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To cite this version:

Claude Husson, Jaime Aguayo, Cécile Revellin, Pascal Frey, Renaud Ioos, et al.. Evidence for homoploid speciation in Phytophthora alni supports taxonomic reclassification in this species complex. Fungal Genetics and Biology, 2015, 77, pp.12-21. 10.1016/j.fgb.2015.02.013 . hal-01269090

HAL Id: hal-01269090

https://hal.science/hal-01269090v1

Submitted on 5 Apr 2019

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Fungal Genetic and Biology
2015 **77:** 12–21
doi:10.1016/j.fgb.2015.02.013

Evidence of homoploid speciation in *Phytophthora alni* supports reclassification in this species complex

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Keywords: *Phytophthora alni*; ploidy; interspecific hybridization; flow cytometry; Real-time PCR; homoploid speciation

Abstract

Alder decline has been a problem along European watercourses since the early 1990's. Hybridization was identified as the main cause of this emerging disease. Indeed, the causal agent, a soil-borne pathogen named *Phytophthora alni* subsp. *alni (Paa)* is the result of interspecific hybridization between two taxa, *Phytophthora alni* subsp. *multiformis (Pam)* and *Phytophthora alni* subsp. *uniformis (Pau)*, initially identified as subspecies of *Paa*. The aim of this work was to characterize the ploidy level within the *P. alni* complex that is presently poorly understood. For that, we used two complementary approaches for a set of 31 isolates of *Paa*, *Pam* and *Pau*: i) comparison of the genome size estimated by flow cytometry and ii) quantification of allele copy number of three single-copy nuclear genes using allele-specific real-time PCR. Relative quantification of alleles of the three single-copy genes showed that the copy number of a given allele in *Paa* was systematically half that of its parents *Pau* or *Pam*. Moreover, DNA content estimated by flow cytometry in *Paa* was equal to half the sum of those in *Pam* and *Pau*. Our results therefore suggest that the hybrid *Paa* is an allotriploid species, containing half of the genome of each of its parents *Pam* and *Pau*, which in turn are considered to be allotetraploid and diploid, respectively. *Paa* is thus the results of an homoploid hybrid speciation. Based on published data and on results from this study, a new formal taxonomic name is proposed for the three taxa *Paa*, *Pam* and *Pau* which are raised to species status and renamed *P. ×alni*, *P. ×multiformis* and *P. uniformis* respectively.

1. Introduction

Polyploidy is defined as the state of having more than two sets of chromosomes, four being the most common. It generally results from the merging of two distinct species genomes (allopolyploidy, interspecific hybridization) or of the genomes of individuals of the same species (autopolyploidy) (Comai, 2005; Parisod et al., 2010; te Beest et al., 2011). Polyploidization and hybridization are therefore closely interrelated processes. The formation of polyploid organisms is pervasive in plants and, to a lesser extent, in animals: recent genomic studies have demonstrated that almost all angiosperms are polyploid or paleopolyploid, i.e. at least one round of polyploidization event occurred in their ancestry (Arrigo and Barker, 2012; Hegarty and Hiscock, 2008; Otto, 2007). Although it may cause cytological problems such as nuclear disruption, cell enlargement, epigenetic instability and the production of aneuploid cells, being polyploid may bring many advantages as, for example, vigor through heterosis or buffering effect on deleterious mutations through gene redundancy (Comai, 2005). Polyploidy is thus associated with speciation and evolution of Eukaryotes, and may enhance the ability of species to colonize new niches and ecosystems (Giraud et al., 2008; Arrigo and Barker, 2012; Hegarty and Hiscock, 2008; Otto and Whitton, 2000; Otto, 2007).

With the exception of the well-described *Saccharomyces* genus, occurrence of polyploidy has been poorly investigated in fungi and fungal-like organisms such as oomycetes (Albertin and Marullo, 2012). Yet, polyploidy is considered to have played an important role in the evolution of fungi, in particular in plant mutualists and plant pathogens (Schardl and Craven, 2003). Although interspecific hybridization events have been described in several fungi and oomycetes, particularly in plant pathogens (Albertin and Marullo, 2012; Stukenbrock and McDonald, 2008; Goss et al., 2011; Bertier et al., 2013; Nagel et al., 2013; Man in ’t Veld et al., 2012), less than 20 fungal species altogether were clearly identified as polyploids, most of them being allopolyploid (Albertin and Marullo, 2012; Nagel et al., 2013; Olson and Stenlid, 2002; Schardl and Craven, 2003).

Interspecific hybridization could lead to emerging fungal diseases, in particular when the hybrid has the capacity to colonize new hosts (Brasier et al., 2001; Schardl and Craven, 2003; Man in ’t Veld et al., 2007; Inderbitzin et al., 2011). A well-described case is the hybrid poplar rust *Melampsora ×columbiana* which has threatened poplar plantations in the USA, and combines the host ranges of its two parents, i.e. *Populus deltoides*, *P. trichocarpa* and *P. ×interamericana* (Newcombe et al., 2000; Newcombe et al., 2001). Even if most described hybrids of fungal and fungal-like plant pathogens seem to have little economic or ecological consequences, their importance remains underestimated (Olson and Stenlid, 2002; Bertier et al., 2013). Indeed, the likelihood of hybridization between allopatric species has been hypothesized to be high because they did not evolve barriers to inter-fertility (Brasier et al., 2000). Moreover, encounters between taxonomic species of allopatric origin could increase in frequency through rising global trade which efficiently disperses pathogens around the world (Brasier, 1995; Brasier, 2000; Desprez-Loustau, 2009). Finally, due to their genome plasticity facilitating genome merging, fungi and oomycetes appears to be good candidates for hybridization (Albertin and Marullo, 2012).

The alder decline observed along European watercourses since the early 1990’s is an example of an interspecific hybridization event in oomycetes through polyploidization that led to important damage in natural ecosystems (Brasier et al., 1995; Gibbs et al., 1999). Although the decline is strongly influenced by climate (Thoirain et al., 2007; Aguayo et al., 2014), interspecific hybridization has been found to be the main cause of this emerging disease. Indeed, based on abnormal cytological behavior and on polymorphic sites in the internal transcribed spacer (ITS) region, the causal pathogen was determined to be a polyploid hybrid taxon. Two different types of variants exhibiting phenotypically and genetically distinct traits were identified and hypothesized to result from genetic breakdown or back-cross products of the initial hybrid (Brasier et al., 1999). The three taxa were then

designated as subspecies, *Phytophthora alni* subsp. *alni* (*Paa*, the most aggressive subspecies) and the 2 variants *P. alni* subsp. *uniformis* (*Pau*) and *P. alni* subsp. *multiformis* (*Pam*) (Brasier et al., 2004). After counting the chromosomes by light microscopy, Brasier et al. (1999) concluded that the hybrid *Paa* was near tetraploid (n=18-22, inferred karyotype ca 4n+2), *Pam* between diploid and tetraploid (n=13-18, inferred karyotype from 2n+4 to 2n+7) and *Pau* near diploid (n=11-13, inferred karyotype ca 2n+2), the latter exhibiting rather high phenotypic and genetic uniformity compared to the two other subspecies.

