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e-Xtra*

Patterns of Genetic Diversification in the Invasive Hybrid Plant Pathogen *Phytophthora* × *alni* and Its Parental Species *P. uniformis*

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ABSTRACT

In pathogenic fungi and oomycetes, interspecific hybridization may lead to the formation of new species having a greater impact on natural ecosystems than the parental species. From the early 1990s, a severe alder (*Alnus* spp.) decline due to an unknown *Phytophthora* species was observed in several European countries. Genetic analyses revealed that the disease was caused by the triploid hybrid *P.* × *alni*, which originated in Europe from the hybridization of *P. uniformis* and *P.* × *multiformis*. Here, we investigated the population structure of *P.* × *alni* (158 isolates) and *P. uniformis* (85 isolates) in several European countries using microsatellite markers. Our analyses confirmed the genetic structure previously observed in other European populations, with *P. uniformis* populations consisting of at most two multilocus genotypes (MLGs) and *P.* × *alni* populations dominated by MLG Pxa-1. The genetic structure of *P.* × *alni* populations in the Czech Republic, Hungary and Sweden seemed to reflect the physical isolation of river systems. Most rare *P.* × *alni* MLGs showed a loss of heterozygosity (LOH) at one or a few microsatellite loci compared with other MLGs. This LOH may allow a stabilization within the *P.* × *alni* genome or a rapid adaptation to stress situations. Alternatively, alleles may be lost because of random genetic drift in small, isolated populations, with no effect on fitness of *P.* × *alni*. Additional studies would be necessary to confirm these patterns of population diversification and to better understand the factors driving it.

Keywords: biological invasion, ecology and epidemiology, genome alterations, subgenome, population biology, population diversity, simple sequence repeats

In fungi (kingdom *Mycota*) and fungus-like oomycetes (kingdom *Stramenopila*), hybridization can be defined as the process of genome fusion between nonconspecific individuals, which occurs both sexually and asexually and generates offspring of mixed ancestry (Abbott et al. 2013; Schardl and Craven 2003; Stukenbrock 2016). When the ploidy level of the hybrid sums that of the two parental species, the process is called allopolyploid speciation, whereas hybridization without change in chromosome number is called homoploid speciation (Giraud et al. 2008; Restrepo et al. 2014). Hybridization plays an important role in the evolution of plant pathogenic fungi and oomycetes (Arnold 2004; Gibson et al. 2014; Kroon et al. 2012), as the newly formed hybrid species may show a better adaptation to the environment and have a greater impact on natural ecosystems than the parental species (so-called heterosis) (Arnold and Martin 2010; Brasier 2000; Gibson et al. 2014; Stukenbrock and McDonald 2008).

Until the 1990s, only a few hybrid plant pathogens, mainly causing diseases to crops, were known (Olson and Stenlid 2002), such as the *Tilletia caries* (DC.) Tul. & C. Tul. × *T. laevis* J.G. Kühn hybrid (Flor 1932) or the *Ustilago hordei* (Pers.) Lagerh × *U. nuda* (C.N. Jensen) Rostr. hybrid (Fullerton and Nielsen 1974). At that time it was mainly thought that fungal hybrids were relatively rare in nature (Barton 2001). Successively, thanks to the development and implementation of new DNA-based techniques (Cooke et al. 2000), it has become evident that hybridization events and hybrid species formation are more frequent than previously supposed (Gibson et al. 2014; Schardl and Craven 2003). Currently, various molecular markers are used to characterize hybrids and their

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populations, including simple sequence repeats (Hegarty and Hiscock 2005), single nucleotide polymorphism (SNPs), and mitochondrial DNA (mtDNA) (Burgess 2015).

Hybridization success strongly depends on the type of isolation (i.e., geographical or reproductive) between the involved species. In sympatric fungal species (i.e., coexisting in the same geographic area), interspecific hybridization is extremely difficult because of the presence of reinforced reproductive barriers (Stukenbrock 2016). Hence, hybridization is more likely to occur between geographically isolated (i.e., allopatric), but not necessarily reproductively isolated, species (Stukenbrock and McDonald 2008; Restrepo et al. 2014). In recent years, international plant trade has led to numerous introductions of microorganisms to new geographic areas (Callaghan and Guest 2015; Fisher et al. 2012; Garbelotto and Gonthier 2013), thereby considerably increasing the opportunities for new interspecific hybridization events to occur. Because of this ample movement of species around the globe, information about the parental species of hybrids is commonly lacking. A well-known example is the oilseed rape pathogen *Verticillium longisporum* (C. Stark) Karapapa, Bainbr. & Heale, whose parental species are of unknown origin (Depotter et al. 2016; Inderbitzin et al. 2011).

Phytophthora de Bary is a cosmopolitan genus of oomycetes, mainly containing obligate plant pathogens that cause damage in both forest and agricultural systems (Erwin and Ribeiro 1996). Recent studies showed that interspecific hybridization can occur between *Phytophthora* species (Bertier et al. 2013; Brasier et al. 1999; Kroon et al. 2012). Besides being successfully created under laboratory conditions, hybrids have also been found in nature on all continents. Naturally formed hybrids were identified in several *Phytophthora* internal transcribed spacer (ITS) clades (Burgess 2015), including clades 1 (Goss et al. 2011; Man In'T Veld et al. 2007), 6 (Burgess 2015; Nagel et al. 2013), 7 (Brasier et al. 2004), and 8 (Bertier et al. 2013). Thus, in the genus *Phytophthora* interspecific hybridization is increasingly considered an important process for the generation of new species (Bertier et al. 2013; Burgess 2015).

