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Strong Genetic Differentiation Between North American and European Populations of *Phytophthora alni* subsp. *uniformis*

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ABSTRACT

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Alder decline caused by *Phytophthora alni* has been one of the most important diseases of natural ecosystems in Europe during the last 20 years. The emergence of *P. alni* subsp. *alni*—the pathogen responsible for the epidemic—is linked to an interspecific hybridization event between two parental species: *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis*. One of the parental species, *P. alni* subsp. *uniformis*, has been isolated in several European countries and, recently, in North America. The objective of this work was to assess the level of genetic diversity, the population genetic structure, and the putative reproduction mode and mating system of *P. alni* subsp. *uniformis*. Five new polymorphic microsatellite markers were used to contrast both geographical populations. The study comprised 71 isolates of *P. alni* subsp. *uniformis* collected from eight European countries and 10 locations in North America. Our results revealed strong differences between continental populations (Fst = 0.88; Rst = 0.74), with no evidence for gene flow. European isolates showed extremely low genetic diversity compared with the North American collection. Selfing appears to be the predominant mating system in both continental collections. The results suggest that the European *P. alni* subsp. *uniformis* population is most likely alien and derives from the introduction of a few individuals, whereas the North American population probably is an indigenous population.

Additional keywords: *Alnus*, oomycetes.

Diseases that increase in severity or expand their range pose important threats to natural ecosystems (4,17,45). Although the emergence of disease can be the result of changes in host or environmental conditions, or evolution in pathogen populations (4), the introduction of exotic pathogens has been shown to be a major cause (4,16,17,46). However, assessing whether the causal organism of an emerging disease is alien is not always straightforward (43,56). This results mostly from a lack of knowledge and data on the biodiversity and ecology of endemic species (17,43). For example, in the genus *Phytophthora,* low genetic variability has often been taken as an indication that specific populations are exotic (see *Phytophthora quercina* [13], *P. nemorosa*, *P. pseudosyringae* [56], and *P. pinifolia* [22]). Indeed, introduction into a new geographical area often results in a population with low variability (36,72) because founder effects caused by the migration of a limited number of individuals result in reduced gene diversity and number of alleles in introduced populations (55). However, low variability cannot be taken as definite proof of the exotic nature of a population as long as the diversity center has not been identified (33,43). Low genetic variability may occur in a native population, especially when selfing is suspected. It is noteworthy that many *Phytophthora* spp. are homothallic and reproduce mainly by selfing (33,43). Self-fertilization will impact population genetic characteristics by increasing linkage disequilibrium (LD) and reducing genetic diversity (33,43). Conversely, some alien populations display high levels of genetic diversity (30,54) as a result of multiple introductions (15,18). Indeed, to study the genetic changes that may have occurred during and after an introduction event, it is important to identify the source of the introduction with as much precision as possible. Moreover, knowledge of the mode of reproduction (sexual versus asexual) and the mating system (selfing versus outcrossing) is of fundamental importance to the evolutionary biology of pathogens (6,34). The reproduction mode and the mating system will affect how diversity is distributed within and among individuals in a population (60), and supply insights into the potential of pathogens to spread and on their ability to evolve (33). Indeed, in *Phytophthora* spp., asexual (zoospores) and sexual (oospores) propagules exhibit contrasted dispersal and survival abilities, with strong epidemiological consequences. The source region will provide the benchmark against which genetic and evolutionary changes can be assessed, representing the variation from which the introduction was actually derived (18). For example, for *P. cinnamomi*, Papua New Guinea has been proposed as the center of origin (19,36). Indeed, this population presented a higher allelic diversity compared with other populations (19,36). However, determining the center of origin of a species is not always obvious. In the case of *P. infestans*, two possible centers of origin have been proposed: either Central Mexico (37) or the Andean region of South America (35).

Alder decline caused by the *P. alni* species complex is a good example of poorly understood pathogen emergence in a natural ecosystem (24,76). This disease has posed a major threat to natural ecosystems in Europe during the last 20 years (74). For example, in eastern France, disease prevalence has reached 17%

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of riparian alder (*Alnus glutinosa*) trees (76). The emergence of this disease is linked to an interspecific hybridization event (9,47). Recent studies have clarified the genesis of the interspecific hybrid *P. alni* subsp. *alni*. *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*, initially identified as genetic variants of *P. alni* subsp. *alni* (9), were shown to be the parental species of the hybrid (47). The hybrid *P. alni* subsp. *alni* is more aggressive than its progenitors and is responsible for disease outbreak (73). However, it is not known when and where hybridization took place. Moreover, the origin of the parental species, *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*, remains unknown. *P. alni* subsp. *alni* is widespread in Europe and has not been identified elsewhere. Although *P. alni* subsp. *multiformis* has been isolated exclusively in Europe, *P. alni* subsp. *uniformis* has been isolated both in Europe and, recently, in North America.

The present work constitutes the first population genetics study of one of the parental species of the hybrid *P. alni* subsp. *alni*. The objectives of this work were to assess the level of genetic diversity, the population genetic structure, and the putative reproduction mode of North American and European populations of *P. alni* subsp. *uniformis*. To this end, we characterized the population structure of two continental collections using new polymorphic microsatellite markers developed for this study.

MATERIALS AND METHODS

Isolate collection and DNA extraction. *P. alni* subsp. *uniformis* isolates used in this study are listed in Table 1. European *P. alni* subsp. *uniformis* isolates were obtained after several field campaigns during the years 1999, 2005, 2008, and 2009. Surveys were carried out in 111 sites across northern France, the Wallonia region of Belgium, and in Hungary. Additional *P. alni* subsp. *uniformis* isolates were obtained from colleagues elsewhere in Europe (Table 1; Fig. 1). North American isolates were collected between the years 2008 and 2012 after a survey of 81 sites across south-central and interior Alaska (1), and Oregon (Fig. 1). Isolates were obtained from collar bark and necrotic root tissues of infected alders and from in situ baiting. Samples were collected from different diseased trees to minimize the risk of sampling the same individual twice. Alder bark and root pieces were placed on V8 juice agar selective medium for *Phytophthora* spp. (27), and growing mycelium was transferred to fresh V8 juice agar medium containing rifampicin (10 mg/liter) as soon as detected. For in situ baiting, rhododendron (*Azalea* spp.) and bearberry (*Arctostaphylos uva-ursi*) leaves and thinleaf alder twigs (*Alnus incana* subsp. *tenuifolia*) were used. As lesions appeared, isolates were transferred to V8 juice agar selective medium. Colonies were subcultured and maintained as above.