However, subsequent analyses of sequence polymorphism at four single-copy nuclear genes (*ASF*-like, *GPA*1, *RAS*-*Ypt* and *TRP*1) and two mitochondrial genes (*cox*1 and *nadh*1) (Ioos et al., 2006), at several microsatellite loci (Ioos et al., 2007a) and at the elicitin genes (Ioos et al., 2007b) showed that *Pau* and *Pam* were not variants of *Paa* but its progenitors. The expression patterns for nuclear encoded isozymes such as glucose-phosphate isomerase (Gpi) and malate dehydrogenase (Mdh-1 and Mdh-2) brought additional support to this scheme (Brasier et al., 2004; Bakonyi et al., 2007). These results were also confirmed by comparison of mitochondrial DNA RFLP pattern between the taxa of the *P. alni* complex (Nagy et al., 2003; Ioos et al., 2006).

The status of *Pau* as a diploid oomycete without hybrid origin was confirmed by Ioos et al. (2006). Furthermore, Aguayo et al. (2013) showed that it is indigenous in North America where it exists independently of *Paa*. By contrast, the ploidy status of *Paa* and *Pam* appeared more complex. A unique allelic pattern was found for the four single-copy nuclear genes mentioned above. Each gene exhibited three different alleles named PAU, PAM1 and PAM2 (Ioos et al., 2006): while for each of the studied genes all *Pau* isolates exhibited a single allele PAU, all *Pam* isolates exhibited both alleles PAM1 and PAM2 and all isolates of the hybrid *Paa* exhibited the three alleles. The authors concluded that *Pam* might be a cryptic polyploid taxon (Ioos et al., 2006; Ioos et al., 2007b). The case of *Paa*, with three alleles at each locus, also did not fit with the near tetraploid status initially described by Brasier et al. (1999). As a result, the ploidy levels of the three taxa of the *P. alni* complex remain uncertain even though this knowledge is critical to characterize genetically populations and to evaluate their evolutionary potential. In parallel, the designation of *Paa*, *Pau* and *Pam* as subspecies does not appear to be correct anymore.

Flow cytometry is a well-proven technique to estimate the DNA content of individual nuclei in plant or animal species (Doležel et Bartoš, 2005; Walker et al., 2006; Jeyaprakash and Hoy, 2009), as well as in fungi and oomycete species, including *Phytophthora* spp. (Bertier et al., 2013; Catal et al., 2010; Vercauteren et al., 2011) and hence ploidy level by using internal standards with known DNA content or ploidy (D'Hondt et al., 2011; Bertier et al., 2013; Burson et al., 2012). More recently, a method based on real-time quantitative PCR has been developed for estimating ploidy levels. Based on the quantification of allele copy number of a single-copy gene DNA using calibrated solutions with known concentrations of the target sequence, the method proved to be efficient with various organisms (Jeyaprakash and Hoy, 2009; Mounsey et al., 2012; Wilhelm et al., 2003).

The aim of this study was therefore to determine the respective ploidy level of the hybrid *Paa* and its progenitors using a combination of two approaches, i.e. comparing their genome size by flow cytometry and the allele copy number for three single-copy nuclear genes using real-time PCR probes specific for the PAU, PAM1 and PAM2 alleles. Finally, based on published data and on results from this study, a new formal taxonomic name is proposed for the three taxa of the *P. alni* complex that were hitherto designated as subspecies.

2. Materials and methods

2.1. Isolate collection

Phytophthora isolates used in this study are detailed in Table 1. *P. alni* strains from France were isolated from inner bark lesions of alder trees using V8 juice agar selective medium as described in Aguayo et al. (2013). The remaining isolates were provided by colleagues from Europe or the USA. Taxonomic identification was performed based on morphological characters and using a set of taxon-specific primers, i.e. SCAR-based primer pairs PA-F/R, PAM-F/R (Ioos et al., 2005) and allele-specific primer pairs TRP-PAU-F/-R, RAS-PAM1-F/-R, RAS-PAM2-F/-R, RAS-PAU-F/R (Ioos et al., 2006). Isolates of *P. ramorum* were added in our studies as a standard since the genome size of this species is known (Tyler et al., 2006; Vercauteren et al., 2011). All isolates were maintained on V8 juice agar medium containing rifampicin (10 mg L^{-1}) at 20°C in darkness until use.

Table 1: Isolates used in this study.

Taxon	Name	Date of isolation	Host	Origin	Supplier (original code)	Analysis using : Flow cytometry	Analysis using : Real-time PCR
Paa	PAA85[a]	1996	*Alnus glutinosa*	France	INRA Nancy (P834)	*	*
Paa	PAA101[a]	2003	*A. glutinosa*	France	INRA Nancy	*	*
Paa	PAA129	2003	*A. glutinosa*	France	INRA Bordeaux (703)	*	*
Paa	PAA354[a]	2008	*A. glutinosa*	France	INRA Nancy	*	*
Paa	PAA563	2009	*A. glutinosa*	Belgium	INRA Nancy	*	
Paa	PAA174[a]	2004	*A. glutinosa*	Germany	INRA Nancy	*	*
Paa	PAA661	2005	*A. glutinosa*	Poland	L. Orlikowski (Paa8)	*	
Paa	PAA782[a]	2009	*A. glutinosa*	Spain	A. Pérez-Sierra (Ps-786)	*	*
Pam	PAM872[a]	2007	*A. glutinosa*	France	INRA Nancy (07dur6)		*
Pam	PAM391[a]	2009	*A. incana*	France	INRA Nancy		*
Pam	PAM393[a]	2009	*A. incana*	France	INRA Nancy	*	*
Pam	PAM396[a]	2009	*A. incana*	France	INRA Nancy	*	*
Pam	PAM444	2009	*A. glutinosa*	France	INRA Nancy		*
Pam	PAM71[a]	-	*A. glutinosa*	Netherlands	W. Man in 't Veld (W1139)	*	*
Pam	PAM90	1998	*A. glutinosa*	Netherlands	C. Brasier (P972)	*	
Pau	PAU60	1999	*A. glutinosa*	France	J.C. Streito (AULO28)	*	
Pau	PAU300[a]	2008	*A. glutinosa*	France	INRA Nancy	*	*
Pau	PAU333[a]	2008	*A. glutinosa*	France	INRA Nancy	*	*
Pau	PAU542[a]	2009	*A. glutinosa*	France	INRA Nancy	*	*
Pau	PAU624[a]	2009	*A. glutinosa*	France	INRA Nancy	*	*
Pau	PAU338	2008	*A. glutinosa*	France	INRA Nancy	*	
Pau	PAU187	2001	*A. glutinosa*	Belgique	A. Chandelier (2276)	*	
Pau	PAU89	2000	*A. cordata*	Italie	P. Capretti (CBS109280)	*	
Pau	PAU142[a]	-	*A. glutinosa*	Slovenia	A. Munda (Phy-A-SLO)	*	*
Pau	PAU768	2010	*A. incana*	USA	G. Adams (QC Bowl 8A)	*	
Pau	PAU781	2009	*A. glutinosa*	Spain	C. Pintos-Varela (685A)	*	
Pau	PAU774	2010	*A. glutinosa*	USA	G. Adams (BLb8atwigs)	*	*
Pau	PAU777	2010	*A. glutinosa*	USA	G. Adams (M54b2)	*	
Pa	PA340	2008	*A. glutinosa*	France	INRA Nancy	*	
Pa	PA454	2005	*A. glutinosa*	France	INRA Nancy		
Pa	PA616	2009	*A. glutinosa*	France	INRA Nancy	*	
P .ramorum	F001	2002	*Rhododendron* sp.	France	INRA Nancy	*	
P. ramorum	F002	2002	*Rhododendron* sp.	France	INRA Nancy	*	

Note. *Paa, Phytophthora alni* subsp. *alni*; *Pam*, *P. alni* subsp. *multiformis*; *Pau*, *P. alni* subsp. *uniformis, Pa, P. alni* isolates that could not be clearly attributed to a taxon *(*see Table 3*)*.