In the early 1990s, a sudden alder (*Alnus* spp.) decline was observed in the United Kingdom (Gibbs 1995) and later on in several other European regions (Brasier et al. 2004). The declining trees (mainly *Alnus incana* (L.) Moench and *Alnus glutinosa* (L.) Gaertn.) showed a sparse crown and bleeding lesions on the root collar and stem (Gibbs 2003). This new lethal disease was attributed to *Phytophthora alni* Brasier & S.A. Kirk sensu lato, a new *Phytophthora* species typically isolated from symptomatic alders. Initially, it was thought that *P. alni* s.l. consisted of a common hybrid variant type and two less frequent types (Brasier et al. 2004). Successively, the three variants were considered as three subspecies, i.e., *P. alni* ssp. *alni* Brasier & S.A. Kirk, *P. alni* ssp. *multiformis* Brasier & S.A. Kirk, and *P. alni* ssp. *uniformis* Brasier & S.A. Kirk (Brasier et al. 2004), and recently described as species (Husson et al. 2015). Brasier et al. (1995) suggested that *P. alni* s.l. originated from interspecific hybridization, but at that time the parental species were unclear. Subsequent studies showed that the parental species of *P.* × *alni* (Brasier & S.A. Kirk) Husson, Ioos & Marçais are *P.* × *multiformis* (Brasier & S.A. Kirk) Husson, Ioos & Marçais and *P. uniformis* (Brasier & S.A. Kirk) Husson, Ioos & Marçais (Husson et al. 2015; Ioos et al. 2007). *P.* × *multiformis* is itself a tetraploid hybrid species whose origin is still unknown (Aguayo et al. 2016; Husson et al. 2015). On the other side, *P. uniformis* is a diploid species, which was possibly introduced to Europe from North America (Aguayo et al. 2013). Because of its frost tolerance, this species is more frequently found in the northern parts of Europe (Redondo et al. 2015), although it has been also reported in the south of the continent (Varela et al. 2012).

In a previous study, Aguayo et al. (2016) documented a low polymorphism and the absence of sexual recombination in *P.* × *alni*, with European populations (mainly from France, Germany, and Hungary) dominated by a single multilocus genotype (Pxa-1). However, since mtDNA patterns of both parental species were found in *P.* × *alni* isolates belonging to the same genotype, the authors concluded that multiple hybridization events occurred independently in several European regions, i.e., Pxa-1 is not a true clone that colonized Europe. Noteworthy, the incidence of Pxa-1 in local populations seemed to increase over time. The population of the parental species *P. uniformis* was also dominated by a single multilocus genotype and was less diverse than expected based on the *P. uniformis* subgenomes present in the *P.* × *alni* population.

In this study, we aimed at determining the genetic population structure of *P.* × *alni* and *P. uniformis* in four European countries that were not analyzed by Aguayo et al. (2016), i.e., Austria, Czech Republic, Lithuania, and Switzerland, and in three previously analyzed countries (Hungary, Spain, and Sweden). Specifically, we addressed the following questions. (i) Do *P.* × *alni* populations in Austria, Czech Republic, Lithuania, and Switzerland show the same diversity pattern reported by Aguayo et al. (2016) for other European countries? (ii) Given that *P.* × *alni* spreads locally through riverine networks (Jung and Blaschke 2004), are populations of this pathogen genetically structured by the river systems? And (iii) is the population structure of the more frost-tolerant parental species *P. uniformis* (Redondo et al. 2015) in Sweden similar to that in central Europe?

MATERIALS AND METHODS

***P.* × *alni* and *P. uniformis* isolates.** In this study, a total of 168 *P.* × *alni* and 90 *P. uniformis* isolates from seven countries (Austria, Hungary, Czech Republic, Lithuania, Spain, Sweden, and Switzerland) were analyzed (Table 1, Supplementary Table S1). The Swiss, Lithuanian, and Czech isolates originated from the culture collection at the Swiss Federal Research Institute (WSL), the Lithuanian State Research Institute Nature Research Centre (NRC), and the Czech Collection of Phytopathogenic Oomycetes (Silva Tarouca Research Institute), respectively, whereas all other isolates were kindly provided by colleagues. Given that both species are not considered quarantine organisms subject to phytosanitary regulations by the Swiss plant protection ordinance (PSV, SR 916.20), a sampling or import permit was not required.