DNA was extracted from 4-day-old pure fresh mycelia grown on V8 juice agar medium using a BioSprint 96 DNA plant kit (Qiagen, Courtaboeuf, France) in combination with a BioSprint 96 automated workstation (Qiagen) following the BS-96DNA-plant protocol, or using a Qiagen DNeasy Plant Mini Kit according to the supplier's instructions. Species identification was based on the DNA sequence homology of the internal transcribed spacer and using species-specific primers designed against the nuclear genes *RAS*-Ypt and *TRP*1, following the protocol of Ioos et al. (47).

For microsatellite development, DNA was extracted from 5-day-old colonies of isolates PAU60, PAU320, and PA340 grown in liquid V8 medium containing rifampicin (10 mg/liter) at 20°C using the Qiagen DNeasy Plant Mini Kit as described above.

Microsatellite development. Microsatellite markers were developed using an enriched library generated with a Roche GS-FLX Titanium pyrosequencing platform. The three lots of DNA extracted from PAU60, PAU320, and PA340 were mixed, and DNA quantity was estimated with a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The enrichment step, data analysis, and automated primer design were described previously by Malausa et al. (58). The enriched microsatellite library generated a total of 34,483 microsatellite sequences, with 550 putative microsatellite loci identified. Choice of microsatellite loci for further tests was constrained to motifs of ≥2 bp in length and a minimum repeat number of 5. Using these criteria, 110 loci were further retained. These 110 primer pairs were tested for amplification on a panel of 10 *P. alni* subsp. *uniformis*, 10 *P. alni* subsp. *multiformis*, and 5 *P. alni* subsp. *alni* European isolates. No North American *P. alni* subsp. *uniformis* isolates were available when loci were tested. Microsatellite loci were amplified in a GeneAmp polymerase chain reaction (PCR) System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA) under the following conditions: an initial denaturing step of 5 min at 95°C; 35 cycles, including 30 s of initial denaturing at 94°C, 90 s of annealing at 60°C, and 60 s of extension at 72°C; and a 30-min final extension step at 60°C. A PCR mix was prepared using the Type-it Microsatellite PCR kit in a 12-µl final reaction volume containing 2 µl of DNA, 5 µl of 2× Type-it mix, 1 µl of 5× Q-solution, 0.2 mM reverse primer, 0.02 mM M13-tailed forward primer, and 0.2 mM fluorescence-labeled M13 primer (5′-CACGACGTTGTAAAACGAC-3′). Amplified PCR products were loaded on an ABI 3730 Genetic Analyzer (Applied Biosystems). Fragments were sized with a LIZ-1200 size standard. Alleles were scored using GeneMapper 4.0 (Applied Biosystems). Loci that showed allelic polymorphism, expected peak size, and correct peak amplification were kept for subsequent tests. Forward primers for the selected perfect microsatellite loci were labeled with a fluorescent tag (FAM, NED, VIC, or PET). PCR conditions for genotyping the 71 *P. alni* subsp. *uniformis* isolates were as described above. PCR was conducted using the Type-it Microsatellite PCR kit in a 12-µl final reaction volume containing 2 µl of DNA, 5 µl of 2× Type-it mix, 1 µl of 5× Q-solution, and 0.2 µM each reverse and forward primers. Six multiplex PCR were run, comprising one to three loci each. PCR products from these multiplex reactions were pooled into two mixes according to their fluorescent tag color and size, and analyzed as described above. Genotyping was replicated for a subset of isolates with independent PCR reactions and sizing of fragments. Reproducibility of molecular data was confirmed.

Data analysis. *Global analysis.* To examine the relationships among isolates, a matrix of genotype distances was constructed with the Polysat R-package (12) using the genetic distance of Bruvo (10). This distance is similar to band-sharing indices used with dominant data but takes into account mutational distances between alleles. The result is a distance ranging from 0 to 1, with 0 indicating identical genotypes and 1 being a theoretical maximum distance if all alleles from one genotype differed by an infinite number of repeats from all alleles in another genotype. A nonmetric multidimensional scaling (MDS) for two axes was then performed (59). In MDS, a small number of axes are chosen explicitly prior to the analysis, and the data are fitted to those dimensions, so that there are no hidden axes of variation. Alternatively, we computed the shared allele distance (D_{as}) (51) using the Populations 1.2.31 program (O. Langella; http://bioinformatics.org/~tryphon/populations/). These kinds of clustering have the important advantage over other clustering methods, such as Bayesian clustering algorithms, that no underlying assumptions such as Hardy-Weinberg (HW) equilibrium or absence of LD between loci are required (52,61). The concordance between the Bruvo and the D_{as} distances was tested by Mantel tests (H_0 = matrices are not correlated) with 10,000 permutations. Estimation of global and across-loci *F*st (based on allele identity under infinite allele model) and *R*st (based on microsatellite allele size under stepwise-mutation model) between the European and North American collections were computed according to Weir and Cockerham (78) and Rousset (70) for *F*st and *R*st estimates, respectively, using SPAGEDI (44). The GENECLASS 2 program

TABLE 1. List of the *Phytophthora alni* subsp. *uniformis* isolates used in this study and multilocus genotype (MLG) assignation for each isolate

