (*Paa*) *i) P. cambivora* x *P. fragariae*-like (Brasier et al., 2004) *ii) P. alni* subsp. *uniformis* (*Pau*) x *P. alni* subsp. *multiformis* (*Pam*) (Ioos et al., 2006a)	*i)* Common phylogenetical clade[a] *ii)* Phylogenetically very close[gh]	*i)* New host *Alnus* spp.[g] *ii)* Same host range than the putative parental species[h]	*i)* n/a *ii)* More aggressive[h]	Epidemic level throughout the European alder population	4n+2[g]	*i)* He x Ho / Ho[g] *ii)* Ho x Ho / Ho[h]	mtDNA characterization suggests sexual crossing[h]	*ii)* Inherited from *Pam* (87% of the *Paa* isolates) or from *Pau* (13% of the *Paa* isolates)[h]	Karyotyping[g], ITS sequencing[g], AFLP patterns[g], Single-copy nuclear gene sequencing[h], Allele-specific PCR, mtDNA RFLP[h], Mitochondrial gene sequencing[h]	*Paa* is stable, thriving on alders and became invasive
P. cactorum x *P. hedraiandra* (Man in't Veld et al., 2006)	Phylogenetically very close[i]	Probable expanded host range (*Allium* spp., *Idesia* sp. *Penstemon* sp.)	*n.i.*	Nurseries and parks in the Netherlands and Germany	Probable trisomy at the *Mdhp* locus, ploidy not investigated	Ho x Ho / Ho	mtDNA characterization suggests sexual crossing	Inherited from *P. hedraiandra* or from *P. cactorum*	Isozyme analysis, ITS sequencing, Mitochondrial gene sequencing	Stable

n/a: not applicable
n.i.: not investigated
[a]Cooke et al, 2000
[b]Sansome et al., 1991
[c]Kroon et al., 2004
[d]Erwin and Ribeiro, 1996
[e]Man in't Veld et al., 1998
[f]Bonants et al., 2000
[g]Brasier et al., 1999
[h]Ioos et al., 2006a
[i]as inferred from ITS sequence alignment retrieved from Genbank
*He: heterothallic
**Ho: homothallic

Conclusions & perspectives

1. - Production d'outils pour l'étude épidémiologique de *P. alni sensu lato.*

Le premier de nos objectifs consistait à mettre au point un outil de détection des trois entités constituant *P. alni sensu lato.* Le test de détection par PCR que nous avons développé dans le cadre de cette étude permet de détecter dans différents substrats chacun des trois taxons constituant *P. alni sensu lato* et de les identifier en combinant l'utilisation de trois couples d'amorces de PCR. Les régions spécifiques ciblées ont été identifiées à partir de trois SCARs qui ne correspondent à aucune région codante. En revanche, le séquençage et l'étude de la distribution des quatre gènes nucléaires relatés dans le chapitre II ont montré que les régions introniques de ces gènes présentaient un intérêt tout particulier en terme de taxonomie, eu égard aux divergences observées entre taxons. Certaines des amorces de PCR allèle-spécifiques qui ont été développés peuvent être valorisées comme des outils de détection et d'identification pour chacun des taxons étudiés. Nous avons ainsi exploité ces régions pour définir des amorces de PCR espèces spécifiques de *P. fragariae* et *P. ramorum*, et il est tout à fait envisageable de définir des amorces de PCR spécifiques à d'autres espèces d'importance économique ou écologique, en suivant le même principe.

Ces outils de détection par PCR sont actuellement utilisés dans le cadre d'études épidémiologiques afin de pouvoir suivre la distribution, la conservation et les fluctuations temporelles de l'inoculum de *P. alni sensu lato.* Les périodes de dissémination et les capacités de conservation de *P. alni* sont encore mal connues et l'utilisation de ces outils de détection devrait apporter des éléments de réponse.

Le test de détection par PCR, incluant un témoin interne d'amplification, est par ailleurs utilisé comme outil de détection dans le cadre officiel de la surveillance du territoire par l'Unité de Mycologie Agricole et Forestière du Laboratoire National de la Protection des Végétaux (LNPV-UMAF). La présence de *P. alni sensu lato* n'est pour l'instant pas rapportée en dehors du continent européen. Le risque écologique considérable que son introduction représenterait pour des pays extérieurs a motivé l'inscription de *P. alni sensu lato* au rang de parasite de quarantaine ou d'alerte pour les pays d'Amérique du Nord (Anonyme, 2003, Seeland et al., 2006). L'utilisation de plants, de substrat et d'eau d'irrigation sains est indispensable pour limiter la dissémination de cet agent pathogène pour lequel aucune méthode d'éradication n'est envisageable à l'heure actuelle.

2. - Relation entre les trois taxons *Paa*, *Pam* et *Pau* et origine de l'hybride *Paa*.

Les premiers travaux de caractérisation menés par Brasier et al. (1999) ont montré que plusieurs entités génétiques différentes de *Phytophthora* sont associées à des symptômes de nécrose sur l'aulne. Ces différentes entités ont été ultérieurement assignées au rang de « sous-espèces », avec l'hypothèse privilégiée présentant *Pam* et *Pau* comme des ségrégants génétiques de *Paa* (Brasier et al., 2004).