DNA extraction. Three different approaches were used to obtain genomic DNA from *P.* × *alni* and *P. uniformis* cultures. (i) Isolates from Austria, the Czech Republic, Lithuania, Spain, and Switzerland were grown on liquid-clarified V8 juice medium (Miller 1955) for 5 to 7 days in the dark at 22°C. The mycelium was subsequently harvested through filtration and washed with sterile H_2O. Thereafter, it was frozen and stored at –20°C until DNA extraction. Mycelia were lyophilized and genomic DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol (Schoebel et al. 2013). (ii) Isolates from Sweden were grown on V8 juice agar for 7 days and DNA was extracted from mycelia harvested from the active growing margin of the cultures using NucleoSpin Plant II (Macherey-Nagel, Hoerdt, France) (Redondo et al. 2015). And (iii) isolates from Hungary were grown in pea-broth (Erwin and Ribeiro 1996) for 7 to 10 days in the dark at 25°C and DNA was extracted from lyophilized mycelium powder using the E.Z.N.A. Fungal DNA Mini Kit (OMEGA Bio-tek, Norcross, GA) or following the slightly modified protocol of Murray and Thompson (1980).

***Phytophthora* species identification.** Prior to genotyping, all isolates were identified to species. Samples from Austria, the Czech Republic (Štěpánková et al. 2013), Hungary (Aguayo et al. 2016), and Sweden (Redondo et al. 2015) have been identified to species before this study. *P. alni* s.l. isolates obtained from Lithuania, Spain, and Switzerland were identified using four sets of specific primers targeting the *TRP*1 (TRP-PAU-F/R) and *RAS-Ypt* (RAS-PAM1-F/R, RAS-PAM2-F/R, RAS-PAU-F/R) genes (Ioos et al. 2006), followed by sequencing of the ribosomal ITS using the ITS-6

(Cooke et al. 2000) and ITS-4 (White et al. 1990) primer set. PCR amplification and sequencing were done as previously described by Schoebel et al. (2013). The obtained sequences were assembled and edited using CLC Main Workbench version software v8 beta 04 (Qiagen). For species identification, sequences (~800 bp) were compared with publicly available sequences in the National Center for Biotechnology Information (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi) database with the BLAST algorithm (with the threshold set to 1e-63). Two sequences were considered to belong to the same species if they showed at least 99% similarity.

Microsatellite genotyping. All *P.* × *alni* and *P. uniformis* isolates were genotyped at the 10 microsatellite loci PA17, PA23 (Ioos et al. 2007), M-PAU3, M-PAU9, M-PAU32 (Aguayo et al. 2013), M-PAU11, M-PAU14, M-PAU15, M-PAU56, and M-PAU72 (Aguayo et al. 2016) using the single-tube nested PCR method developed by Schuelke (2000). This method is based on using fluorescently-labeled M13-tailed primers. PCR reactions were conducted using the Type-it Microsatellite PCR Kit (Qiagen), following a modified manufacturer's protocol. Modifications included the use (i) of less forward primer than reverse primer (0.1 and 0.2 µM, respectively; Schuelke (2000)), and (ii) of only 3 µl of Master Mix per reaction (Burokienė et al. 2015; Schoebel et al. 2013). PCR conditions were set as follows: initial denaturation for 5 min at 95°C, followed by 28 cycles with denaturation for 30 s at 95°C, annealing for 90 s at 60°C, extension for 30 s at 72°C, followed by M13-tag binding reaction of 8 cycles with denaturation for 30 at 95°C, annealing for 90 s at 55°C, extension for 30 s at 72°C, and a final extension for 30 min at 60°C (Schoebel et al. 2013). PCR amplifications were performed on Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, U.S.A.). PCR products were run on ABI 3130 DNA Analyzer with the GeneScan 500 LIZ Size Standard for fragment analysis. All alleles were scored manually using the software GeneMapper v. 3.7 (Applied Biosystems). Since *P.* × *alni* is a triploid organism, at each microsatellite locus three alleles are expected. However, if only two peaks were visible in the electropherogram, the third allele was considered missing. We adopted this approach because determining the third allele based on the peak height ratio (Aguayo et al. 2016) was not always possible (Supplementary Fig. S1). All detected multilocus genotypes (MLGs) were compared with those published by Aguayo et al. (2016) and named accordingly (Supplementary Table S1). To assure reproducibility of allele calling, genotyping was replicated for 19 isolates belonging to newly detected (i.e., not reported in Aguayo et al. 2016), rare MLGs with independent PCR reactions and sizing of fragments. All electropherograms are available from the corresponding authors upon request.

Population diversity analyses. *P.* × *alni* and *P. uniformis* isolates were assigned to MLGs with the software GENODIVE v. 2.0b27, using the stepwise mutation model as distance index under the assumption that allele repeat lengths differ by ancestry. The distance threshold for assigning individuals to the same MLG was set at zero (0) to differentiate closely related MLGs (Meirmans and Van Tienderen 2004). Genotypic richness, diversity, and evenness of *P.* × *alni* populations were calculated using poppr v. 2.6.1 (Kamvar et al. 2014). Genotypic richness was estimated by the number of observed (MLGs) and expected MLGs (eMLGs), i.e., corrected by sample size based on a rarefaction procedure. To avoid distorted genotypic diversity values due to uneven sample sizes, a corrected Simpson's index (D) was calculated as $D = ((N/N - 1)) \times \lambda$, where N is the sample size and λ is Simpson's index (Kamvar et al. 2014). Genotype evenness was evaluated using the E_5 index. This measure indicates the distribution of genotype abundance across a population, where 1 shows that genotypes are equally abundant in the population and 0 indicates the population is dominated by one genotype. E_5 is less susceptible to the effect of sample size (Grünwald et al. 2003). To roughly assess the evolution over time of the genotypic diversity in *P.* × *alni* populations (i.e., number of eMLGs and incidence of the in Europe prevalent MLG Pxa-1), a simple linear regression analysis was performed. For the time component, the maximal number of years between the first official isolation of *P.* × *alni* in a country and the sampling for this study was considered.