Isolate	Supplier	Reference[a]	Country	Location	Isolation year	MLG[b]
PAU768	G. Adams	QC Bowl 8A	United States (Alaska)	Quartz Creek	2010	A5
PAU769	G. Adams	QC Bowl 10	United States (Alaska)	Quartz Creek	2010	A6
PAU770	G. Adams	QC Bowl 12	United States (Alaska)	Quartz Creek	2010	A1
PAU771	G. Adams	QCb10	United States (Alaska)	Quartz Creek	2010	A5
PAU846	G. Adams	PAU420 / Gen-7 Bowl-2 1-2	United States (Alaska)	Quartz Creek	2009	A2
PAU772	G. Adams	CLBowl9B	United States (Alaska)	Cooper Landing	2010	A3
PAU773	G. Adams	CLb8	United States (Alaska)	Cooper Landing	2010	A3
PAU845	G. Adams	PAU330 / Gen-9 Bowl-4 1-1	United States (Alaska)	Cooper Landing	2009	A3
PAU774	G. Adams	BLb8atwigs	United States (Alaska)	Birch Lake	2010	A4
PAU775	G. Adams	BLb8twigs	United States (Alaska)	Birch Lake	2010	A2
PAU776	G. Adams	BLb9	United States (Alaska)	Birch Lake	2010	A1
PAU825	G. Adams	BLR15-3	United States (Alaska)	Birch Lake	2011	A1
PAU826	G. Adams	DC30T1-2	United States (Alaska)	Birch Lake	2011	A1
PAU836	G. Adams	BLR30-3	United States (Alaska)	Birch Lake	2011	A1
PAU837	G. Adams	DC30T3	United States (Alaska)	Dave's Creek	2011	A1
PAU843	G. Adams	PAU290 / Gen 3-B2- 2-1	United States (Alaska)	Dave's Creek	2009	A2
PAU844	G. Adams	PAU291 / Gen 3-B2- 3-4	United States (Alaska)	Dave's Creek	2009	A7
PAU851	G. Adams	PAU491 / Gen 4-B2- 1-1	United States (Alaska)	Dave's Creek	2009	A1
PAU777	G. Adams	M54b2	United States (Alaska)	Mile 54	2010	A3
PAU778	G. Adams	SRb3twigs	United States (Alaska)	Slana River	2010	A1
PAU779	G. Adams	LTR b9	United States (Alaska)	Little Tok River	2010	A4
PAU847	G. Adams	PAU53 / PAM-M-2sc	United States (Alaska)	Panguingue River	2008	A1
PAU848	G. Adams	PAU95 / stem 60-60- 7/9-3-1	United States (Alaska)	Fairbanks	2009	A1
PAU815	E. Hansen	118-R-IK.1	United States (Oregon)	Yachats	2011	A1
PAU816	E. Hansen	118-R-IJ.3	United States (Oregon)	Yachats	2011	A1
PAU817	E. Hansen	118-R-IJ.4	United States (Oregon)	Yachats	2011	A1
PAU849	E. Hansen	110-R-IN-1	United States (Oregon)	Reedsport	2012	A1
PAU187	D. de Merlier	2276	Belgium	Mons	2001	E1
PAU188	D. de Merlier	2277	Belgium	Habay-la-Neuve	2001	E1
PAU558	J. Aguayo	…	Belgium	Harmignies	2009	E1
PAU561	J. Aguayo	…	Belgium	Harmignies	2009	E1
PAU87	D. de Merlier	2271	Belgium	Ligneuville	2001	E1
PAU302	C. Husson	…	France	Baerendorf	2008	E1
PAU320	O. Caël	…	France	Baerendorf	2008	E1
PAU368	O. Caël	…	France	Baerendorf	2009	E1
PAU540	J. Aguayo	…	France	Bischholtz	2009	E1
PAU541	J. Aguayo	…	France	Bischholtz	2009	E1
PAU542	J. Aguayo	…	France	Bischholtz	2009	E1
PAU338	O. Caël	…	France	Bischoffsheim	2008	E1
PAU60	J.C. Streito	AUL028	France	Girmont	1999	E2
PAU496	R. Ioos	…	France	Métairies-Saint-Quirin	2005	E1
PAU497	R. Ioos	…	France	Métairies-Saint-Quirin	2005	E1
PAU498	R. Ioos	…	France	Métairies-Saint-Quirin	2005	E1
PAU526	R. Ioos	…	France	Métairies-Saint-Quirin	2005	E1
PAU528	R. Ioos	…	France	Métairies-Saint-Quirin	2005	E1
PAU529	R. Ioos	…	France	Métairies-Saint-Quirin	2005	E1
PAU530	R. Ioos	…	France	Métairies-Saint-Quirin	2005	E1
PAU531	R. Ioos	…	France	Métairies-Saint-Quirin	2005	E1
PAU624	J. Aguayo	…	France	Métairies-Saint-Quirin	2009	E3
PAU625	J. Aguayo	…	France	Métairies-Saint-Quirin	2009	E1
PAU626	J. Aguayo	…	France	Métairies-Saint-Quirin	2009	E1
PAU627	J. Aguayo	…	France	Métairies-Saint-Quirin	2009	E1
PAU628	J. Aguayo	…	France	Métairies-Saint-Quirin	2009	E1
PAU629	J. Aguayo	…	France	Métairies-Saint-Quirin	2009	E1
PAU630	J. Aguayo	…	France	Métairies-Saint-Quirin	2009	E1
PAU632	J. Aguayo	…	France	Métairies-Saint-Quirin	2009	E1
PAU633	J. Aguayo	…	France	Métairies-Saint-Quirin	2009	E1
PAU333	O. Caël	…	France	Wingen-sur-Moder	2008	E1
PAU538	J. Aguayo	…	France	Wingen-sur-Moder	2009	E1
PAU539	J. Aguayo	…	France	Wingen-sur-Moder	2009	E1
PAU300	C. Husson	…	France	Wolfskirchen	2008	E1
PAU808	S. Werres	BBA 7/03	Germany	–	2002	E1
PAU811	S. Werres	BBA70434	Germany	–	1997	E1
PAU668	Z. Nagy	155-a	Hungary	Hanság	1999	E1
PAU669	Z. Nagy	155-b, CBS117377	Hungary	Hanság	1999	E1
PAU670	Z. Nagy	155-c	Hungary	Hanság	1999	E1
PAU89	P. Capretti	Ph 68, CBS109280, P1234*	Italy	Northern Tuscany	2000	E2
PAU142	A. Munda	Phy-A-Slo	Slovenia	Ljubljana	2003	E1
PAU780	C. Pintos-Varela	685A	Spain	Ribadavia	2009	E1
PAU84	C. Olsson	CH161/P875*	Sweden	Gothenburg	1996	E2
PAU807	C. Olsson	CH162	Sweden	Gothenburg	1996	E1

[a] Original code for the isolates; * indicates reference isolates in Brasier et al. (9).
[b] *P. alni* subsp. *uniformis* MLG assignation for each isolate.

(65) was used to detect first-generation migrants. The test uses a Monte-Carlo resampling method that identifies statistical thresholds beyond which individuals are likely to be F_0 immigrants (64). The goal of this test is to distinguish between residents that have a genotype that is most likely to occur in a population other than the one in which the individual was sampled by chance, and F_0 immigrants that are misassigned because they originated somewhere other than where they were sampled (64). Additionally, to study the relationship among genotypes, a minimum spanning network (MSN) was constructed using the Bruvo's distance. MINSPNET (26) was used to create the network, which was visualized using the NEATO program in the GRAPHVIZ package (32).

Within-continent characterization. Identification of multilocus genotypes (MLGs) was performed using a Visual Basic module developed by Goyeau et al. (40). This procedure, designed specially to deal with clonal organisms, allows the identification of MLGs, scoring the number of variant alleles. Clonal richness, $R = G - 1/N - 1$ (20) was computed (with G = number of MLGs found and N = sample size). Clonal evenness (V)—an index that reflects equitability in the distribution of clonal membership among samples—was computed using GENCLONE 1.0 (5). Clonal richness and clonal evenness indices reflect the extent of clonal reproduction in populations (5,42). Genotypic diversity (D_g) was computed with MULTILOCUS 1.3 (2). This index is independent of clonal richness and can be defined as the probability that two individuals taken at random have different MLGs. The allelic richness (Ar)—an index that represents the number of alleles corrected for sample size (25)—and the expected and observed heterozygosities were computed with FSTAT 2.9.3.2 (39). Fisher's exact tests were run to test the significance of genetic differentiation and Ar. Fisher's exact tests were used because they are robust when the number of individuals is low (40,68). Because clonal amplification of genotypes could influence data interpretation, analyses of genetic structure should be performed with and without repeated identical MLGs (42,75). Indeed, when duplicate MLGs are removed, rare alleles that distinguish MLGs have increased weight in the analysis. This may also underrepresent common alleles, because identical MLGs, composed of identical common alleles, can be expected to occur by chance following recombination. On the other hand, if duplicate MLGs are not removed, a single individual may be represented several times in the data set if asexual reproduction occurs (49). Thus, all the following indices were computed considering both all individuals and one representative isolate of each MLG per sampling site (referred to as the "MLG data set"). Departure from HW equilibrium was studied by two methods. First, global tests using the complete enumeration method (57), well adapted for low numbers of alleles by locus, were performed using GENEPOP (68). Because an exact P value is computed by complete enumeration, no standard value is calculated. The null hypothesis tested was a random union of gametes. Second, unbiased estimates of $F$is (78) and $R$is (70) were computed with SPAGEDI.