[a] isolates used for primer/probe specificity tests.

2.2. *Phytophthora* zoospore suspensions

Approximately thirty 5-mm agar plugs taken from the growing edge of a 3-day-old colony were placed into Petri dishes containing river water filtered through a 10-µm Omnipore™ membrane filter (JCWP04700, Millipore, Molsheim, France). Water collected from river is essential for inducing *P. alni* zoospores production. Plates were incubated at 20°C in darkness and the river water was changed daily. As soon as sporangia were produced, i.e. 2-3 days later, plugs were transferred into a new plate containing 4°C ultra-pure water and exposed 1-2 h to daylight at room temperature in order to stimulate zoospore release. The zoospore suspensions were then filtered through a 45 µm sieve to remove mycelium, transferred into a 50 mL Falcon® tube, vortexed for 1 min and finally stored for 1 hour at 4°C to induce zoospores encystement. Zoospores were then retrieved by filtering the suspension through a 10-µm Omnipore™ membrane filter, and filters were placed into a 14 mL Falcon® tube containing 3 mL Tris-Hcl EDTA buffer (10 mM Tris-HCl pH 8, 10 mM EDTA, 100 mM NaCl, Anderson et al., 2010). The tubes were vortexed for 1 min to release zoospores from filters and to increase zoospore concentration in the buffer. The concentration of zoospores was determined by using a haemocytometer under a microscope and the suspension was diluted to appropriate concentrations according to the technique used.

2.3. DNA extraction

For six isolates of each of *Paa, Pau* and *Pam* (Table 1), 2 mL microtubes containing 200 µL Tris-Hcl EDTA buffer and 10,000, 20,000, 40,000 or 80,000 zoospores were produced. DNA extractions from the zoospore suspensions or from pure fresh mycelia grown on V8 agar medium (for taxonomic identification) were performed using the DNeasy Plant mini-kit (Qiagen, Courtaboeuf, France). One 3-mm tungsten carbide bead and twenty 2-mm glass beads were added into the microtubes and the samples were ground for 30 s at a frequency of 30 Hz using a bead-beater (TissueLyser, Qiagen, France). AP1 buffer (400 µL) was added in each tube and the samples were ground again for 30 s. DNA was then extracted following the manufacturer's instructions except that incubation period with the AP1 lysis buffer was extended to 1 hour at 65°C. Finally, DNA was eluted in a final volume of 200 µL AE buffer. Each series of DNA extraction included samples from the three taxa. Since the aim was not to characterize individual strains, but the *P. alni* taxa, only one replicate per strain and zoospore concentration was performed.

2.4. Design of allele-specific primer pairs and probes for the three single-copy genes

Sequences of single-copy nuclear genes *ASF*-like, *GPA*1 and *RAS-Ypt* from *Pam* (PAM71 and PAM54) and *Pau* (PAU60) deposited in Genbank by Ioos et al. (2006) were analyzed using Sequencher 4.7 (Gene Codes Corporation, USA). Primer pairs and dual-labelled probes specific to alleles PAU, PAM1 and PAM2 were designed for each single-copy gene using Primer 3 software (Rozen and Skaletsky, 2000) and Beacon Designer software (Premier Biosoft, Palo Alto, CA, USA) (Table 2).

Specificity of primer pairs was assessed by conventional PCR on DNA samples of 15 *Paa*, *Pam* and *Pau* isolates (Table 1). PCRs were carried out in a 20 µL final reaction volume containing ultrapure water, 1x *Taq* DNA polymerase buffer (Sigma-Aldrich, Lyon, France), 2 mM $MgCl_2$, 0.45 µM of each primer, 200 µM dNTP, 0.7 unit of *Taq* DNA polymerase (Sigma-Aldrich) and 2 µL of template DNA (2-10 ng). They were performed by using a PTC-200 Peltier Thermal Cycler (MJ Research, Bio-Rad, Marnes-la-Coquette, France) and included an initial denaturation at 95°C for 3 min followed by 35 cycles at 95°C for 30 s, 62°C for 30 s, 72°C for 60 s and a final extension at 72°C for 10 min. PCR products were separated by a 1-h electrophoresis in 1% agarose gels in TBE buffer at 4 V cm^{-1} and visualized by U.V. exposition following ethidium bromide staining. The fragment sizes were estimated by

comparison with a 100-bp DNA ladder (Invitrogen). Images were recorded with a GelDoc 2000 system (Biorad).

Table 2: List and characteristic of forward (-F) and reverse (-R) primers and probes (-P) developed in this study, except * which were designed in Ioos et al. (2006).

Name	Sequence (5' - 3')	DNA template region gene	allele
AL-ASF-PAU-F	ACCGCCACAACATCCACT	*ASF*-like	ASF-PAU
AL-ASF-PAU-R	GCACGGGACCTACGAGGA	*ASF*-like	ASF-PAU
AL-ASF-PAM1-F	CACCACCACCACAATATCTCC	*ASF*-like	ASF-PAM1
AL-ASF-PAM1-R	ACAAGCACTTCCTCCAGCA	*ASF*-like	ASF-PAM1
AL-ASF-PAM2-F	ACCGCCATCACCACCATA	*ASF*-like	ASF-PAM2
AL-ASF-PAM2-R	CACGAGAACTTCCTCCAGCA	*ASF*-like	ASF-PAM2
AL-ASF-P	FAM- AGGACGAGAGCCGCGACCAG-BHQ1	*ASF*-like	ASF-PAU/-PAM1/-PAM2
AL-GPA-PAU-R	GTCCTCGCAACTCCCACTAT	*GPA*1	GPA-PAU
AL-GPA-PAM1-R	ACTGCGAGCAAGTCTACGG	*GPA*1	GPA-PAM1
AL-GPA-PAM2-R	CACCGCGAACAAGTCAAC	*GPA*1	GPA-PAM2
AL-GPA-F	AAGAACGAGGAGGCTCACC	*GPA*1	GPA-PAU/-PAM1/-PAM2
AL-GPA-P	FAM-CTGCTGCTGCTGGGAGCTGG-BHQ1	*GPA*1	GPA-PAU/-PAM1/-PAM2
AL-RAS-PAU-F	AACATGGCTACGGTTTGCTC	*RAS-Ypt*	RAS-PAU
AL-RAS-PAU-R	GTCCCACTACAGTTCCGAATAACC	*RAS-Ypt*	RAS-PAU
AL-RAS-PAM1-F	GCTGACATTATCGTGCTCGT	*RAS-Ypt*	RAS-PAM1
AL-RAS-PAM1-R	GAAAGCGGACACCAGTAAGC	*RAS-Ypt*	RAS-PAM1
AL-RAS-PAM2-F	ACTCCGATTGCTGACGTTGT	*RAS-Ypt*	RAS-PAM2
AL-RAS-PAM2-R	GCGTACGTCAGTAAGCTCCA	*RAS-Ypt*	RAS-PAM2
AL-RAS-P	FAM-ACGATCGAGCTGGACGGCA-BHQ1	*RAS-Ypt*	RAS-PAU/-PAM1/-PAM2
TRP-PAU-F*	GTGCGTCGCTAGCCCATCA	*TRP*1	TRP-PAU
TRP-PAU-R*	CGCCTACAGAGCATCATAG	*TRP*1	TRP-PAU
RAS-PAM1-F*	AGAGGGATATATTTGAGGTT	*RAS-Ypt*	RAS-PAM1
RAS-PAM1-R*	GTTGGACCCGGGACGGTCTTC	*RAS-Ypt*	RAS-PAM1
RAS-PAM2-F*	AGAGGGATATATTTGCGGCT	*RAS-Ypt*	RAS-PAM2
RAS-PAM2-R*	TCAGCAATCGGAGAGCAAGCT	*RAS-Ypt*	RAS-PAM2
RAS-PAU-F*	ATTTACTTGCAGCCGCAGGCT	*RAS-Ypt*	RAS-PAU
RAS-PAU-R*	ACCTAGGGCAGACAAGCTAGTC	*RAS-Ypt*	RAS-PAU