Les résultats qui ont été obtenus dans le cadre de ma thèse ont bien confirmé la cohérence de la séparation des trois entités *Paa*, *Pam* et *Pau*. En revanche, ils laissent supposer que *Pam* et *Pau* ne peuvent pas être des ségrégants directs issus de *Paa*, principalement eu égard à la distribution dichotomique du type mitochondrial. *A contrario*, toutes les données de caractérisation obtenues par l'étude de la distribution allélique de gènes simple copie, de l'ADN mitochondrial, des profils de gènes de familles multigéniques (élicitines) ou encore les génotypes résolus grâce aux marqueurs microsatellites, militent pour une implication directe ou indirecte de *Pam* et *Pau* en tant que parents potentiels de l'hybride *Paa*. La terminologie de « sous-espèce » qui est employée actuellement ne semble donc pas être pertinente pour la qualification de ces trois entités. Le terme de « taxon » devrait être utilisé préférentiellement pour qualifier chacune d'entre-elles, jusqu'à ce que de nouvelles descriptions taxonomiques soient réalisées, tenant compte de l'ensemble des données de caractérisation obtenues et publiées.

A ce titre, le rang d'espèce apparaîtrait parfaitement justifié en ce qui concerne le taxon *Pau*, au vu de ses caractéristiques culturales, génétiques et cytologiques relativement stables. Il est par ailleurs intéressant de noter que lors des prospections menées pour la récolte d'isolats, nous avons identifié un site particulier le long de la Sarre Rouge (Nitting, Moselle) présentant des alignements d'aulnes ripicoles mais aussi des aulnes dispersés à quelques dizaines de mètres de la rivière, y compris le long de ruisseaux affluents. Certains de ces aulnes présentaient des nécroses, parfois sévères, mais sans qu'un dépérissement notable des arbres ne se manifeste. Les nombreux isolements réalisés sur différents sujets ont mis en évidence la présence unique de *Pau*, à l'exclusion des deux autres taxons. Comparativement à *Paa*, *Pau* semble moins avantagé écologiquement puisqu'il est rapporté moins agressif sur l'aulne dans certaines circonstances expérimentales (Brasier & Kirk, 2001) et produit significativement moins de sporanges *in vitro* (R. Ioos, observations personnelles). En d'autres termes et d'après ces observations sur le terrain, il parait ici encore plus parcimonieux de proposer que *Pau* est un plutôt un taxon préexistant à *Paa* qu'un de ses ségrégants, puisque dans le cas contraire, il serait logique d'isoler aussi *Paa* sur ce site ou *Pau* est prédominant.

Pau peut être une espèce endémique ou introduite. Son mode de reproduction qui semble uniquement végétatif ne permet pas la génération d'une variabilité génétique très importante, ce qui semble confirmé par le génotypage microsatellite des quelques isolats étudiés. Sans l'étude d'un nombre significatif d'isolats de *Pau* d'autres régions européennes, il sera difficile de savoir si ce taxon a été récemment introduit, ce qui devrait se traduire par un effet de fondation, ou s'il est endémique. Les arbres phylogénétiques générés à partir des séquences des quatre gènes nucléaires ainsi que l'étude des profils génétiques des gènes d'élicitines montrent que *Pau* est très proche de l'espèce polyphage *P. cambivora*. Il est donc probable que ces deux espèces aient un ancêtre commun, ou que l'un des taxons soit l'ancêtre de l'autre. Si cette dernière hypothèse était retenue, il est plus probable que *P.*

cambivora soit l'espèce ancestrale eu égard à la variabilité génétique observée, à sa polyphagie et à sa description bien plus ancienne (Petri, 1917, syn. *Blepharospora cambivora*).

L'origine de *Pam* semble quant à elle plus complexe. Nos résultats suggèrent que ce taxon résulte lui-même d'un événement d'hybridation ou d'une autopolyploïdisation. Il n'est pas possible de trancher définitivement entre ces deux hypothèses au vu des données que nous avons obtenues. Pour chacun des quatre gènes nucléaires la présence systématique de deux allèles, certes divergents, mais appartenant au même groupe phylogénétique, ne permet pas de choisir entre l'auto et l'allo-polyploïdie. En effet, la topologie des arbres phylogénétiques obtenus pour chacun des quatre gènes nucléaires étudiés, en particulier la séparation des séquences de type PAM1 et PAM2, serait identique dans le cas d'une autopolyploïdisation (gènes portés par des chromosomes homéologues issus du doublement du matériel nucléaire d'un unique taxon) ou d'une allopolyploïdisation (gènes portés par des chromosomes homéologues issus du rassemblement du matériel nucléaire de deux taxons distincts). Il est évident que la découverte des taxons « donneurs » respectivement porteurs des allèles homéologues PAM1 et PAM2 permettrait de trancher en faveur de l'allopolyploïdie suite à une hybridation entre ces deux taxons, mais à l'heure actuelle, leur existence n'est pas démontrée. Il est de plus possible que ces hypothétiques taxons donneurs type « PAM1 » et type « PAM2 » *i)* n'aient pas été isolés suite à un biais d'échantillonnage, *ii)* présentent une écologie différente (autre gamme d'hôtes), *iii)* présentent une origine géographique différente, ou encore iv) soient malheureusement éteints.

Les résultats apportés par cette étude ne permettent pas encore de définir « l'âge » exact de l'hybride. Bien qu'il soit acquis que les premiers enregistrements de dépérissement causés par *Paa* remontent à 1993 (Gibbs, 1995), il n'est pas exclu que *Paa* préexistât à cette description, sans avoir provoqué de dégâts remarquables. Le progrès très rapide des dépérissements massifs à travers l'Europe depuis le début des années 1990 peut s'expliquer par des modifications environnementales qui auraient favorisé le développement de l'hybride ou/et par une dissémination accélérée du parasite via les transports de terre ou de plant infectés à travers l'Europe (Jung & Blaschke, 2004). Le ou les sites dans lesquels se seraient produits les différentes hybridations restent par ailleurs inconnus, bien que l'implication de pépinières soit évoquée par Jung & Blaschke (2004). L'utilisation des marqueurs microsatellites développés dans le cadre de notre travail sur un échantillonnage conséquent d'isolats pourrait à terme permettre de mieux comprendre la structure des populations de *Paa* et de ses progéniteurs et d'éventuellement répondre à ces questions.