LD was assessed by two approaches. First, we studied the significance of non-random association or gametic disequilibrium among pairs of loci for each population. This test looks for the association between diploid genotypes at both loci. For a pair of diploid loci, no assumption is made about the gametic phase in

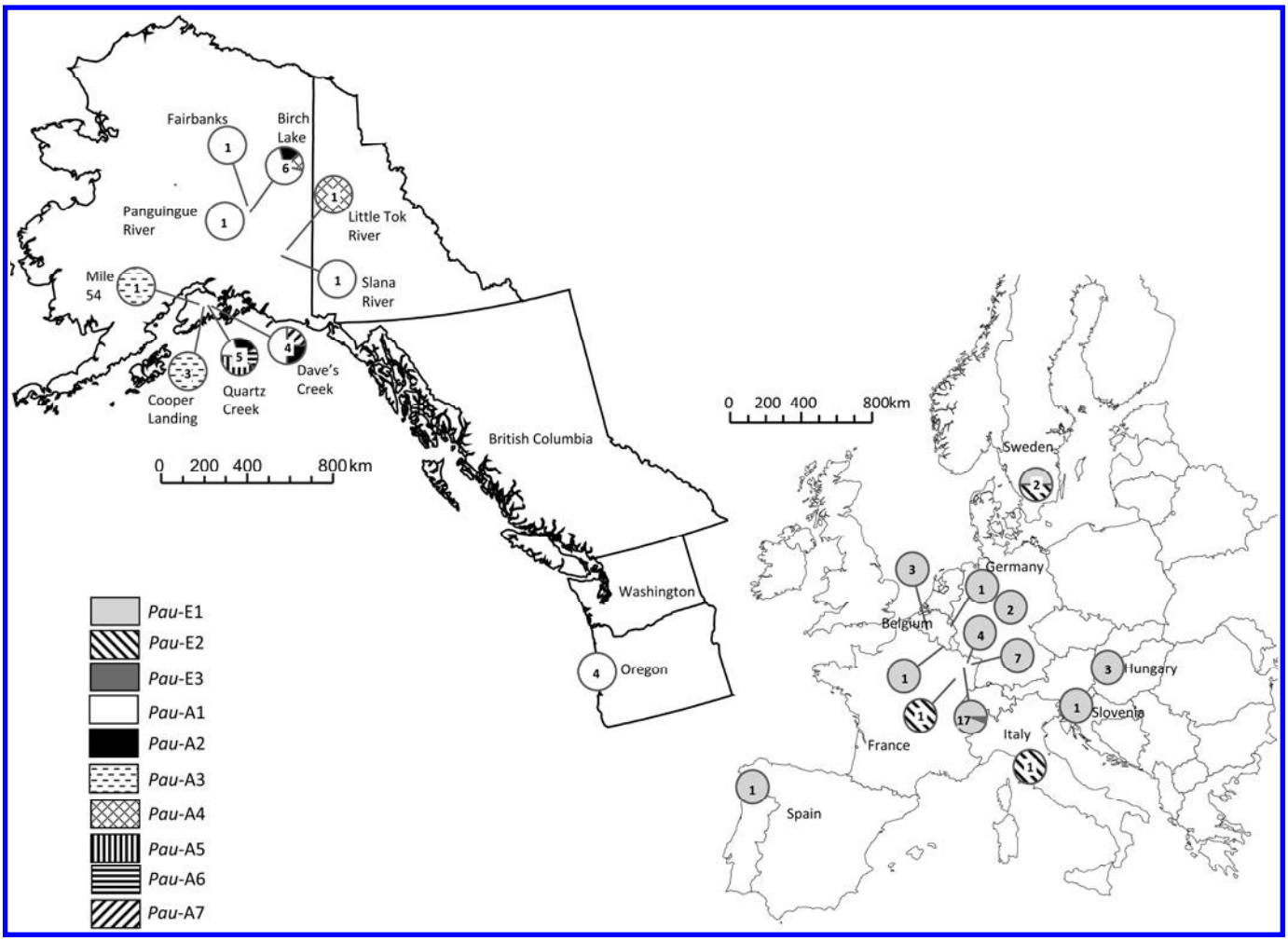

Fig. 1. Location of the sampling sites in North America and Europe, and spatial distribution of *Phytophthora alni* subsp. *uniformis* multilocus genotypes. Numbers inside the pie charts indicate the sample size for each location.

double heterozygotes (particularly in HW equilibrium). The test was performed with GENEPOP, considering as null hypothesis that genotypes at one locus are independent of genotypes at the other locus. The default test statistic was the log likelihood ratio statistic (G test). Contingency tables were created for all pairs of loci in each sample, and then a G test was computed for each table using a Markov chain algorithm (68). Second, using MULTILOCUS 1.3, we computed the measure of multilocus LD, $\bar{r}_s$—an alternative measure of multilocus LD that analyzes the allelic values directly and sums over all individuals. The analysis is similar to a two-way analysis of variance (ANOVA) on allelic values, which tests for a significant between-individual effect. Because *P* values are obtained from randomizations, no assumption of normality is associated generally with ANOVA tests. This index was computed by bootstrapping alleles 1,000 times among individuals, independently for each locus.

Mantel tests were performed to test the significance of the correlation between geographical or temporal and genetic distances. First, a matrix of Euclidean pairwise geographical distances was built for both European and North American collections. Second, in order to check the temporal genetic structure, a pairwise matrix of the differences between sampling years was computed. A normalized Mantel statistic was obtained by combining the binary matrix describing the geographical or temporal relationships and the corresponding Bruvo's genetic distances. All tests were performed for all the isolates and the MLG data set with 10,000 permutations.