2.5. Quantification of allele copy number of three single-copy genes

Quantification of allele copy number in the three taxa was performed by real-time PCR assays in a 20 µl reaction volume consisting of ultra-pure water, 10 µL of Brilliant III Ultra-Fast QPCR Master Mix (Agilent, Les Ulis, France) 0.3 µM of each primer, 0.1 µM of dual-labelled probe, 0.15 U of Uracil-N-Glycosylase (UNG), 30 nM of ROX reference dye and 2 µl of template DNA. Real-time PCR was carried out in a Stratagene Mx3005P and using the Mx-Pro software version 4.1 (Agilent). The PCR thermal cycling program included an initial step for UNG activation for 2 min at 50°C, a *Taq* polymerase activation step for 3 min at 95°C followed by 45 cycles of denaturation at 95°C for 15 s, and annealing / elongation at 65°C for 50 s.

The experiment was designed in order to compare without bias the allele copy number between *Paa* and its parents, *Pam* and *Pau*, for each of the three studied genes, *ASF*-like, *GPA*1 and *RAS-Ypt*. Therefore, to avoid any extraction bias, an equal number of strains of *Paa* and of either *Pau* or *Pam* was included in each DNA extraction (5 different DNA

extractions). Twenty-four DNA samples were studied for each taxon, i.e. 6 isolates * 4 different concentrations (from 10,000 to 80,000 zoospores in 200 µL). PCR was repeated twice for each DNA sample. Real-time PCR assays were performed on 96-well plates containing either all the DNA samples of *Paa* and *Pau* or all those of *Paa* and *Pam* (2 taxa x 24 DNA samples x 2 replicates for each plate). The former were amplified with primers and probes specific for allele PAU while the latter were amplified with primers and probes specific for alleles PAM1 or PAM2 (Table 2). The amplification efficiency of the two studied taxa for each PCR assay was computed as the mean efficiency estimated by the Mx-Pro software with the current default baseline algorithm. Only the PCR assay that exhibited less than 5% difference in the amplification efficiency of the two compared taxa were retained for further analysis.

The Ct values were analyzed by the following Bayesian model with WinBUGS 1.4:

$$CTi = \mu + \alpha_{1i} \times Extraction_i + \alpha_{2i} \times Primer_i + \alpha_{3i} \times Parent_i + \alpha_{4i} \times Primer_i \times Parent_i + (Sl + \beta_{1i} \times Extraction_i + \beta_{2i} \times Primer_i + \beta_{2i} \times Parent_i) \times concentr_i + Isolate_i + \varepsilon_i$$

with µ and Sl, the mean intercept and slope, $Extraction_i$, the DNA extraction number of the sample (1-5), $Primer_i$, the primer pair used (9 combinations of the 3 genes and of the 3 alleles), $Parent_i$, whether the taxon was the hybrid *Paa* or *Pam/Pau* (0-1), $concentr_i$, the zoospore concentration and $Isolate_i$ a random isolate effect that followed a normal distribution.

Non informative priors were assigned to the model parameters with a normal distribution N(0, 1e04) and to the variances, residual and isolate random variable, with a gamma distribution G(0.001, 0.05). Three parallel chains with different initial parameter values were run for 7500 iteration following a burn-in of 3000 with a thinning of 10. The convergence was checked by Gelman-Rubin test (R_{hat} were close to 1).

The ratio of allele copy number in *Paa* versus the parents, *Pau* or *Pam*, was computed for each primer pair (gene x allele combination) as $(1 + E)^{\alpha_{3i}}$, with E the amplification efficiency and α_{3i}, the coefficient computed for the "Parent" effect. Indeed, the amplification during a PCR run, N_{Ct} /$N_{0,}$ can be expressed as $(1 + E)^{Ct}$, with N_o, the initial allele copy number, N_{Ct}, the copy number at the threshold and Ct, the threshold cycle. As primer pairs were amplified in the same run for *Paa* and the parents, N_{Ct} is the same. The ratio of allele copy number in the parent *Pam* or *Pau* versus *Paa* for a primer pair will thus be $(1 + E)^{Ct_{Paa}-Ct_{Parent}}$. A preliminary analysis showed that the interaction between the "Primer" effect or the "concentr" effect and the "Parent" effect were not significant. Thus, for each DNA extraction, primer pair, and zoospore concentration, the difference in Ct between *Paa* and either parent is equal to α_{3i}. The ratio of allele copy number in the parental taxa versus *Paa, i.e.* $(1 + E)^{\alpha_{3i}}$, was monitored during the MCMC run. These ratios will be named respectively *Pau*:*Paa* and *Pam*:*Paa* hereafter.

2.6. Estimation of nuclear DNA content by flow cytometry

Suspensions of about 2.10^5 zoospores per mL were obtained for each *Phytophthora* isolate (Table 1). They were filtered through a 10 µm Partec CellTrics® filter and stained by adding 4.6 µL Propidium Iodide (PI, Partec, Munster, Germany) and 2 µL RNase (100 mg mL^{-1}) per mL. After incubation at 4°C overnight, PI-stained nuclei of zoospores were analyzed with a Cyflow blue flow cytometer (Partec GmbH) operating a 20-mW solid-state laser (488 nm). Data analysis was performed using Flomax 2.7 software (Partec GmbH). Forward angle light scattering (FSC), right angle light scattering (SSC) and red fluorescence (emission 630 nm) were measured simultaneously. Each measurement was repeated twice per isolate.

Fluorescence intensity histograms were constructed to exhibit a peak of fluorescence linearly correlated with the DNA content in the G1 phase of the cell cycle of the sample analyzed (Doležel and Bartoš, 2005). An analysis of variance (ANOVA) was conducted to

test the effect of taxon on the peak position, with isolate effect taken into account as a random variable. The model was fitted in a Bayesian framework under WinBUGS 1.4. Non informative priors were assigned to model parameters with a normal distribution N(0, 1e04) and a gamma distribution G(0.001, 0.05) for variances (residual and isolate random variable). Three parallel chains with different initial parameter values were run for 2000 iteration following a burn-in of 2000 with a thinning of 10. The convergence was checked by Gelman-Rubin test (R_{hat} were close to 1).