Enfin, la question de la ploïdie exacte des différentes entités de *P. alni sensu lato* reste sujette à questions. Si nos résultats sont en accord avec les observations de Brasier et al. (1999) concernant la diploïdie de *Pau*, il n'en est pas de même pour *Pam* et par conséquence pour *Paa*. L'observation de

génotypes complexes sur les chromatogrammes de génotypage microsatellite pour les isolats de *Pam* suggère que ce taxon est plus proche de la tétraploïdie que de la diploïdie. La combinaison de nos résultats (publication 3) et des observations microscopiques de Brasier et al. (1999) pourrait suggérer que *Pam* est homoploïde et proche de la diploïdie, mais les génotypages de loci microsatellites et la comparaison des profils d'expression de gènes d'élicitines incitent plutôt à penser que *Pam* possède beaucoup plus de matériel génétique que *Pau,* référence diploïde. Par voie de conséquence, les mêmes observations contradictoires s'opposent dans le cas de *Paa,* puisque nos résultats suggèrent que ce taxon pourrait être proche de l'hexaploïdie.

Nous avons donc mis en place quelques travaux préliminaires pour développer une technique d'estimation de la ploïdie basée sur la technique de la cytométrie de flux, en utilisant les zoospores unicellulaires des différents taxons. Deux séances de mise au point de cette mesure ont été réalisées à l'INRA de Dijon avec l'aide du Dr C. Revellin, mais il est apparu que cette technologie et l'appareil utilisé nécessitent une quantité importante de matériel biologique (zoospores), ce qui ne nous a pas encore permis pour l'instant d'effectuer des mesures fiables.

3. - Hybridation artificielle entre *Pam* et *Pau.*

La possibilité d'une hybridation entre *Pam* et *Pau* et de la création d'un taxon de type *Paa* mérite d'être testée expérimentalement, et permettrait de confirmer *in vivo* une hypothèse générée « *in silico* » à partir de travaux de caractérisation génétique. Au cours de cette étude, nous avons tenté d'hybrider artificiellement plusieurs isolats de *Pam* avec plusieurs isolats de *Pau.* Avant que nos investigations sur l'ADN mitochondrial n'orientent plus définitivement nos hypothèses sur le mécanisme d'hybridation vers la voie sexuelle, nous avions tenté de réaliser des hybridations somatiques *in vitro,* en utilisant le protocole développé par Ersek et al. (1995). Les zoospores de trois isolats de *Pam* ont été placées en conditions favorisant la fusion protoplastique avec les zoospores de quatre isolats de *Pau* et deux isolats de *P. cambivora,* soit 18 « croisements » possibles. Pour chacune des 18 tentatives de fusion, 98 repiquages monozoospores ont été effectués. Ces 1728 descendants ont ensuite été criblés par PCR allèle-spécifiques pour identifier des isolats hybrides potentiels. Aucun des isolats testés n'a présenté de constitution hybride stable d'après nos tests de criblage.

En revanche, suite aux résultats obtenus par caractérisation de l'ADN mitochondrial des isolats de *Paa, Pau* et *Pam,* nous avons mis en place en février 2006 une expérimentation en serre visant à tenter de réaliser des hybridations sexuelles *in planta.* Pour cela, nous avons placé en contact des inocula de *Pam* et *Pau* sur graines de millet dans le substrat de culture de plants d'aulnes. Des plants d'aulnes ont été par ailleurs aussi mis en contact avec des inocula témoins « mono-taxon » de différents isolats de *Paa,* de *Pam* et de *Pau.* Les pots de cultures ont subi deux ennoyages de 24 et 48 h, respectivement en mars et en mai 2006, afin de favoriser la dissémination des inocula et de favoriser leur rencontre. Les premières observations montrent que dans nos conditions les isolats de *Paa* sont bien plus agressifs sur

les plants d'aulnes, provoquant des nécroses à progression très rapide et ont déjà abouti à la mort de certains sujets six mois après l'inoculation. En revanche, aucun plant d'aulne inoculé par un mélange *Pam+Pau,* par *Pam* seul, ou par *Pau* seul, ne présente de dépérissement aussi sévère que ceux inoculés par *Paa,* ce qui confirme certains tests expérimentaux d'agressivité par blessure (Brasier & Kirk, 2001). Il est donc possible d'imaginer qu'en laissant les inocula évoluer, un hybride de type *Paa* nouvellement généré produirait des symptômes sévères sur le plant d'aulne, ce dernier se comportant ainsi comme un filtre biologique pour la sélection de *Paa.* Des isolements sur nécroses sont prévus dans le courant de l'automne 2006, puis les mois suivants afin de suivre l'évolution de ce pathosystème artificiellement reconstitué.

4. - Particularités du statut hybride chez *Paa.*

Le statut allopolyploïde de *Paa,* déjà suggéré par la particularité des profils AFLP et le polymorphisme observés au niveau des ITS rapportés par Brasier et al. (1999) a été confirmé sans ambiguïté par nos résultats. La présence systématique de trois allèles divergents pour chacun des quatre gènes nucléaires étudiés ainsi que la présence de plus de quatre allèles pour certains loci microsatellites, pour un individu donné, traduit bien la coexistence de génomes distincts au niveau d'un même individu. Il présente certaines particularités mais partage aussi certaines caractéristiques avec les autres taxons hybrides de *Phytophthora* d'origine naturelle.