RESULTS

Sample collection and species identification. Surveys in alder stands in France, Belgium, and Hungary allowed us to isolate 34 *P. alni* subsp. *uniformis* from a total of 516 *P. alni* sensu lato isolates (6.6%). Among these *P. alni* subsp. *uniformis* isolates, 50% were collected from one single site (Métairies-Saint-Quirin) in northeastern France. Ten other isolates were obtained from colleagues in other European locations (Table 1; Fig. 1). In North America, the yield was approximately one *P. alni* subsp. *uniformis* isolate per 100 *Phytophthora* colonies. Neither *P. alni* subsp. *alni* nor *P. alni* subsp. *multiformis* were isolated from North American sites. PCR amplification with species-specific primers for nuclear genes *RAS*-Ypt and *TRP*1 confirmed the *P. alni* subsp. *uniformis* status of all isolates (data not shown). In total, 71 isolates (44 from Europe and 27 from North America) were analyzed in this study.

Microsatellite development. Among the 110 microsatellite loci tested, 37 (33.6%) amplified as expected. Among these loci, 32 (86%) were monomorphic and 5 (14%) were polymorphic in *P. alni* subsp. *uniformis*, with 2 to 3 alleles per locus and an average of 1.5 alleles per locus (Table 2). Only one locus was informative among European isolates (M-PAU3), whereas three loci exhibited some polymorphism within North American isolates (M-PAU3, M-PAU9, and M-PAU55). All five loci were highly informative to discriminate between continental collections (Table 3).

Between-continent genetic analysis. Genotyping of the 71 *P. alni* subsp. *uniformis* isolates permitted their assignment into 10 MLGs, including 7 from North America and 3 from Europe (Table 3). No MLGs were shared between the North American and European collections. The European collection exhibited one major MLG (*Pau*-E1, 91% of the isolates) and two minor MLGs (*Pau*-E2 and *Pau*-E3). The 27 North American isolates were distributed into 7 MLGs (Table 3). MLG *Pau*-A1 was dominant in North America, accounting for 50% of the isolates. Private alleles

TABLE 2. Characteristics of the microsatellite markers used in this study

					Europe (n = 44)[d]				North America (n = 27)[d]			
Locus	Primer sequences (5′–3′)	GenBank[a]	Repeat[b]	Alleles[c]	*N*	Ar	H_e	H_o	*N*	Ar	H_e	H_o
M-PAU3	F1: TAAGAGACCTCCGGCAGAGA R1: AAAGCGAACACGAAGTCCAC	JX462795	$(GA)_{10}$	105/107/113	3	2.6	0.15	0.02	2	2.0	0.33	0.04
M-PAU9	F1: TCATGGCGCTGATCAAGTAG R1: TAGTGGAGACTTACGGGGTT	JX462796	$(AC)_9$	93/95	1	…	…	…	2	2.0	0.11	0.04
M-PAU32	F1: TCAGCTCCTGTATCATCAATCG R1: AAGTTGCCGGTGAGTTGG	JX462797	$(CA)_{10}$	90/92	1	…	…	…	1	…	…	…
M-PAU53	F1: TCTGACGAAGACCTCGACCT R1: CTCGAGATTGCCTTGCTGTC	JX462798	$(CT)_8$	183/185	1	…	…	…	1	…	…	…
M-PAU55	F1: ACATTGCTCATTCAGATGCG R1: GTGGAGGAGCACTTCATGGT	JX462799	$(GT)_8$	224/226	1	…	…	…	2	2.0	0.37	0.11

[a] GenBank accession number.
[b] Repeat motif.
[c] Alleles observed per locus.
[d] Abbreviations: n = number of individuals per collection, N = number of alleles observed by collection, Ar = allelic richness per locus, H_e = mean expected heterozygosity per locus, and H_o = mean observed heterozygosity per locus.

TABLE 3. Multilocus genotypes (MLGs) identified for the European and North American collections of *Phytophthora alni* subsp. *uniformis*

MLG[a]	N[b]	Isolates (%)	M-PAU3	M-PAU9	M-PAU32	M-PAU53	M-PAU55
Pau-E1	40	90.9	107/107	93/93	92/92	185/185	226/226
Pau-E2	3	6.8	113/113	93/93	92/92	185/185	226/226
Pau-E3	1	2.3	105/107	93/93	92/92	185/185	226/226
Total Europe	44	…	…	…	…	…	…
Pau-A1	14	51.9	107/107	95/95	90/90	183/183	226/226
Pau-A2	4	14.8	107/107	95/95	90/90	183/183	224/224
Pau-A3	4	14.8	105/105	95/95	90/90	183/183	226/226
Pau-A4	2	7.4	107/107	95/95	90/90	183/183	224/226
Pau-A5	1	3.7	105/105	93/93	90/90	183/183	226/226
Pau-A6	1	3.7	107/107	93/95	90/90	183/183	224/226
Pau-A7	1	3.7	105/107	95/95	90/90	183/183	224/224
Total North America	27	…	…	…	…	…	…

[a] *P. alni* subsp. *uniformis* MLG.
[b] Number of individuals per MLG.

were observed between continental collections (Table 4). Four alleles were shared by isolates from the two collections, four alleles were unique to the North American isolates, and three alleles were unique to the European isolates.

The MDS performed using Bruvo's distance between isolates clearly differentiated the European and North American collections (Fig. 2). A similar pattern was obtained when D_{as} distance was used (data not shown). This was confirmed by a Mantel test which indicated that both distances were significantly correlated ($r = 0.90$, $P < 0.001$). Consistently, high global *F*st (*F*st = 0.88; $P < 0.001$) and *R*st (*R*st = 0.74; $P < 0.001$) values were found between both collections. Significant *F*st and *R*st values were obtained for all loci (Table 5). Indices computed for the MLG data set showed the same pattern, with high and significant global and across loci *F*st (*F*st = 0.82; $P < 0.001$) and *R*st (*R*st = 0.63; $P < 0.001$) values (Table 5). Tests performed with GENECLASS did not detect any first-generation migrants in either population. The MSN (Fig. 3) showed that the European cluster was linked to the North American cluster by a Bruvo distance of 0.125 between the MLGs *Pau*-E1 and *Pau*-A5. This distance was larger than the range of distances among MLGs within both North America (0.034 to 0.045) and Europe (0.045 to 0.068), possibly indicating lack of sampling of missing intermediate genotypes.