To evaluate the genetic relationships among the *P.* × *alni* MLGs, a minimum spanning network (MSN) was constructed using poppr and visualized using igraph ver. 1.1.2 (Csardi and Nepusz 2006). The MSN was based on Bruvo's genetic distance (Bruvo et al. 2004) under the assumption of combined and averaged genome addition and loss model. Each node represented a different MLG and node size was proportional to the abundance of the specific MLG.

Population differentiation analyses. Analyses of population differentiation were only conducted for *P.* × *alni* in the Czech Republic, Hungary, and Sweden, i.e., in the three countries with the largest sample sizes. Given that after introduction the pathogen mainly spreads downstream with streams and floods (Jung and Blaschke 2004), for analyses isolates were assigned to corresponding basins (i.e., an area of land drained by small rivers), rivers (i.e., a larger river collecting water from different basins), and river systems (i.e., a main river that drains part of a country and in which the rivers flow) (Fig. 1). Discriminant analysis of principal components (DAPC) (Jombart et al. 2010) was performed to describe clusters of genetically related *P.* × *alni* MLGs across the Czech Republic, Hungary, and Sweden. DAPC was conducted

TABLE 1. Diversity of the *Phytophthora* × *alni* and *P. uniformis* populations in the seven European countries included in this study

			Phytophthora × *alni*						*Phytophthora uniformis*		
Country	First detection of *P. alni* s.l.	Sampling year/period	N[a]	MLG[b]	eMLG[c] (± SE)	Pxa-1[d] (%)	D[e]	E_5[f]	N	Pu-1[g]	Pu-2[h]
Austria (AU)	1996 (Brasier et al. 2004)	2007–2014	9	4	2.93 (± 0.59)	33.3	0.805	0.93	2	2	0
Czech Republic (CZ)	2001 (Černý et al. 2003)	2006–2014	49	20	2.75 (± 0.89)	53.1	0.699	0.34	4	4	0
Hungary (HU)	1999 (Szabó et al. 2000)	2001–2009	39	7	1.97 (± 0.77)	74.4	0.442	0.45	0	n.a.[i]	n.a.
Lithuania (LT)	1999 (Jovaišienė 2002)	2014	9	2	1.44 (± 0.5)	88.9	0.222	0.59	0	n.a.	n.a.
Spain (SP)	2009 (Solla et al. 2010)	2010–2012	4	2	2.00 (± 0.0)	75.0	0.5	0.93	12	12	0
Sweden (SW)	1996 (Redondo et al. 2015)	2013–2015	41	9	3.21 (± 0.67)	24.4	0.852	0.84	67	46	21
Switzerland (CH)	2008 (Meier et al. 2009)	2015	7	1	1.00 (± 0.0)	100.0	0.000	n.a.	0	n.a.	n.a.
Total	–	–	158	31	2.69 (± 0.88)	55.1	0.687	0.32	85	64	21

[a] N, sample size.
[b] MLG, number of observed multilocus genotypes.
[c] eMLG, expected number of multilocus genotypes in a population of N = 4 (i.e., smallest population being analyzed).
[d] Pxa-1, incidence of the most common MLG Pxa-1 (Aguayo et al. 2016).
[e] D, modified Simpson's index.
[f] E_5, evenness index.
[g] Pu-1, MLG Pu-1 (Aguayo et al. 2016).
[h] Pu-2, MLG Pu-2 (Aguayo et al. 2016).
[i] n.a., not applicable.

using adegenet v.2.1.1 (Jombart 2008) with prior known *P.* × *alni* population information. To evaluate population differentiation in all river systems and rivers, an analysis of molecular variance (AMOVA) was conducted (Excoffier et al. 1992) using poppr. In most studies, population differentiation is measured using Wright's F-statistics (Holsinger and Weir 2009). However, due to its sensitivity to population size, migration, and mutation rate estimates (Fu et al. 2003; Meirmans and Hedrick 2011), this statistic is not the best suited for polyploid organisms. In fact, compared with diploid organisms, polyploid organisms have higher total numbers of chromosome copies and thus different migration and mutation measures (Meirmans et al. 2018). The Φ (phi) statistic (Ronfort et al. 1998) implemented in AMOVA was suggested in evaluation of polyploid organism population structure studies as a good alternative to Wright's F-statistics (Meirmans et al. 2018). The total *P.* × *alni* genetic variation (in the Czech Republic, Hungary, and Sweden) was partitioned at three levels: (i) within river basins, (ii) among river basins within one river system, and (iii) among river systems. The analysis was performed only with clone-corrected data.

The association between the presence of the two *P. uniformis* MLGs in Sweden (described in Results) and latitude and river basin was tested with a logistic regression. We used the presence/absence of each MLG as a binomial response and the latitude and river basin as explanatory variables. Since latitude and winter temperature in Sweden are correlated ($P < 0.0001$), we further tested the association between winter temperature and the presence of the *P. uniformis* MLGs by repeating the analysis using the average February air temperature for the last 20 years (values obtained from Redondo et al. 2015) instead of latitude.