Tous comme les deux autres hybrides naturels bien documentés qui ont tous deux impliqué *P. cactorum* (Man in't Veld et al., 1998, 2006), *Paa* semble avoir été généré par hybridation sexuelle, et au moins à deux reprises puisque deux types mitochondriaux sont observés. Malgré la possibilité de produire artificiellement des hybrides de *Phytophthora* par fusion de zoospores, les exemples naturels suggèrent que la voie sexuelle semble privilégiée par rapport à la voie somatique, bien que dans certains écosystèmes artificiels, il y ait de fortes probabilités de rencontre entre zoospores de différentes espèces (Bonants et al., 2000). Il est possible d'imaginer que l'hybridation somatique puisse aussi se produire naturellement, mais que les hybrides générés soient moins stables et disparaissent suite au déséquilibre génétique lié à l'interaction entre les différents types mitochondriaux.

Comme les deux autres exemples d'hybrides impliquant *P. cactorum, Paa* a été généré par deux entités phylogénétiquement proches. Cette proximité semble être un prérequis à la production d'hybrides stables, et reflète probablement la nécessaire similarité entre deux génomes pour co-exister dans une même cellule. A ce titre, ces exemples naturels supportent parfaitement l'hypothèse de Brasier (1995) et Burnett (2003) qui présentent l'introduction d'espèces exotiques de *Phytophthora* comme un danger potentiel en terme d'hybridation. En effet, des espèces ayant divergé d'un ancêtre commun et ayant évolué en allopatrie peuvent présenter des génomes très proches et leur

rapprochement artificiel par l'activité humaine peut autoriser leur hybridation puisque ces espèces n'exprimeront entre elles pas de barrières pré-zygotiques suffisantes.
Nous avons par ailleurs émis l'hypothèse que les différents génomes présents dans *Paa* semblaient s'exprimer, basés sur les profils d'expression des gènes d'élicitines, ce qui montre que pour vette famille de gènes, le processus de pseudogénisation souvent observés chez les végétaux allopolyploïdes (Volkov et al., 1999) ne s'est pas (encore) mis en place. De telles investigations n'ont malheureusement pas encore été entreprises sur les autres hybrides naturels de *Phytophthora* décrits, et il ne peut être tiré de règle générale de ce phénomène observé chez *Paa*.

L'apparition de la pathogénicité envers l'aulne avait été évoquée par Brasier et al. (1999) comme un caractère autapomorphe, conséquence de réarrangements génétiques suite à l'hybridation de *P. cambivora* et un taxon proche de *P. fragariae*, ces deux taxons n'étant pas pathogènes sur l'aulne. Cette nouvelle fonction adaptative pourrait être l'apanage des hybrides, leur procurant une supériorité écologique vis-à-vis des espèces parentales qui leur permettent de subsister. Une gamme d'hôte élargie ou différente et/ou une agressivité supérieure aux espèces parentales correspond à la définition du « super-pathogène » telle que proposée par Brasier (1995). Nos résultats orientant plutôt l'origine de *Paa* comme un taxon issu de l'hybridation entre *Pam* et *Pau* élimine la possibilité d'une acquisition d'une nouvelle pathogénicité, puisque ces derniers sont pathogènes sur l'aulne. Les deux autres exemples d'hybrides naturels (*P. cactorum* x *P. nicotianae* et *P. cactorum* x *P. hedraiandra*) ont montré une gamme d'hôtes élargie par rapport à celles rapportées pour les espèces parentales (Man in't Veld et al., 1998, 2006 ; Bonnants et al., 2000). Toutefois, des tests de pathogénicité *in vitro* n'ont pas été effectués pour vérifier l'absence de pathogénicité des espèces parentales envers ces « nouveaux » hôtes desquels ont été isolés les hybrides (Man in't Veld, communication personnelle), et dans ce cas l'acquisition d'une nouvelle pathogénicité reste encore à l'état d'hypothèse.
En revanche, le cas de *Paa* est exemplaire d'un renforcement de l'agressivité d'un hybride vis-à-vis des espèces parentales. Cet avantage évolutif permettrait d'expliquer en partie pourquoi *Paa* est bien plus fréquemment isolé en Europe que *Pam* et *Pau* (Brasier, 2003 ; R. Ioos, observations personnelles) et il pourrait être envisagé que l'hybride supplante et remplace progressivement les espèces parentales (Schardl & Craven, 2003). Néanmoins, d'autres critères comme par exemple la capacité de conservation, la production d'inoculum et la compétition avec d'autres microorganismes devraient aussi être étudiés pour mieux appréhender l'éventail des éventuels avantages adaptatifs générés suite à une telle hybridation.

Enfin, les différentes approches que nous avons utilisées pour caractériser le statut hybride de *Paa* apparaissent très complémentaires. Goodwin & Fry (1994) ont montré qu'il était nécessaire de s'appuyer sur des marqueurs génétiques non ambigus pour démontrer l'existence d'une hybridation entre deux espèces de *Phytophthora*. Il semble aussi important, d'après nos résultats, d'effectuer les

études comparatives ou phylogénétiques en se basant le plus possible sur des séquences issues de produits clonés, et sur un nombre adéquat de sous-clones. L'existence de plusieurs allèles d'un même gène pourrait passer inaperçue (Rauscher et al., 2002) ou difficilement interprétable si des séquences issues de produits bruts de PCR étaient utilisées.

La combinaison de marqueurs nucléaires (hérités des deux taxons parentaux) et de marqueurs mitochondriaux (hérités d'un des taxons parentaux en cas d'hybridation sexuelle) permet par ailleurs d'orienter les relations entre les différents protagonistes. Dans notre cas de figure, il est apparu indispensable d'examiner un nombre significatif d'isolats, puisque l'existence du mitotype « U » chez *Paa* n'avait pas été mise en évidence par Nagy et al. (2003) faute d'un d'échantillonnage suffisant.

Le recours à des marqueurs génétiques est indispensable à la caractérisation du statut hybride. Il est possible d'imaginer que d'autres hybrides interspécifiques de *Phytophthora* existent ou aient existé mais restent ou soient restés cryptiques eu égard à une gamme d'hôte ou une agressivité « ordinaires ».