TABLE 4. Private alleles and allele frequency detected for European and North American collections

	Europe		North America	
Locus	Allele	Frequency	Allele	Frequency
M-PAU3	113	0.068	…	…
M-PAU9	…	…	95	0.944
M-PAU32	92	1.000	90	1.000
M-PAU53	185	1.000	183	1.000
M-PAU55	…	…	224	0.240

Within-continent genetic analysis. The gene diversity (H_e) per locus for all isolates ranged from $H_e = 0$ to $H_e = 0.37$, with an average gene diversity value of $H_e = 0.16 \pm 0.16$ and $H_e = 0.03 \pm 0.07$ for the North American and European isolates, respectively (mean ± standard deviation [SD]) (Table 6). Observed heterozygosity (H_o) for all isolates ranged from $H_o = 0$ to $H_o = 0.1$, with an average value of $H_o = 0.04 \pm 0.04$ for the North American collection and $H_o = 0.004 \pm 0.01$ for the European collection (mean ± SD) (Table 6). Clonal richness (*R*), genotypic diversity (D_g), and evenness (*V*) were low for the European collection ($R = 0.05$, $D_g = 0.17$, $V = 0.14$) compared with the North American collection ($R = 0.23$, $D_g = 0.74$, $V = 0.62$) (Table 6). Both collections exhibited significant deviation from HW proportions ($P < 0.001$ for both collections,). Similar results were obtained for the MLG data set ($P = 0.003$ and $P < 0.001$ for the European and the North American MLG data sets, respectively). Consistently, global *F*is and *R*is estimates computed for all individuals and for the MLG data set were positive and significant (Table 6). The Ar

TABLE 5. Global and per locus *F*st and *R*st between European and North American populations of *Phytophthora alni* subsp. *uniformis*[a]

	*F*st		*R*st	
Locus	All individuals ($n = 71$)	MLG ($n = 37$)	All individuals ($n = 71$)	MLG ($n = 37$)
Per locus				
M-PAU3	0.09*	0.03*	0.13*	0.18*
M-PAU9	0.95*	0.91*	0.95*	0.91*
M-PAU32	1.00*	1.00*	1.00*	1.00*
M-PAU53	1.00*	1.00*	1.00*	1.00*
M-PAU55	0.27*	0.30*	0.27*	0.30*
Multilocus	0.88*	0.82*	0.74*	0.63*

[a] MLG = multilocus genotype and * indicates statistically significant ($P < 0.05$).

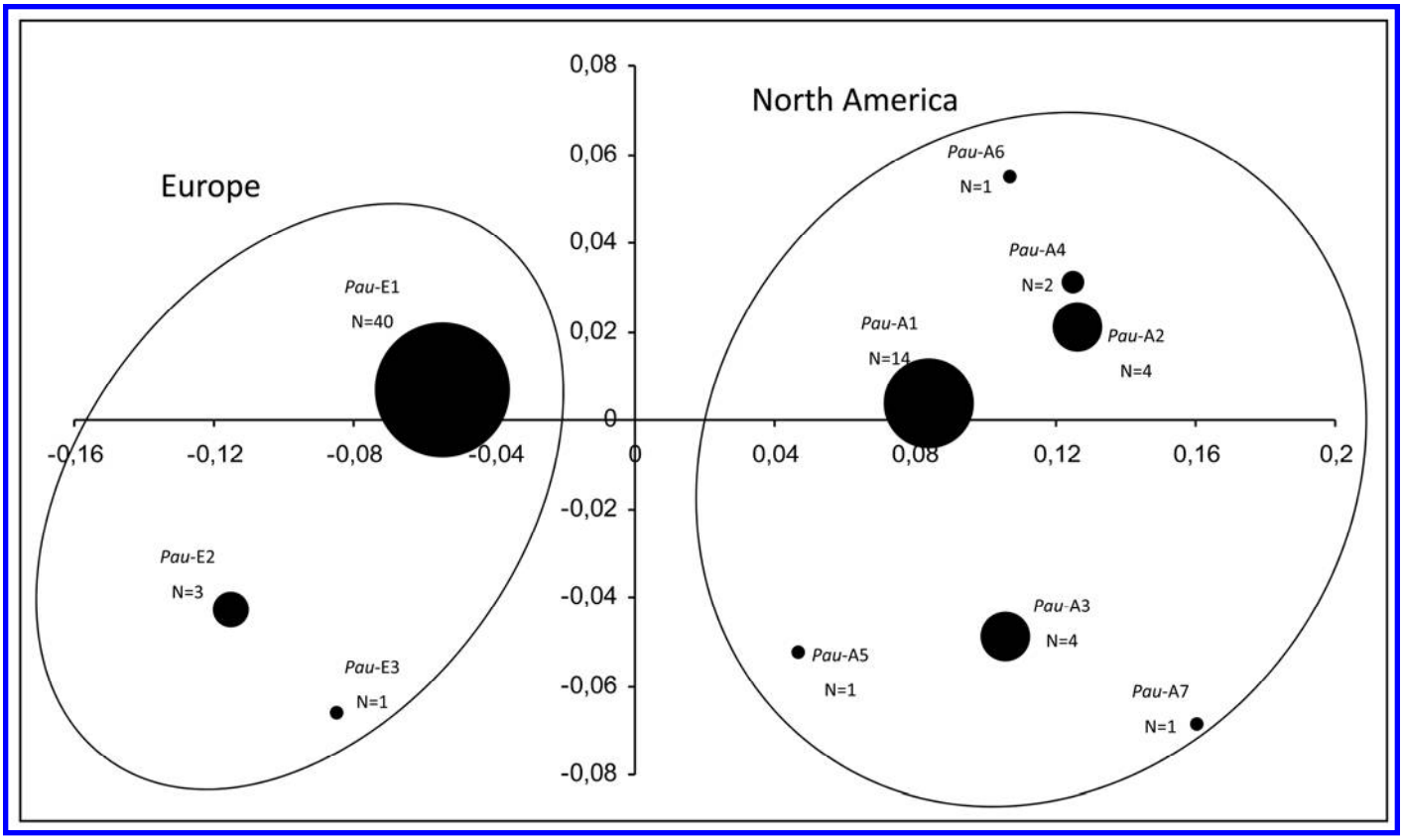

Fig. 2. Nonmetric multidimensional scaling for two axes of *Phytophthora alni* subsp. *uniformis* multilocus genotypes (MLGs) on the basis of the Bruvo's distance. Circle areas are proportional to the number of individuals, except for MLGs *Pau*-E1 and *Pau*-A1.

corrected for 27 isolates was higher for the North American collection compared with the European collection ($P < 0.001$). This difference was lower and not significant when computed for the MLG data set ($P > 0.05$).