RESULTS

Population diversity. A total of 258 *P. alni* s.l. isolates (168 *P.* × *alni* isolates and 90 isolates of *P. uniformis*) from seven different European countries were genotyped at 10 microsatellite loci (Supplementary Table S1). For 243 isolates (158 isolates of *P.* × *alni* and 85 *P. uniformis* isolates), alleles were successfully amplified at all loci. In total, 23 alleles were identified in the analyzed *P.* × *alni* and *P. uniformis* populations, with one (*P.* × *alni* and *P. uniformis*) to three (*P.* × *alni*) alleles per locus. Compared with previous studies (Aguayo et al. 2013, 2016; Ioos et al. 2007), no new alleles were found.

P. × alni. The 158 *P.* × *alni* isolates that were successfully genotyped at all loci belonged to 31 different MLGs, 19 of which had not been found before. The most common MLG was Pxa-1 (87 isolates), which was present in all countries with an incidence ranging from 24.4% (Sweden) to 100% (Switzerland) (Table 1). Pxa-1 was followed by MLG Pxa-6 (nine isolates), which was found in the Czech Republic, Hungary, Spain and Sweden. Pxa-3 and Pxa-9 included eight isolates each, but were only found in Sweden. A total of seven MLGs (Pxa-1, Pxa-2, Pxa-6, Pxa-8, Pxa-19, Pxa-27, and Pxa-28) were present in more than one country, whereas 24 MLGs were restricted to a specific country (Table 2, Supplementary

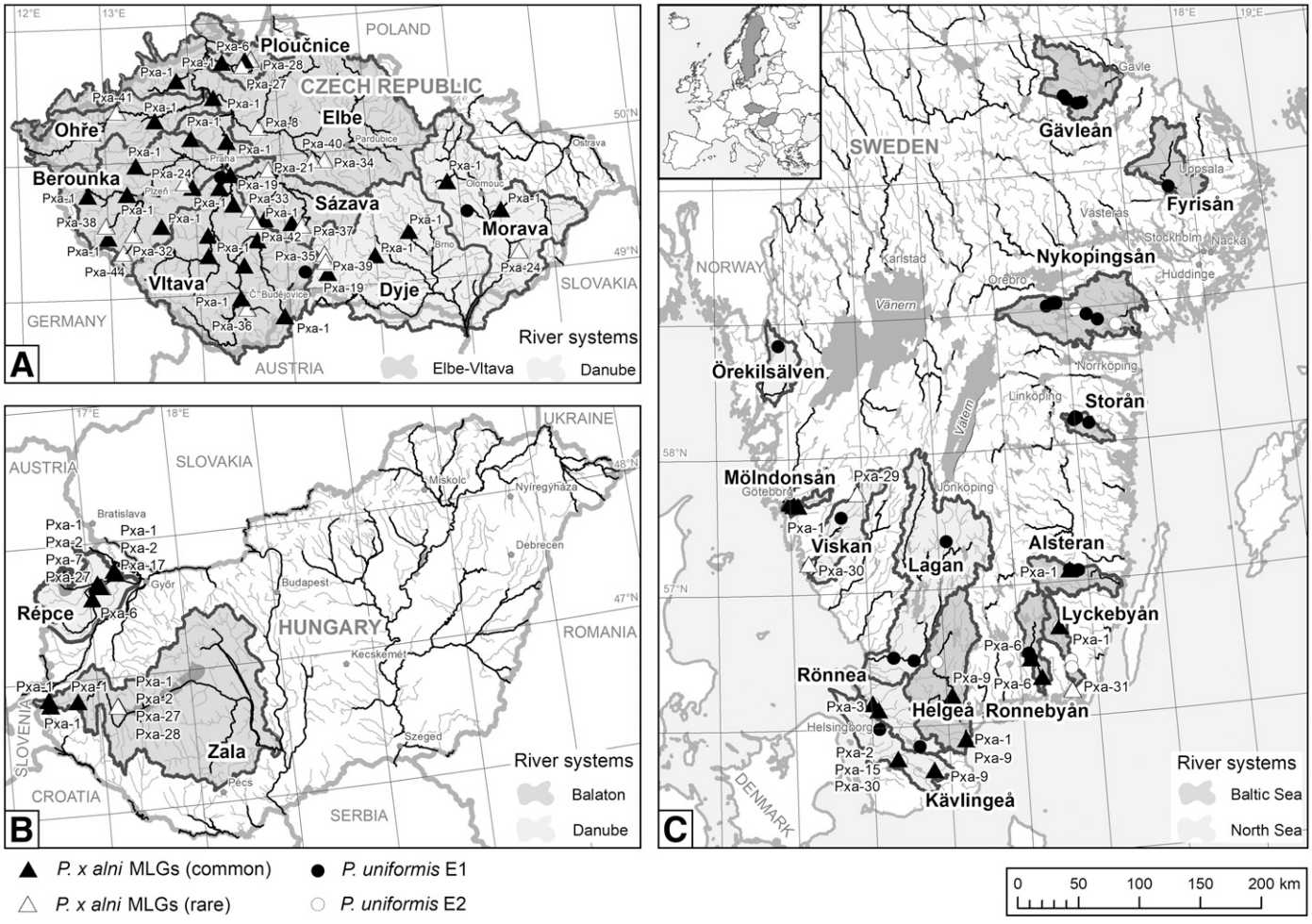

Fig. 1. Main water systems in the **A,** Czech Republic, **B,** Hungary, and **C,** Sweden from which *Phytophthora* × *alni* and *P. uniformis* samples were genotyped in this study and geographic origin (including water basin) of the samples. Common *P.* × *alni* multilocus genotypes (MLGs) refers to MLGs with more than two isolates and rare *P.* × *alni* MLGs to MLGs with one to two isolates.