Les différentes études de surveillance menées au sujet des *Phytophthora* reposent principalement sur l'identification via des critères morphologiques ou sur l'utilisation d'outils de détection spécifiques (PCR). Dans ces deux cas de figure, la mise en évidence d'un taxon hybride est très peu probable et faute de l'apparition d'un caractère autapomorphe ou d'une agressivité exacerbée, comme ce fut le cas pour *Paa*, un taxon hybride naturel restera probablement souvent confondu avec une de ses espèces parentales.

Références bibliographiques

Anonymous (2000) Council directive 2000/29/EC of 8 may 2000 on protective measures against the introduction into the community of organisms harmful to plants or plant products and against their spread within the community. OJ N° L169, 2000.7.10, 1-112.

Anonymous (2002) Commission Decision (2002/757/EC) of 19 September 2002 on provisional emergency phytosanitary measures to prevent the introduction into and the spread within the Community of *Phytophthora ramorum* Werres, De Cock & Man in 't Veld sp. nov. OJ N° L252, 2002-09-20, 3739.

Anonymous (2003) Import requirements of non-manufactured wood and other non-propagative wood products, except solid wood packaging material, from all areas other than the continental United States. Directive D-02-012, September 15, 2003.

Arnold M. L. (2004) Transfer and origin of adaptations through natural hybridization: were Anderson and Stebbins right? *The Plant Cell* 16, 562-570.

Barbara D.J., Morton A., and Miller N.J. (2005) Isolation of microsatellite markers from an interspecific hybrid isolate of the fungal plant pathogen *Verticillium dahliae*. *Molecular Ecology Notes* 4, 854-856.

Bonants P.J.M., Hagenaar-de Weerdt M., Man in't Veld W.A., and Baayen R. (2000) Molecular characterization of natural hybrids of *Phytophthora nicotianae* and *P. cactorum*. *Phytopathology* 90, 867-874.

Boullard B. (1969) Notes de biologie forestière à propos des aulnes. *La forêt privée* 65, 25-31.

Boullard B. (1990) *Guerre et paix dans le règne végétal.* Ed. Ellipse/Marketing. 336 pp.

Brasier C.M. (1992) Evolutionary Biology of *Phytophthora*. Part I: Genetic system, sexuality and the generation of variation. *Annual Review of Phytopathology* 30, 153-171.

Brasier C.M. (1995) Episodic selection as a force in fungal microevolution, with special reference to clonal speciation and hybrid introgression. *Canadian Journal of Botany* 73, 1213-1221.

Brasier C.M. (2000) The rise of the hybrid fungi. *Nature* 405, 134-135.

Brasier C.M. (2001) Rapid evolution of introduced plant pathogens via interspecific hybridization. *Bioscience* 51, 123-133.

Brasier C.M. (2003) The hybrid alder *Phytophthora*: genetic status, pathogenicity, distribution and competitive survival. In: Gibbs, J.N., Van Dijk, C., and Webber, J.F. (Eds.) *Phytophthora* disease of alder in Europe. *Forestry Commission Bulletin* 126, 39-54. HMSO, Edinburgh.

Brasier C.M., Cooke D.E.L., and Duncan J.M. (1999) Origin of a new *Phytophthora* pathogen through interspecific hybridization. *Proceedings of the National Academy of Sciences of the USA* 96, 5878-5883.

Brasier C.M. and Kirk S. (2001) Comparative aggressiveness of standard and variant hybrid alder *Phytophthora*, *Phytophthora cambivora* and other *Phytophthora* species on bark of *Alnus*, *Quercus* and other woody hosts. *Plant Pathology* 50, 218-229.

Brasier C.M., Kirk S.A., Delcán J., Cooke D.E.L., Jung T., and Man in't Veld W.A. (2004) *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. *Mycological Research* 108, 1172-1184.

Brasier C.M., Rose J., and Gibbs J.N. (1995) An unusual *Phytophthora* associated with widespread alder mortality in Britain. *Plant Pathology* 44, 999-1007.

Bruno D. and Brinegar C. (2004) Microsatellite markers in coast redwood (*Sequoia sempervirens*). *Molecular Ecology Notes* 4, 482-484.

Burnett J.H. (2003) *Fungal populations and species*, Oxford University Press, UK. 368 pp.

Cech T. and Hendry S. (2003) A review of diebacks and declines of alder (*Alnus* spp.) in Europe. In: Gibbs, J.N., Van Dijk, C., and Webber, J.F. (Eds.) *Phytophthora disease of alder in Europe*. Forestry Commission Bulletin N°126, 15-24. HMSO, Edinburgh.

Christiansen D.G. (2005) A microsatellite-based method for genotyping diploid and triploid water frog of the *Rana esculenta* hybrid complex. *Molecular Ecology Notes* 5, 190-193.

Christiansen D.G., Fog K., Pedersen B.V., and Boomsma J.J. (2005) Reproduction and hybrid load in all-hybrid populations of *Rana esculenta* water frogs in Denmark. *Evolution* 59, 1348-1361.

Claessens H. (2003) The alder population in Europe. In: Gibbs, J.N., Van Dijk, C., and Webber, J.F. (Eds.) *Phytophthora disease of alder in Europe*. Forestry Commission Bulletin N°126, 5-14. HMSO, Edinburgh.

Cooke D.E.L., Drenth A., Duncan J.M., Wagels G., and Brasier C.M. (2000) A molecular phylogeny of *Phytophthora* and related Oomycetes. *Fungal Genetics and Biology* 30, 17-32.

Delcán J. and Brasier C.M. (2001) Oospore viability and variation in zoospore and hyphal tip derivatives of the hybrid alder Phytophthoras. *Forest Pathology* 31, 65-83.