Examination of LD by correlation between pairs of loci revealed complete dependence among all pairs of loci for the European collection. For the North American collection, when tests could be performed, zero of three pairs of loci exhibited no

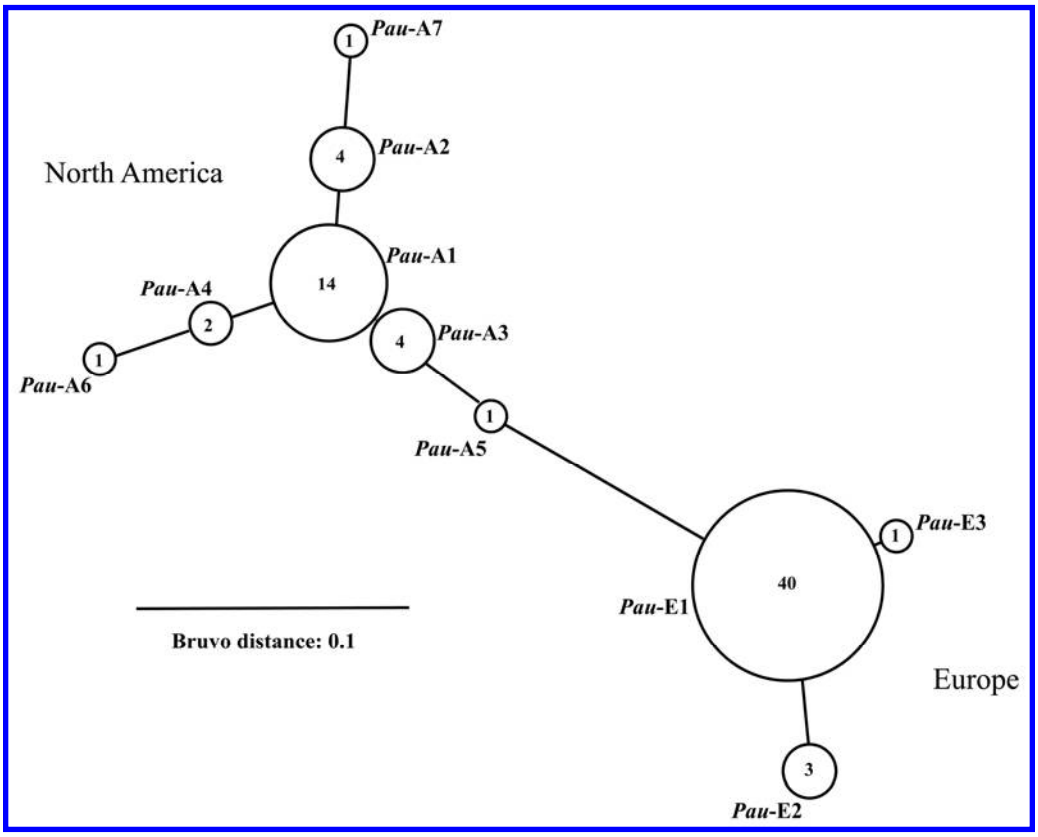

Fig. 3. Minimum spanning network showing the relationships among the 10 multilocus genotypes (MLGs) found in Europe and North America. Branch sizes are proportional to Bruvo's genetic distance, and surface areas of the circles are proportional to the numbers of isolates in each MLG (indicated inside the circles). Names of the MLGs are indicated.

TABLE 6. Comparison of the genetic structure of North American and European populations of *Phytophthora alni* subsp. *uniformis* revealed by microsatellite markers[a]

	North America		Europe	
Statistics[b], locus	All individuals ($n = 27$)	MLG ($n = 17$)	All individuals ($n = 44$)	MLG ($n = 20$)
R	0.37	nc	0.05	nc
V	0.74	nc	0.14	nc
D_g	0.62	nc	0.17	nc
H_e	0.16 (± 0.16)	0.19 (± 0.19)	0.03 (± 0.07)	0.10 (± 0.23)
H_o	0.04 (± 0.04)	0.06 (± 0.10)	0.004 (± 0.01)	0.02 (± 0.04)
Ar	1.60 (± 0.55)	1.60 (± 0.55)	1.32 (± 0.72)	1.38 (± 0.85)
Pairwise LD	0/3	0/3	nc	nc
$\bar{r}_s$	–0.04	–0.01	nc	nc
*F*is per locus				
M-PAU3	0.73	1.00*	0.85*	0.81*
M-PAU9	0.67	0.64	nc	nc
M-PAU32	nc	nc	nc	nc
M-PAU53	nc	nc	nc	nc
M-PAU55	0.72*	0.26	nc	nc
*F*is multilocus	0.71*	0.62*	0.85*	0.81*
*R*is per locus				
M-PAU3	0.73	0.83*	0.85*	0.84*
M-PAU9	0.67	0.65	nc	nc
M-PAU32	nc	nc	nc	nc
M-PAU53	nc	nc	nc	nc
M-PAU55	0.71	0.62	nc	nc
*R*is multilocus	0.78*	0.67*	0.85*	0.84*

[a] When possible, statistics were computed considering all individuals, or multilocus genotype MLG dataset only; nc = cannot be computed and * = statistically significant ($P < 0.05$).

[b] *R* = clonal richness, *V* = clonal evenness, D_g = genotypic diversity, H_e = unbiased expected heterozygosity (mean ± standard deviation [SD]), H_o = observed heterozygosity (mean ± SD), Ar = allelic richness (mean ± SD), LD = pairwise linkage disequilibrium, and $\bar{r}_s$ = multilocus linkage disequilibrium.

pairwise LD when all individuals were considered. The same results were obtained for both collections when the MLG data set was considered (Table 6). The $\bar{r}_s$ multilocus estimate of LD computed for the North American collection was negative and not significant ($\bar{r}_s$ = –0.04; P = 0.82). Similar results were obtained for the North American MLG data set ($\bar{r}_s$ = –0.01; P = 0.90). For the European isolates, $\bar{r}_s$ could not be computed, because polymorphism was extremely low and determined only by one locus, suggesting complete linkage across loci.

The geographical distribution of the MLGs for European and North American isolates are shown in Figure 1. Mantel tests showed no evidence for spatial structure in either collections (r = 0.09, P = 0.19 for the European collection and r = –0.11, P = 0.99 for the North American collection). The MLG data set exhibited similar results (r = –0.15, P = 0.39 for the European collection and r = –0.19, P = 0.84 for the North American collection). No evidence for temporal structure in the genetic diversity was found in either collections (r = 0.040, P = 0.27 for the European collection and r = –0.26, P = 0.99 for the North American collection). The same pattern was found when tests were performed for the MLG data set (r = 0.013, P = 0.35 and r = –0.24, P = 0.87 for the European and for the North American MLG data sets, respectively).

DISCUSSION

Isolate sampling and microsatellite development. Isolate collection for this study accounted for more than 10 years of field work in Europe and North America. The low recovery rate of *P. alni* subsp. *uniformis* is in accordance with the published literature (53). The low number of polymorphic loci found and the low number of alleles per locus are consistent with results obtained for other oomycetes (19,66,71,77). Thus, the use of a high-throughput methodology involving pyrosequencing of a microsatellite-enriched library yielded results comparable with those found by Ioos et al. (48) on *P. alni* subsp. *alni* using the classical method of cloning and sequencing of a microsatellite-enriched DNA library. This low number of polymorphic microsatellites in *P. alni* subsp. *uniformis* is in accordance with results of Dutech et al. (23), who showed that obtaining microsatellite markers with an acceptable level of polymorphism is generally more difficult from fungi and oomycetes than from other organisms.