Table S1). The number of MLGs observed in a country ranged from 1 (Switzerland) to 20 (Czech Republic). After a rarefaction procedure ($n = 4$, corresponding to the smallest population being analyzed), the highest genotypic richness was observed in Sweden (eMLG = 3.21 ± 0.673). Genotypic diversity assessed with the corrected Simpson's index showed the highest values in Sweden, Austria, and the Czech Republic and the lowest in Switzerland and Lithuania (Table 1). Overall evenness of *P.* × *alni* MLGs across the seven European countries was low ($E_5 = 0.32$). However, considerable differences in the E_5 values were observed among countries (Table 1). Genotypic diversity in *P.* × *alni* populations showed a significant ($P = 0.019$) positive trend with increasing age of the population (Fig. 2A), whereas the incidence of Pxa-1 decreased significantly ($P = 0.007$) over time (Fig. 2B).

The MSN based on Bruvo's genetic distance showed four branches of MLGs starting from the dominant Pxa-1 (Fig. 3). Two branches included mainly MLGs from Austria and the Czech Republic, and two branches comprised MLGs mainly from Sweden. Noteworthy, Swedish MLGs grouped according to their geographic origin, with those from Eastern Sweden (Pxa-6, Pxa-9, and Pxa-31) on one branch and those from Western Sweden (Pxa-3, Pxa-15, Pxa-29, and Pxa-30) on the other branch.

P. uniformis. The 85 *P. uniformis* isolates that could be successfully genotyped belonged to the MLGs Pu-E1 (64 isolates) and Pu-E2 (21 isolates) that were previously described by Aguayo et al. (2013). Pu-E1 was found in all four countries from which *P. uniformis* isolates were genotyped, whereas Pu-E2 was only found in Sweden in the rivers Helgeå, Kävlingeå, Lagan,

TABLE 2. Allelic patterns at the 10 microsatellite loci of the 19 rare (i.e., including only one to four isolates) *Phytophthora* × *alni* multilocus genotypes (MLGs) newly detected in this study

Rare MLG	Country[a]	Closest MLG[b]	N	Microsatellite loci[c]										Allele loss[d]	Subgenome[e]
				M-PAU3	M-PAU9	M-PAU11	M-PAU14	M-PAU15	PA17	PA23	M-PAU32	M-PAU56	M-PAU72		
Pxa-27	HU, CZ	Pxa-1	4	–	–	–	–	95/95 (~~92~~/95)	–	–	–	–	–	1	*P.* × *multiformis*
Pxa-28	HU, CZ	Pxa-1	2	–	–	–	–	–	–	157/157 (157/~~171~~)	–	–	–	1	*P.* × *multiformis*
Pxa-29	SW	Pxa-30	1	–	–	–	84/89 (84/89/~~99~~)	–	–	–	–	–	–	1	*P.* × *multiformis*
Pxa-30	SW	Pxa-2	2	–	–	–	–	–	–	–	–	91/98 (91/~~94~~/98)	162/174 (162/174/~~177~~)	2	*P.* × *multiformis*
Pxa-31	SW	Pxa-6	2	–	–	–	89/89 (~~84~~/89)	–	–	–	–	–	–	1	*P.* × *multiformis*
Pxa-32	CZ	Pxa-1	1	–	–	–	–	92/92 (92/~~95~~)	–	–	–	–	–	1	*P. uniformis*
Pxa-33	CZ	Pxa-8	1	–	–	–	–	–	–	–	107/107 (107/~~113~~)	–	–	1	*P. uniformis*
Pxa-34	CZ	Pxa-6	1	133/133 (127/127)	–	209/209 (~~173~~/209)	–	–	–	–	–	–	162/174 (162/174/~~177~~)	2	*P.* × *multiformis* and *P. uniformis*
Pxa-35	CZ	Pxa-1	1	–	–	–	–	–	–	171/171 (~~157~~/171)	–	–	–	1	*P.* × *multiformis*
Pxa-36	CZ	Pxa-1	1	–	–	–	–	–	–	–	113/113 (~~107~~/113)	–	–	1	*P.* × *multiformis*
Pxa-37	CZ	Pxa-33	1	–	–	–	–	95/95 (~~92~~/95)	–	–	–	–	–	1	*P.* × *multiformis*
Pxa-38	CZ	Pxa-7	1	–	–	–	–	–	–	–	–	–	162/162 (162/~~177~~)	1	*P.* × *multiformis*
Pxa-39	CZ	Pxa-21	1	–	–	–	–	–	–	–	–	91/94 (91/94/~~98~~)	162/177) (162/~~174~~/177)	2	*P.* × *multiformis*
Pxa-40	CZ	Pxa-7	1	–	–	–	–	95/95 (~~92~~/95)	–	–	–	–	–	1	*P.* × *multiformis*
Pxa-41	CZ	Pxa-27	1	–	–	–	–	–	–	–	–	–	162/174 (162/174/~~177~~)	1	*P.* × *multiformis*
Pxa-42	CZ	Pxa-33	1	–	–	173/173 (173/~~209~~)	–	–	–	–	–	–	–	1	*P.* × *multiformis*
Pxa-43	LT	Pxa-6	1	–	–	–	–	–	–	–	–	91/91 (91/~~94~~/~~98~~)	–	2	*P.* × *multiformis*
Pxa-44	CZ	Pxa-38	1	–	–	–	89/89 (~~84~~/89/99)	95/95 (~~92~~/95)	–	–	113/113 (~~107~~/113)	91/91 (91/~~94~~)	–	5	*P.* × *multiformis*
Pxa-45	AU	Pxa-1	1	–	–	209/209 (~~173~~/209)	–	–	–	–	–	–	–	1	*P. uniformis*
Total allele loss			–	0	0	3	4	5	0	2	3	5	5	27	–