De Lucas J.R., Domingez A.I., Mendoza A., and Laborda F. (1998) Use of flow-cytometry to distinguish between haploid and diploid strains of *Aspergillus fumigatus*. *Fungal Genetics Newsletters* 45, 7-9.

De Merlier D., Chandelier A., Debruxelles N., Noldus M., Laurent F., Dufays E., Claessens H., and Cavelier M. (2005) Characterization of alder *Phytophthora* isolates from Wallonia and development of SCAR primers for their specific detection. *Journal of Phytopathology* 153, 99-107.

Dobrowolski M.P., Tommerup I.C., Blakeman H.D., and O'Brien P.A. (2002) Non-mendelian inheritance revealed in a genetic analysis of sexual progeny of *Phytophthora cinnamomi* with microsatellite markers. *Fungal Genetics and Biology* 35, 197-212.

Domanski S. and Kowalski T. (1987) Fungi occurring on forests injured by air pollutants in the Upper Silesia and Cracow industrial regions X. Mycoflora of dying young trees of *Alnus incana*. *European Journal of Forest Pathology* 17, 337-348.

Dussart G. (1999) The ecological implication of loss of alder trees. Appendix 3. Consolidated Progress Report of the EU concerted Action FAIR5-CT97-3615.

Érsek T., English J., and Schoelz J.E. (1995) Creation of species hybrids of *Phytophthora* with modified host ranges by zoospore fusion. *Phytopathology* 85, 1343-1347.

Gibbs J. (1995) *Phytophthora* root disease of alder in Britain. *EPPO Bulletin* 25, 661-664.

Gibbs J. (2003) Management and control of *Phytophthora* disease of alder. In: Gibbs, J.N., Van Dijk, C., and Webber, J.F. (Eds.) *Phytophthora disease of alder in Europe*. Forestry Commission Bulletin N°126, 73-78. HMSO, Edinburgh.

Gibbs J.N., Lipscombe M.A., and Peace A.J. (1999) The impact of *Phytophthora* disease on riparian populations of common alder (*Alnus glutinosa*) in southern Britain. *European Journal of Forest Pathology* 29, 39-50.

Gibbs J.N., Strouts R., Rose J., and Brasier C.M. (1994) An unusual *Phytophthora* associated with disease of common alder. Report on Forest Research. HMSO, London, 27-28.

Goodwin S.B., and Fry W.E. (1994) Genetic analyses of interspecific hybrids between *Phytophthora infestans* and *Phytophthora mirabilis*. *Experimental Mycology* 18, 20-32.

Gregory S., Mackaskill G., and Winter T. (1996) *Crown thinning and dieback of alder in Northern Britain.* Forestry Commission Research Information Note 283. Forestry Commission, Edinburgh.

Gross B.L. and Rieseberg L.H. (2005) The ecological genetics of homoploid hybrid speciation. *Journal of Heredity* 96, 241-252.

Husson C., Thoirain B., Caël O., Ioos R., and Marçais B. (2004) Epidemiology of the *Phytophthora*-induced alder decline in northeastern France. 3rd IUFRO Forest *Phytophthora* Research Workshop, Freising, Germany, 11-17 Sept. 2004.

Ioos R., Husson C., Andrieux A., and Frey P. (2005) SCAR–based PCR primers to detect the hybrid pathogen *Phytophthora alni* and its subspecies causing alder disease in Europe. *European Journal of Plant Pathology* 112, 323-335.

Ivors K., Garbelotto M., De Vries I., Ruyter-Spira C., Hekkert B.T., Rozenzweig N., and Bonants P. (2006) Microsatellite markers identify three lineages of *Phytophthora ramorum* in US nurseries, yet single lineages in US forest and European nursery populations. *Molecular Ecology* 14, 1493-1505.

Jung T. and Blaschke M. (2004) *Phytophthora* root and collar rot of alders in Bavaria: distribution, modes of spread and possible management strategies. *Plant Pathology* 53, 197-208.

Jalas J. and Suominen J. (1976) Atlas Florae Europaeae II: 3: *Salicaceae* to *Balanophoraceae*. Cambridge University Press, Cambridge.

Kamoun S., Hraber P., Sobral B., Nuss D., and Govers F. (1999) Initial assessment of gene diversity for the oomycete pathogen *Phytophthora infestans* based on expressed sequences. *Fungal Genetics and Biology* 28, 94-106.

Kroon L.P.N.M., Bakker F.T., van den Bosch G.B.M., Bonants P.J.M., and Flier W.G. (2004) Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology* 41, 766-782.

Lacourt I. and Duncan J.M. (1997) Specific detection of *Phytophthora nicotianae* using the polymerase chain reaction and primers based on the DNA sequence of its elicitin gene *ParA1*. *European Journal of Plant Pathology* 103, 73-83.

Lederer W. and Seemüller E. (1991) Occurrence of mycoplasma-like organisms in diseased and non-symptomatic alder trees (*Alnus* spp.). *European Journal of Forest Pathology* 21, 90-96.

Lees A.K., Wattier R., Shaw D.S., Sullivan L., Williams N.A., and Cooke D.E.L. (2006) Novel microsatellite markers for the analysis of *Phytophthora infestans* populations. *Plant Pathology* 55, 311-319.

Lonsdale D. (2003) *Phytophthora* disease of alder: source of inoculum, infection and host colonisation. In: Gibbs, J.N., Van Dijk, C., and Webber, J.F. (Eds.) *Phytophthora disease of alder in Europe*. Forestry Commission Bulletin N°126, 65-72. HMSO, Edinburgh.

Mallet J. (2005) Hybridization as an invasion of the genome. *Trends in Ecology and Evolution* 20, 229-237.