Genetic characterization of North American and European *P. alni* subsp. *uniformis*. North American *P. alni* subsp. *uniformis* exhibited a moderate genotypic diversity and evenness. Our data suggest that selfing is probably the predominant mating system of North American *P. alni* subsp. *uniformis*. Indeed, the population exhibited significant deviation from HW proportions and high global *F*is and *R*is values, as expected for a homothallic organism (3,21,29,36). Self-fertilization has frequently been considered important in homothallic oomycetes (36). However, strict selfing cannot explain the diversity exhibited by the North American population. Self-fertilization reduces the amount of heterozygosity by one-half in every generation; thus, there should be almost no heterozygosity in homothallic *Phytophthora* spp. that have been established for more than a few generations (36). This was not the case for North American *P. alni* subsp. *uniformis*, which exhibited gene diversity similar to heterothallic, outcrossing oomycetes (36,41). Moreover, our results did not show complete LD, suggesting that the genetic structure of North American populations of *P. alni* subsp. *uniformis* can be explained by a mixed mating system, including selfing and, probably, rare outcrosses. Indeed, it has been shown that outcrossing is important in generating and maintaining variation in homothallic oomycetes (28,29,41). However, the balance between selfing and outcrosses is not easy to establish, and may be dynamic and highly dependent on spatiotemporal conditions (34).

The genetic characteristics of the European collection of *P. alni* subsp. *uniformis* were different from the North American population. Although northeastern France is over-represented in our sampling, we have studied *P. alni* subsp. *uniformis* isolates from eight different countries scattered throughout Europe, and the extremely low genotypic diversity and evenness across large areas and different environmental conditions is significant. All the variation observed in Europe was attributed to a single locus (M-PAU3). However, the reproductive mode and the mating system of European *P. alni* subsp. *uniformis* are less clear than that of North American *P. alni* subsp. *uniformis*. Low genotypic diversity and gene diversity, coupled with high and significant *F*is and *R*is values, and the strong LD detected for all loci tested suggest that the European *P. alni* subsp. *uniformis* are selfing. However, strict clonality after a strong bottleneck resulting from an introduction of *P. alni* subsp. *uniformis* in Europe cannot be rejected. This has been described for other pandemics of plant pathogens that were subjected to strong founder effects after introduction (37,38,50,67). The possibility of sexual reproduction in Europe would have some important epidemiological consequences, such as the production of resistant spores (24,53). Delcán and Brasier (14) showed that European *P. alni* subsp. *uniformis* were able to produce viable oospores in controlled laboratory conditions, although germination was not observed. However, in vitro observations do not allow strong inferences regarding the reproductive mode in natural conditions (6).

Neither population exhibited any spatial or temporal structure. This can be explained by the existence of an efficient long-distance dispersal means through rivers. Moreover, in Europe, planting of seedlings from infected nurseries has been shown to be an important mechanism of long-distance spread (53). This may explain the widespread occurrence of *P. alni* subsp. *uniformis* in Europe.

Genetic differences between North American and European populations of *P. alni* subsp. *uniformis*. Our study revealed strong differences between the American and European collections of *P. alni* subsp. *uniformis* (global *F*st = 0.89 and *R*st = 0.74). The genetic differentiation between collections was caused by the presence of private alleles within each population. No evidence of gene flow could be detected between populations. Such high levels of genetic differentiation within one species have already been reported (63,69), and could be caused by genetic drift through founder effects accompanying the spread of the disease, which may result in strongly differentiated populations (69). Furthermore, the mating system (predominantly selfing) of *P. alni* subsp. *uniformis* is likely to reinforce the genetic structure between continental populations, because selfing will tend to decrease the effective population size (11).

We hypothesize that *P. alni* subsp. *uniformis* is likely to be alien in Europe, and may have derived from the introduction of only a few individuals, whereas the American population of *P. alni* subsp. *uniformis* is probably indigenous. The differentiation between collections is large and does not point to a North American origin of the European *P. alni* subsp. *uniformis* (large *F*st and *R*st, presence of private alleles, and no evidence of first-generation migrants in either collection). However, a North American origin of European *P. alni* subsp. *uniformis* remains possible. A single connection between both collections visualized with the computed MSN suggests a single and not a multiple introduction of *P. alni* subsp. *uniformis* in Europe from a putative North American source population. However, the MSN indicates that some intermediate genotypes were missing. Indeed, sampling in North America was not exhaustive and, thus, private alleles observed in Europe could well have been missed in this limited sample.

Concluding remarks. This study provides new insights into the origin of the interspecific hybrid *P. alni* subsp. *alni*, which is primarily responsible for the outbreak of alder decline in Europe. The origin of *P. alni* subsp. *alni* could be explained by three nonexclusive scenarios: (i) a recent hybridization event directly following the introduction of one (or two) of the parental species,

P. alni subsp. *uniformis* and *P. alni* subsp. *multiformis*; (ii) long-time coexistence of both parental species which, after the apparition of favorable environmental conditions (e.g., climatic conditions), would have succeeded in mating in nature; or (iii) an ancient hybridization event giving raise to *P. alni* subsp. *alni*, followed by a long coexistence of *P. alni* subsp. *alni*, *P. alni* subsp. *uniformis*, and *P. alni* subsp. *multiformis*, and a recent emergence of the *P. alni* subsp. *alni*-induced alder decline triggered by more favorable environmental conditions. Our results suggest that the introduction of *P. alni* subsp. *uniformis* would have enabled hybridization with *P. alni* subsp. *multiformis* and, consequently, may be a major cause of the emergence of alder decline in Europe. However, when and where the *P. alni* subsp. *uniformis* invasion and subsequent hybridization took place, and its recurrence, remain unknown. Moreover, whether *P. alni* subsp. *multiformis* is exotic or indigenous to Europe is also an open question. It seems that *P. alni* subsp. *alni* may have arisen after multiple hybridizations of the parental species in alder nurseries used for riparian restoration projects in Europe (33,47,53). Further studies in population genetics and epidemiology of *P. alni* subsp. *alni*, *P. alni* subsp. *uniformis*, and *P. alni* subsp. *multiformis* are needed to address these hypotheses.

Species involved in interspecific hybridization need to meet some genetic criteria and to share the same environment for successful hybridization to occur (62). The genetic barriers to hybridization are weaker in phylogenetically close species that have evolved in allopatry (7). Therefore, the chances of interspecific hybridization increase when two allopatric species accidentally come into secondary contact (7,8,62), as probably occurred for *P. alni* subsp. *alni*. The risk of hybridization between previously allopatric pathogen species is likely to increase as the international trade of plant material intensifies, and their associated pathogens are introduced into new geographical areas (8,31).

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