[a] AU, Austria; CZ, Czech Republic; HU, Hungary; LT, Lithuania; and SW, Sweden.
[b] According to the minimum spanning network based on Bruvo's genetic distance (Bruvo et al. 2004).
[c] For the locus/loci where the rare MLG differ from the genetically closest MLG, alleles are given (strikethrough: allele(s) lost in the rare MLG).
[d] Number of alleles lost compared with the genetically closest MLG.
[e] Subgenome from which the alleles were lost.

Lyckebyån, and Nykopingsån (Fig. 1C) with an overall incidence of 31.3% (Table 1). The presence of Pu-E2 increased in southern and warmer areas of Sweden (latitude $P = 0.003$; February temperature $P = 0.0016$), whereas Pu-E1 was widespread across the country. There was no clear association between river basin and frequency of the two *P. uniformis* MLGs.

Allelic patterns in rare *P.* × *alni* MLGs. Nineteen out of the 31 MLGs of *P.* × *alni* detected in this study were represented by one to four isolates and were not reported previously by Aguayo et al. (2016) (Table 2). The highest incidence of these MLGs was observed in the Czech Republic (14 MLGs), whereas in the other four countries they were rather scarce. Allele patterns at the 10 microsatellite loci showed that these MLGs were characterized by a loss of alleles at one to four loci compared with their genetically closest MLG (Table 2). In total, 27 alleles were lost at seven loci, with most MLGs (14 out of 19) lacking one allele at one locus. The