Man in't Veld W.A., Veenbaas-Rijk W.J., Ilieva E., De Cock A.W.A.M., Bonants P.J.M., and Pieters R. (1998) Natural hybrids of *Phytophthora nicotianae* and *P. cactorum* demonstrated by isozyme analysis and random amplified polymorphic DNA. *Phytopathology* 88, 922-929.

Man in't Veld W.A., de Cock A.W.A.M., Summerbell R.C. (2006) Natural hybrids of resident and introduced *Phytophthora* species proliferating on new hosts. *European Journal of Plant Pathology*. In press.

Marcone D., Firrao G., Ragozzino A., and Locci R. (1994) Detection of MLOs in declining alder trees in Southern Italy and their characterization by RFLP analysis. *European Journal of Forest Pathology* 24, 217-228.

Mullis K.B. and Faloona F.A. (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology*. 155, 335-350.

Moricca S. (2002) *Phomopsis alnea*, the cause of dieback of black alder in Italy. *Plant Pathology* 51, 755-764.

Moriondo F. (1958) Una nuova malattia dell'ontano in Toscana. *L'Italia Forestale* 13, 204-207.

Nagy Z.A., Bakonyi J., Ersek T. (2003) Standard and Swedish variant types of the hybrid alder *Phytophthora* attacking alder in Hungary. *Pest Management Science* 59, 484-492.

Olson A. and Stenlid J. (2002) Pathogenic fungal species hybrid infecting plants. *Microbes and infection* 4, 1353-1359.

Olsson C.H.B. (1999) Occurrence of the alder (*Alnus glutinosa* L.) decline in Sweden and affinities of the causal *Phytophthora* pathogen as assessed by isozyme analysis. In: Agraria 161, Acta Universitatis Agriculturae Sueciae. *Diagnosis of root infecting* Phytophthora *spp.*

Panabières F., Amselem J., Galiana E., and Le Berre J.-Y. (2005) Gene identification in the Oomycete pathogen *Phytophthora parasitica* during in vitro vegetative growth through expressed sequence tags. *Fungal Genetics and Biology* 42, 611-623.

Panabières F., Ponchet M., Allasia V., Cardin L., and Ricci P. (1997) Characterization of border species among *Pythiaceae*: several *Pythium* isolates produce elicitins, typical proteins from *Phytophthora* spp. *Mycological Research* 101, 1459-1468.

Pandey M., Gailing O., Fischer D., Hattemer H.H., and Finkeldey R. (2004) Characterization of microsatellite markers in sycamore (*Acer pseudoplatanus* L.). *Molecular Ecology Notes* 4, 253-255.

Peace T. (1962) *Pathology of trees and shrubs*. Clarendon Press, Oxford.

Ponchet M., Panabières F., Milat M.-L., Montillet J.-L., Suty L., Triantaphylides C., Tirilly Y., and Blein J.-P. (1999) Are elicitins cryptogams in plant-Oomycete communications? *Cellular and Molecular Life Sciences* 56, 1020-1047.

Prospero S., Black J.A., and Winton L.M. (2004) Isolation and characterization of microsatellite markers in *Phytophthora ramorum*, the causal agent of sudden oak death. *Molecular Ecology Notes* 4, 672-674.

Ramsden C., Bériault K., and Bogart J.P. (2006) A nonlethal method of indentification of *Ambystoma laterale*, *A. jeffersionanum* and sympatric unisexuals. *Molecular Ecology Notes* 6, 261-264.

Rauscher J.Y., Doyle J.J., and Brown A.H.D. (2002) Internal transcribed Spacer repeat-specific primers and the analysis of hybridization in the *Glycine tomentella* (Leguminosae) polyploidy complex. *Molecular Ecology* 11, 2691-2702.

Santini A., Barzanti G.P., and Capretti P. (2001) A new *Phytophthora* root disease of alder in Italy. *Plant Disease* 85, 560.

Santini A., Barzanti G.P., and Capretti P. (2003) Susceptibility of some mesophilic hardwoods to alder *Phytophthora*. *Journal of Phytopathology* 151, 406-410.

Seeland T. M., Ostry M.E., Venette R., and Juzwik J. (2006) Document internet disponible au 25/08/06. http://ncrs.fs.fed.us/pubs/gtr/gtr_nc270.pdf

Shardl C.L. and Craven K.D. (2002) Interspecific hybridization in plant-associated fungi and oomycetes: a review. *Molecular Ecology* 12, 2861-2873.

Si-Ammour A. (2002) Molecular analysis of the A*rabidopsis-Phytophthora* pathosystem. PhD Thesis, University of Fribourg, Switzerland.

Streito J.-C. (2003) *Phytophthora* disease of alder: identification and distribution. In: Gibbs JN, Van Dijk C and Webber JF (eds.) *Phytophthora* disease of alder in Europe. Forestry Commission Bulletin N°126, 25-38. HMSO, London.

Streito J.-C., Jarnouen de Villartay G., and Tabary F. (2002a) Methods for isolating the alder *Phytophthora*. *Forest Pathology* 32, 193-196.

Streito J.-C., Legrand P., Tabary F., and Jarnouen de Villartay G. (2002b) *Phytophthora* disease of alder (*Alnus glutinosa*) in France: investigations between 1995 and 1999. *Forest Pathology* 32, 179-191.

Surico G., Mugnai L., Pastorelli R., Giovannetti L., and Stead D.E. (1996) *Erwinia alni*, a new species causing bark cankers of alder (*Alnus* Miller) species. *International Journal of Systematic Bacteriology* 46, 720-726.

Thoirain B., Husson C., and Marçais B. (2006) Risk factors for the *Phytophthora*-induced decline of alder in North-Eastern France. *Phytopathology*, In press.