Relative DNA content, i.e. ratio between the peak positions of each taxon of *P. alni* complex and *P. ramorum* and ratio of the DNA content in *Paa* to the sum of DNA content in *Pau* and *Pam* were computed and monitored during the MCMC run. The absolute nuclear DNA content and genome size of each taxon of the *P. alni* complex were then determined by comparison with a reference species with known genome size, i.e. *P. ramorum*.

Table 3: Taxonomic identification based on the allele-specific primers of Ioos et al. (2006) and SCAR-based primers of Ioos et al. (2005).

Isolates	RAS-PAM1-F/R	RAS-PAM2-F/R	RAS-PAU-F/R	TRP-PAU-F/R	PAM-F/R	PA-F/R	Taxon
PAA85, PAA101, PAA129, PAA174, PAA324, PAA563, PAA661, PAA782	**+**	**+**	**+**	**+**	**+**	**+**	*Paa*
PAU60, PAU89, PAU142, PAU187, PAU300, PAU333, PAU338, PAU542, PAU624	**-**	**-**	**+**	**+**	**-**	**+**	*Pau*
PAM71, PAM90, PAM391, PAM393, PAM396, PAM444, PAM872	**+**	**+**	**-**	**-**	**+**	**+**	*Pam*
PA340	+	+	+	+	-	+	.
PA454	-	-	-	+	-	+	.
PA616	+	+	+	-	+	+	.

Note. *Paa*, *Pam* and *Pau* are *P. alni* subsp. *alni*, *multiformis* and *uniformis* respectively; PA340, PA454 and PA616 are *P. alni* isolates that could not be clearly attributed to one of the three taxo.

3. Results

3.1. Taxonomic identification of the isolate collection

While most tested isolates unambiguously belonged to one of the three taxa described by Brasier et al. (2004) using the set of primer pairs designed by Ioos et al. (2005, 2006), three isolates, PA340, PA454 and PA616, did not present conventional pattern (Table 3). Although these isolates clearly fitted in the *P. alni* complex, based on morphological features and SCAR-based primers pairs, they could not be assigned to a defined taxon. For example, PA340 exhibited a pattern close to *Pau* but exhibited additional alleles PAM1 and PAM2 at locus *RAS-Ypt*. On the opposite PA616 was close to *Pam* but exhibited an additional allele PAU at locus *RAS-Ypt*.

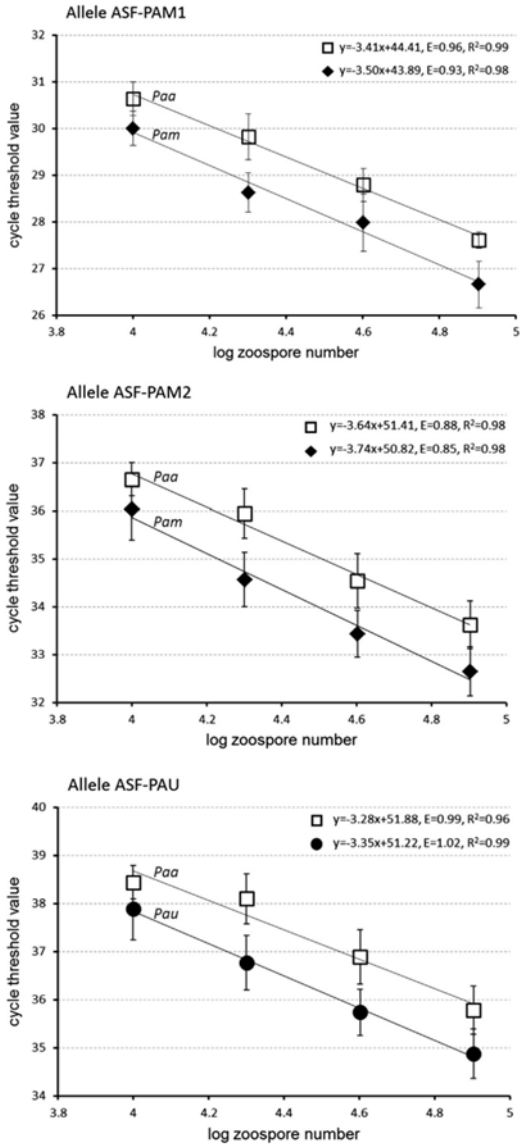

Fig. 1: Cycle threshold values yielded by real-time PCR amplification of the three alleles of the *ASF*-like gene, ASF-PAM1, ASF-PAM2 and ASF-PAU. The taxa are indicated above the standard curves. *Paa*, *Pam* and *Pau* are *P. alni* subsp. *alni*, *multiformis* and *uniformis* isolates, respectively. E, amplification efficiency. Errors bars, 95% confidence intervals.

3.2. Quantification of allele copy number of three single-copy genes

A unique probe for each of the three genes (*ASF*-like, *GPA*1 and *RAS-Ypt*) and a unique forward primer for the *GPA*1 gene were designed due to the close similarity of the sequences of the three single-copy genes (Table 2).

The specificity of primer pairs designed for real-time PCR was first confirmed by conventional PCR on a set of 15 genomic DNA samples belonging to the three *P. alni* taxa. The primer pairs AL-ASF-PAU-F/-R, AL-GPA-F/AL-GPA-PAU-R and AL-RAS-PAU-F/-R yielded an amplicon of the expected size for the *Paa* and *Pau* isolates, i.e. 154, 142 and 196 bp, respectively. No amplicon was obtained for *Pam* isolates. PCRs using primer pairs AL-ASF-PAM1-F/-R, AL-GPA-F/AL-GPA-PAM1-R, AL-RAS-PAM1-F/-R on one hand, and AL-ASF-PAM2-F/-R, AL-GPA-F/AL-GPA-PAM2-R, AL-RAS-PAM2-F/-R on the other hand

amplified a DNA fragment of the expected size for the *Paa* and *Pam* isolates (138, 127, 147, 141, 128, 152 bp, respectively) and no amplicon for the *Pau* isolates.

Subsequently, a series of nine allele-specific real-time PCR reactions were carried out and analyzed, each of them quantifying a unique allele from each of the studied gene (three alleles, PAU, PAM1 and PAM2 x three genes). For all studied alleles, DNA from the parental taxa *Pau* or *Pam* yielded mean Ct values that were on average 1 cycle lower than the DNA from the *Paa* hybrid (Fig. 1). The parent status (*Paa* versus *Pau* or *Pam*), the extraction and the primer pair all had a significant effect on the Ct value (p-values all under 0.001). Zoospore concentration, primer pair x zoospore concentration and extraction x zoospore concentration interactions and the isolate random factor also induced significant variability (p-values all under 0.001). By contrast, the parent status x primer pair and the parent status x zoospore concentration interactions had no significant effect on the Ct value (respective p-value of 0.364 and 0.151).

The *Pau*:*Paa* or *Pam:Paa* ratios ranged from 1.95 to 2.29 depending on the primer pair (Table 4). As none of the interactions involving the parent factor significantly influenced the Ct, a global parent species:*Paa* ratio could be estimated as 2.1 (95% CI [1.6 - 2.8]) meaning that the copy number of an allele PAU, PAM1 or PAM2 in *Pau* or *Pam* was twice as high as in *Paa*, regardless of the gene studied.

Table 4: Ratios of allele copy numbers in the three taxa *Paa*, *Pau* and *Pam* (95% confidence intervals). *Paa*, *Pam* and *Pau* are *P. alni* subsp. *alni*, *multiformis* and *uniformis* respectively.

Gene	*Pau:Paa* ratio for allele PAU	*Pam:Paa* ratio for allele PAM1	*Pam:Paa* ratio for allele PAM2
ASF-like	2.29 (1.66, 3.17)	2.21 (1.63, 3.03)	2.10 (1.58, 2.83)
*GPA*1	1.94 (1.50, 2.53)	2.10 (1.58, 2.83)	2.06 (1.55, 2.74)
RAS-Ypt	2.02 (1.54, 2.67)	2.01 (1.53, 2.66)	2.06 (1.56, 2.75)

3.3. Flow cytometry analysis

Flow cytometry analyzes were carried out on 8 *Paa*, 4 *Pam* and 13 *Pau* isolates. Since the zoospore suspensions were produced using river water filtered through a 10 µm filter, numerous PI-stained organisms, such as bacteria or protists, were detected in FSC versus SSC plot (Fig. 2, left panel). Based on their densities and sizes, zoospore nuclei were targeted in a selective gate. For each sample, 250 to 3000 PI-stained zoospore nuclei were analyzed. A peak of fluorescence was clearly observed in PI fluorescence histograms for all samples (Fig. 2, right panel). The taxon factor significantly influenced peak position (all p-value < 0.001). The peak positions observed for *Paa* were in an intermediate position between those observed for *Pau* and *Pam* (Fig. 3). The isolates PA340 and PA616 that could not be attributed clearly to one of the taxon had a peak position very similar to *Paa*. Estimated DNA content of *Paa* was half the sum of DNA content of its parents with the ratio (*Paa* peak position / (*Pam* + *Pau* peak positions)) equal to 0.51 (95% CI [0.49 – 0.53]). Given that DNA content of *P. ramorum* zoospore nucleus has been estimated by flow cytometry to be 0.146 pg (Vercauteren et al., 2011), the mean nuclear DNA content of *Paa*, *Pam* and *Pau* were respectively estimated at 0.384 pg (95% CI 0.327, 0.458), 0.453 (95% CI [0.385, 0.540]), and 0.303 pg (95% CI [0.257, 0.361]).

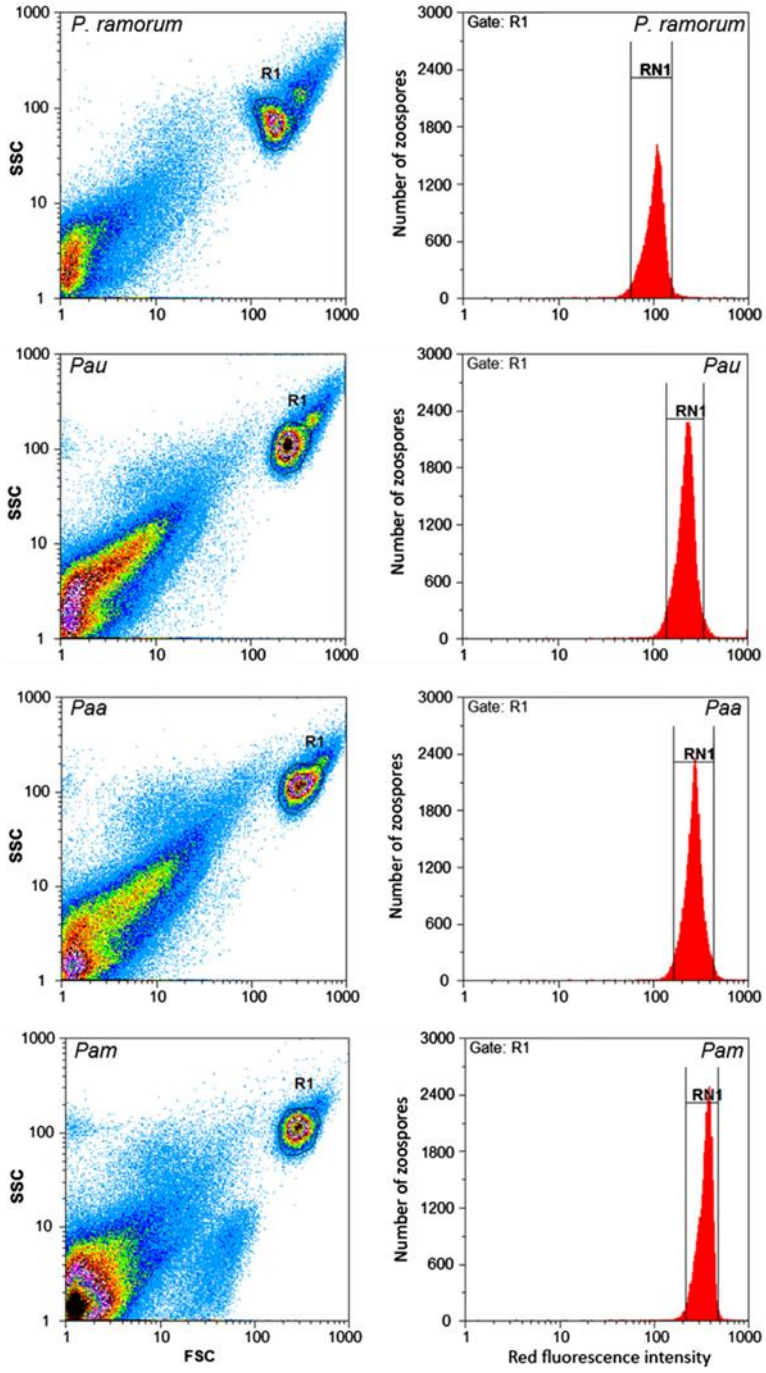

Fig. 2: Flow cytometry analysis of PI-stained zoospore nuclei of *Phytophthora* sp. isolates. *P. ramorum* isolate F001; *Pau*, *P. alni* subsp. *uniformis* isolate PAU777 ; *Paa*, *P. alni* subsp. *alni* isolate PAA174 ; *Pam*, *P. alni subsp. m*ultiformis PAM71. On the left: forward scattering (FSC) versus side scattering (SSC). On the right: histogram of red fluorescence intensity (logarithmic scale). R1, gate used for measuring mean red fluorescence intensity (RN1) emitted by stained nuclei of zoospores in the G1 phase of the cell cycle.

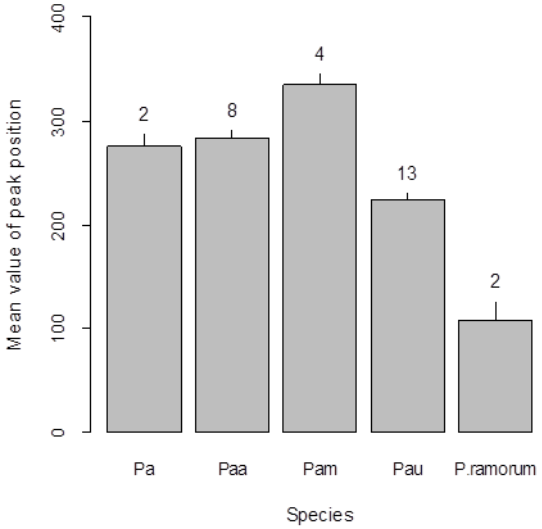

Fig. 3: Red fluorescence emission peak position measured by flow cytometry. *Paa*, *Pam* and *Pau* are *P. alni* subsp. *alni*, *multiformis* and *uniformis* isolates, respectively. *Pa* isolates could not be assigned clearly to one of the taxon. Errors bars: 95% confidence intervals. The number of isolates studied is indicated above the bars.