Truong C., Palmé A.E., Felber F., and Naciri-Graven Y. (2005) Isolation and characterization of microsatellite markers in the tetraploid birch, *Betula pubescens* ssp. *tortuosa*. *Molecular Ecology Notes* 5, 96-98.

Turok J., Erikson G., Kleinschmit J., Canger S. (1996) *Noble hardwood network. Report of the first meeting.* International Plant Genetic Resource Institute, Rome.

Van der Planck J.E (1968) Disease resistance in plants. Academic press. New York and London. 206 p.

Volkov R.A., Borisjuk N.V., Panchuk I.I., Schweizer D., and Hemleben V. (1999) Elimination and rearrangement of parental rDNA in the allotetraploid *Nicotiana tabacum*. *Molecular Biology and Evolution* 16, 311-320

Weising K., Nybom H., Wolff K., and Meyer W. (1995) *DNA fingerprinting in plants and Fungi.* CRC press, USA.

White J. and Gibbs J.N. (2000). The value of alders to Britain. *Quarterly Journal of Forestry* 94, 23-28.

Whittaker, S.L., Assinder, S. J., and Shaw, D.S. (1994). Inheritance of mitochondrial DNA in *Phytophthora infestans*. *Mycological Research* 98, 569-575.

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Titre : Caractérisation génétique de *Phytophthora alni* Brasier & S.A. Kirk, hybride interspécifique agent du dépérissement de l'aulne en Europe.

Résumé.

Une maladie émergente causant le dépérissement de l'aulne est causée par un complexe de trois taxons du genre *Phytophthora* (Oomycète) : *P. alni* subsp. *alni* (*Paa*), *P. alni* subsp. *multiformis* (*Pam*) et *P. alni* subsp. *uniformis* (*Pau*).

La première partie de cette étude a consisté à mettre au point des outils de détection spécifiques de ces trois taxons. À partir de SCARs générés par RAPD, nous avons défini trois couples d'amorces de PCR dont l'utilisation combinée permet la détection et l'identification spécifique de *Paa*, *Pam* et *Pau* dans différents substrats (plante, eau, sol).

Nous avons ensuite étudié la présence et la distribution allélique pour quatre gènes nucléaires contenant des introns sur une collection de *P. alni* et d'espèces proches. L'ADN mitochondrial a également été étudié par RFLP et séquençage de deux gènes. Nous avons montré que *i) Pau* ne semble pas avoir été généré par hybridation, *ii) Pam* présente deux allèles fortement divergents pour chaque gène nucléaire et résulterait donc d'une réticulation ou d'une autopolyploïdisation, *iii) Paa* cumule les allèles présents chez *Pam* et *Pau* et a probablement été créé par hybridation entre *Pam* et *Pau* ou des taxons très proches.

De plus, nous avons étudié le profil d'expression des gènes codant pour des élicitines, famille multigénique spécifique du genre *Phytophthora*. L'additivité des profils de *Pau* et *Pam* vis-à-vis de *Paa* a confirmé nos premiers résultats et montrent que chez ce taxon allopolyploïde, des génomes distincts peuvent être co-exprimés.

Enfin, afin d'étudier la variabilité génétique de ces différents taxons, des marqueurs microsatellites ont été isolés chez *Paa* et caractérisés. Les génotypes obtenus montrent une faible variabilité chez les trois taxons. Ils confirment nos hypothèses quant à l'origine de *Paa,* indiquent que *Paa* aurait été généré à plusieurs reprises et suggèrent que *Pam* est aussi un taxon allopolyploïde.

Mots clés : hybridation interspécifique, *Phytophthora*, émergent, allopolyploïde, aulne, marqueur moléculaire, ADN mitochondrial, marqueur microsatellite, gène simple copie, intron, détection.

Title: Genetic characterization of *Phytophthora alni* Brasier & S.A. Kirk, interspecific hybrid causing the disease of alder in Europe.

Abstract.

An emergent disease of alder is caused by a complex of three taxa belonging to the genus *Phytophthora* (Oomycetes): *P. alni* subsp. *alni* (*Paa*), *P. alni* subsp. *multiformis* (*Pam*) and *P. alni* subsp. *uniformis* (*Pau*).

The first part of this study focused on the development of specific detection tools for these three taxa. Based on SCARs generated with RAPD, we designed three PCR primer pairs which can be combined to specifically detect and identify *Paa*, *Pam* and *Pau* in different substrates (plant tissue, water, soil).

Second, we studied the occurrence and the allelic distribution for several nuclear single-copy genes containing introns on a wide collection of *P. alni* and close species. Mitochondrial DNA was also studied through RFLP and gene sequencing. We demonstrated that *i) Pau* may not result from a hybridization event, *ii)* two divergent alleles for each of the nuclear genes are observed in *Pam*, which suggests this taxon may have been generated by a reticulation or by autopolyploidisation, *iii) Paa* combines the alleles observed in *Pam* and *Pau* and was probably generated by hybridization between *Pam* and *Pau* or *Pam*- and *Pau*-like taxa.

In addition, we studied the expression of elicitin genes, a multigenic family specific to the genus *Phytophthora*. The cumulative patterns of *Pau* and *Pam* in regard with *Paa* confirmed our first results and demonstrated that for that allopolyploid taxa, distinct genomes may be co-expressed.

Last, in order to study the genetic variability of the different taxa, microsatellite markers were isolated in *Paa* and characterized. The genotypes we resolved demonstrate a low level of variability for the three taxa. They confirm our hypotheses in regard with *Paa* origin, indicate that *Paa* was generated by several hybridization events, and suggest that *Pam* is also an allopolyploid taxon.

Key words : interspecific hybridization, Phytophthora, emergent, allopolyploid, alder, molecular marker, mitochondrial DNA, microsatellite marker, single-copy gene, intron, detection.