4. Discussion

4.1. Ploidy determination in the *P. alni* complex

This study brings new insights on the hybridization process that led to *Paa* through quantitative features. Relative quantification of allele copy number of the three single-copy genes showed that the copy number of a given allele in *Paa* was systematically half that of its progenitors *Pau* or *Pam*. This ratio is particularly reliable since it was confirmed for three alleles (PAU, PAM1, PAM2) of three different single-copy genes (*ASF*-like, *GPA*1, *RAS-Ypt*), regardless of zoospore concentration and primer pairs / probes used. Our results suggest therefore that the hybrid *Paa* contains half of the genome of each parent *Pam* and *Pau*. This result is confirmed by the DNA content estimate assessed by flow cytometry on a panel of *Paa, Pam* and *Pau* isolates. The total DNA content of *Paa* was equal to half the sum of those of *Pam* and *Pau*, suggesting that the *Paa* genome is the addition of half the *Pam* and half the *Pau* genomes.

Ioos et al. (2006) suggested that *Pam* could be either a tetraploid taxon or a diploid taxon resulting from homoploid speciation i.e. a hybridization that occurred without change in chromosome number. We showed that *Pam* has transmitted to *Paa* half of its genome while transmitting at the same time both the PAM1 and PAM2 alleles at each of the three single copy genes studied. Moreover, the copy number of each allele is twice in *Pam* than in *Paa*. This does not fit with *Pam* being a diploid, and leads us to conclude that *Pam* is probably a tetraploid. In addition, the sequences of the PAM1 and PAM2 alleles for the four genes studied by Ioos et al. (2006) were highly divergent with 2.2% to 8.6% base pair polymorphism, which suggests that *Pam* is the result of an interspecific hybridization between two unknown *Phytophthora* species, which we will refer to as *Pm1* and *Pm2* for convenience. *Pam* could have resulted either from a fusion of unreduced (2n) gametes of both *Pam1* and *Pam2*, or from a genome duplication following a fusion of normally reduced gametes (Fig. 4). Finding the putative progenitors of *Pam* remains a challenge. Survey of *Phytophthora* communities in rivers or soil of poorly investigated regions might help to

identify *Pm1* and *Pm2* and would shed light on the evolutionary history of the *P. alni* complex.

The diploid level of *Pau* that was inferred in previous studies (Ioos et al., 2006; Aguayo et al., 2013) is in agreement with our results since a low variation of nuclear DNA content within *Pau* was found. We therefore assumed that the origin of *Paa* has resulted from the hybridization between the diploid *Pau* and the allotetraploid *Pam*. Given that *Paa* possesses half of the genome of both parents, it can be regarded as an allotriploid hybrid or an aneuploid, near-triploid hybrid as Brasier et al. (1999) reported the occurrence of missing chromosomes. As a result, it is not an allopolyploid species resulting from the merging of the parental genomes (Restrepo et al. 2014); Paa can be instead considered to be a homoploid hybrid since it was formed through hybridization without chromosome doubling (Abbott et al., 2010; Buerkle, 2000; Rieseberg, 1997).

4.2. Estimation of the total DNA content of the three taxa

According to the terminology defined by Greilhuber et al. (2005) and the flow cytometry analysis performed in this study, the 2C genomic DNA content was 0.384 pg for *Paa*, 0.453 pg for *Pam* and 0.303 pg for *Pau*. This means that the monoploid genome size (1Cx-value) of *Paa*, *Pam* and *Pau* would be of 119 Mbp, 112 Mbp and 144 Mbp, respectively (Doležel et al., 2003). It is higher than the genome size of *P. ramorum* (65 Mbp, Tyler et al., 2006, or 71Mbp, Vercauteren et al., 2011) or *P. sojae* (95 Mbp, Tyler at al., 2006) but lower than that of *P. infestans* (240 Mbp, Haas et al., 2009).

Although *Paa* isolates were collected throughout Europe, no intraspecific DNA content variation was found, confirming that the hybridization event is quite recent.

4.3. Triploidy and homoploid speciation in fungi or fungal-like species

Not surprisingly, most of triploids reported in the literature belong to the plant kingdom (Ramsey and Schemske, 1998). They occur either within species (autotriploidy) or between two distinct species (allotriploidy) and result from the fusion of a normally reduced gamete (n) from a diploid species with a 2n gamete from a diploid (unreduced gamete) or from a tetraploid species (Hegarty and Hiscock, 2008), as found in this work for *Paa*. The formation of the allotriploid *Paa* is quite remarkable since very few triploids have been described in fungi or fungal-like species, most of them being autotriploids, as in the genus *Candida*, *Saccharomyces* and *Allomyces* (Albertin and Marullo, 2012).

Although much less frequent than allopolyploid speciation in plants, homoploid hybrid speciation has also been described in plants, in particular *Helianthus* sp. and *Senecio* sp., and in animals (Abbott et al., 2010; Hegarty et al., 2009; Mallet, 2007; Liu et al., 2014). In fungi, homoploid speciation has rarely been demonstrated (Restrepo et al. 2014): itwas described for yeast (Grey et al., 2002), *Microbotryum* sp. (Devier et al., 2010) and *Melampsora* sp. (Newcombe et al., 2000; Frey et al., 2005). A major problem for homoploids as well as for triploids is sterility or, at least, a severe reduction in fertility that occurs when parental chromosomes are too dissimilar to correctly pair up at meiosis (Buerkle et al., 2000; Hegarty and Hiscock, 2008). This explains the low oospore (*Phytophthora* sexual spore) viability observed in *Paa*, the high level of abortion and the absence of germination in the over 4000 oospores analyzed (Delcán and Brasier, 2001). Because it results in new genotypic combinations and often occurs together with chromosome loss and chromosomal rearrangements, homoploid speciation also often leads to ecological divergence from the progenitors, with adaptation of the hybrid to new habitats (Abbott et al., 2010; Bertier et al., 2013). Indeed, all well-described homoploid hybrids occur in habitats that are distinct from their parental species such as the three diploid *Helianthus* hybrid species *H. anomalus*, *H. deserticola* and *H. paradoxus* which colonize more extreme habitats than their parents, or the fish *Gila seminude*, native to the United States, which is found in close but distinct rivers than its parental species (Gross and Rieseberg, 2005). For *P. alni,* the hybridization event produced an aggressive pathogen, *Paa*, which invaded European riparian alder forests while

both progenitors remain infrequent and have not been associated to severe alder decline (Aguayo et al., 2013; Brasier et al., 2004; Jung and Blaschke 2004). Moreover, the ecological niche of the hybrid and its parents appear to be different as *Pau* is present along small watercourses at higher elevation (Štěpanková et al., 2013) and at higher latitude (Redondo et al., 2015) compared to *Paa*. We found comparable results in the Marais Poitevin in western France for *Pam*; among 111 stands investigated throughout the catchment, *Pam* was detected exclusively upstream (n = 7), while *Paa* was found only in the lower part of the river catchment (n = 63, Husson, unpublished results).

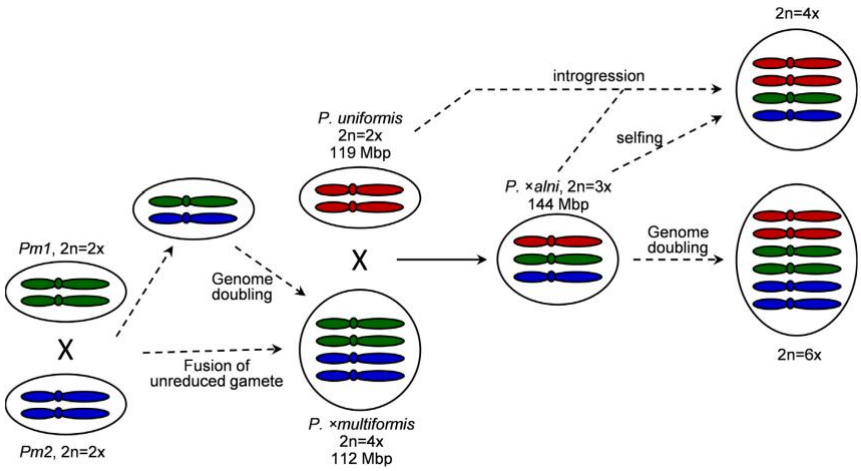

Fig. 4: Hypothetical scenarios for the interspecific hybridization events leading to the triploid species *Phytophthora ×alni*, for the origin of the progenitor *P. ×multiformis* and about possible future evolutions of *P. ×alni*. Dashed lines indicate hypothetical events involving undescribed species, including *Pm1* and *Pm2* as hypothetical parents of *P. ×multiformis*. In red, allele PAU; in green, allele PAM1; in blue, allele PAM2. Ploidy level and monoploid genome size (1Cx-value) are indicated for each species. Adapted from Hegarty and Hiscock (2008).

The potential for evolution of a triploid homoploid hybrid depends on its degree of fertility (Ramsey and Schemske, 1998). It may contribute to the formation of a tetraploid either by selfing or by backcrossing to the diploid parent (Comai, 2005). Such a mechanism could occur for *P. alni* if the triploid *Paa* would backcross with the diploid parent *Pau*, yielding an allotetraploid progeny (Fig. 4). It may be worthy to note that few isolates could not be clearly attributed to one of the taxa, sharing properties of the three taxa. These isolates represented about 0.5% of a collection of 519 tested isolates (31 *Pam*, 34 *Pau* and 451 *Paa*, Aguayo, 2012). We did not clearly determine their nature. A hypothesis would be that they represent evolution after *Paa* formation, either by subsequent loss of chromosomes or backcross with one of the parents. The fact that the unassigned isolates PA340 and PA616 exhibit a total DNA content close to that of *Paa*, and that they differ from *Paa* only by a few alleles missing, is consistent with the hypothesis of a chromosome loss. A large variation in DNA content seems to be common in *Phytophthora* hybrids (Bertier et al., 2013). Another possible evolution after the formation of a triploid homoploid hybrid would be genome duplication leading to an allohexaploid (Fig. 4). This evolution could restore the fertility of the hybrid as each chromosome would then have an exact homolog (Giraud et al., 2008; Hegarty and Hiscock, 2008). Such restoration of fertility in *Paa* would, in turn, further increase its evolutionary potential. Moreover, absence of viable sexual spores has been shown to be associated with poor overwintering of *P. alni* after cold winters, with important epidemiological consequences (Aguayo et al, 2014). Thus, detecting these possible

evolutions of *Paa* is critical, and the methods used in this study, i.e. quantitative PCR and flow cytometry, would be a useful tools for that purpose.

4.4. Taxonomy

Formerly called alder *Phytophthora* (Brasier et al., 1995), the causal pathogen of alder decline was later taxonomically described as *P. alni* (Brasier et al., 2004). Due to the phenotypic and genotypic distinctive traits within the *P. alni* complex, the taxa were split into three subspecies called *P. alni* subsp. *alni* and its variants *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis*, which were both considered to have been generated by genetic breakdown or by backcrosses involving *Paa* (Brasier et al., 1999, 2004).

However, both *Pam* and *Pau* have been found as standalone species, *Pau* as a native species in North America and *Pam* in different rivers of France (Aguayo 2012; Aguayo et al, 2013). Moreover, numerous molecular analyses, based on sequence polymorphism of four single-copy nuclear, two mitochondrial and six elicitin genes (Ioos et al., 2006, 2007b), on nine microsatellite loci (Ioos et al., 2007a) and on expression pattern of isozymes and mitochondrial DNA (Bakonyi et al., 2007; Nagy et al., 2003), clearly showed that *Pau* and *Pam* are the parental species of the hybrid *Paa*.

Based on these results and on the present study, which clarifies the ploidy level of *Paa* and *Pam*, we propose to raise *Paa*, *Pau* and *Pam* to species status and to rename them as follows:

Phytophthora ×*alni* (Brasier & S. A. Kirk) Husson, Ioos & Marçais, nothosp. nov.

Basionym *Phytophthora alni* subsp. *alni* Brasier & S. A. Kirk, Mycological Research 108(10): 1172-1186. 2004

Phytophthora uniformis (Brasier & S. A. Kirk) Husson, Ioos & Aguayo, comb. nov.

Basionym *Phytophthora alni* subsp. *uniformis* Brasier & S. A. Kirk, Mycological Research 108(10): 1172-1186. 2004

Phytophthora ×*multiformis* (Brasier & S. A. Kirk) Husson, Ioos & Frey, nothosp. nov.

Basionym *Phytophthora alni* subsp. *multiformis* Brasier & S. A. Kirk, Mycological Research 108(10): 1172-1186. 2004

These species can be identified following Ioos et al (2006) using the allele-specific primer pairs TRP-PAU-F/-R, RAS-PAM1-F/-R and RAS-PAM2-F/-R, *P. uniformis* being detected with TRP-PAU-F/-R, *P.* ×*multiformis* with RAS-PAM1-F/-R and RAS-PAM2-F/-R and *P.* ×*alni* with all three primers pairs. Finally, it has to be pointed out that, although very infrequent, some isolates cannot be clearly attributed to one of the three species using these tools.

Acknowledgements

This work was supported by grants from INRA, the Région Lorraine (France), the Agence Nationale de la Recherche (ANR 07-BDIV-003, Emerfundis program), the FEDER (Interreg IV-A Grande Région Ecolirimed program). We thank the colleagues listed in Table 1 for sharing *Phytophthora* sp. isolates. We are grateful to Prof. Dr. Johann Greilhuber (University of Vienna), Cyril Dutech (INRA Bordeaux-Aquitaine) and to Katherine Hayden for helpful comments and Thomas Munier for technical assistance. The UMR1136 is supported by a grant overseen by the French National Research Agency (ANR) as part of the “Investissements d’Avenir” program (ANR-11-LABX-0002-01, Laboratory of Excellence ARBRE).

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