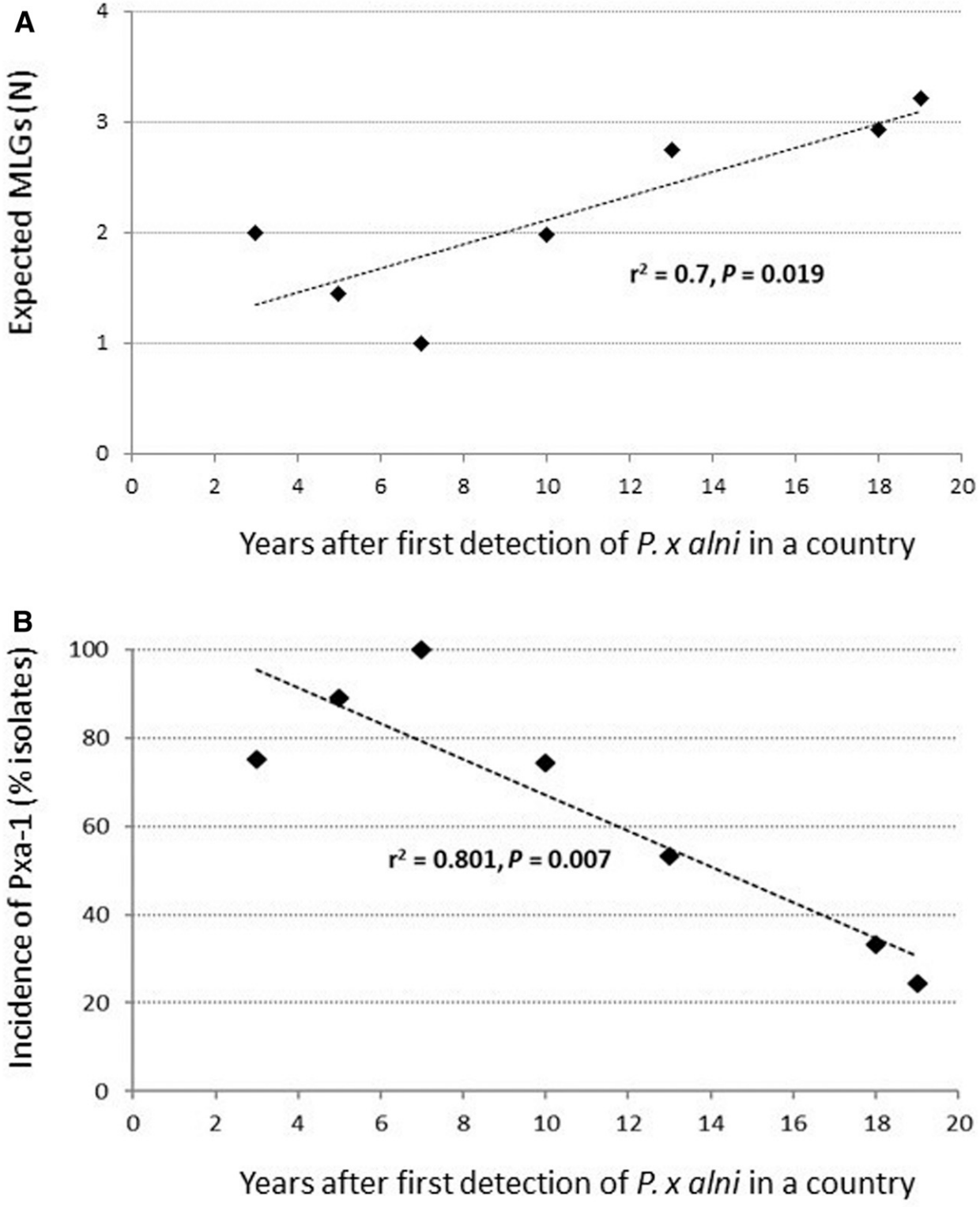

Fig. 2. Relationship between **A,** the number of expected multilocus genotypes (eMLGs) and **B,** the incidence (% isolates) of Pxa-1 in *Phytophthora* × *alni* populations and the age of the populations (i.e., years between first isolation of the pathogen in a country and sampling for this study).

highest number of missing alleles (five) was observed at loci M-PAU15, M-PAU56, and M-PAU72, whereas at loci M-PAU3, M-PAU9, and PA17 no alleles were lost.

Since Aguayo et al. (2016) previously assigned *P.* × *alni* alleles to the corresponding *P.* × *multiformis* or *P. uniformis* subgenomes, we could link allele loss to the specific parental subgenome. Fifteen new MLGs formed after allele loss in the *P.* × *multiformis* subgenome, three MLGs after allele loss in the *P. uniformis* subgenome and one MLG (Pxa-34) after allele loss in both subgenomes (Table 2). In Hungarian, Lithuanian, and Swedish *P.* × *alni* populations, alleles were lost only in the *P.* × *multiformis* subgenome. MLGs with allele loss in the *P. uniformis* subgenome were found in Austria and the Czech Republic, whereas the only MLG with allele loss in both subgenomes originated from the Czech Republic. The dominant genotype Pxa-1 had the highest number of genetically close rare MLGs (Table 2): four MLGs formed after allele loss in its *P.* × *multiformis* subgenome and two MLGs after allele loss in its *P. uniformis* subgenome.

Population differentiation. The total number of *P.* × *alni* MLGs detected in single river basins in the Czech Republic, Hungary, and Sweden ranged from one to five (Table 3). The widespread MLG Pxa-1 was present in 14 out of 18 basins with an incidence ranging from 16% (Helgeå, Sweden) to 100% (Mölndalsån and Alsteran, Sweden). This MLG was not found in three basins (Kävlingeå, Ronneå, Viskan) of the North Sea river system and one basin (Ronnebyån) of the Baltic Sea river system in Sweden. All river basins except Viskan harbored one to four previously described MLGs. Moreover, in 12 out of 18 basins, one to three new MLGs were identified. These were particularly frequent in Czech river basins, whereas they were rarer in Hungarian and Swedish basins (Table 3).

DAPC revealed a partial grouping of rare MLGs according to the country of origin (Fig. 4). The first axis discriminated between MLGs from Sweden and from the other two countries (Hungary and Czech Republic). The second axis separated the Swedish MLGs in two groups, one including MLGs found in the Baltic Sea river system (Eastern Sweden) and one with MLGs of the North Sea river system (Western Sweden). AMOVA showed that the component "among river systems" significantly accounted for 11.1% of the genetic variation associated with microsatellites (Table 4).

DISCUSSION

Our analyses revealed an overall higher genotypic diversity in *P.* × *alni* than in *P. uniformis*, confirming previous results by Aguayo et al. (2013, 2016). The 85 successfully genotyped *P. uniformis* isolates belonged to two MLGs (Pu-E1 and Pu-E2), one of which (Pu-E2) was only found in the southernmost rivers of Sweden. In the previous study by Aguayo et al. (2013), this particular MLG was detected one time each in France, Italy, and Sweden. However, as only two Swedish *P. uniformis* isolates were genotyped at that time, it is not possible to draw conclusions about a possible increase in the incidence of Pu-E2 in Sweden. Since *P. uniformis* seems to be well adapted to cold winter conditions (Redondo et al. 2015), it was previously hypothesized that populations from cold European regions might harbor additional genetic diversity (Aguayo et al. 2016). Our analyses of 67 Swedish *P. uniformis* isolates did not reveal any new alleles and suggest that *P. uniformis* populations in Northern Europe are not more diverse than those in warmer parts of the continent.

The overall *P.* × *alni* population across the seven European countries considered in this study showed a low genotypic diversity,

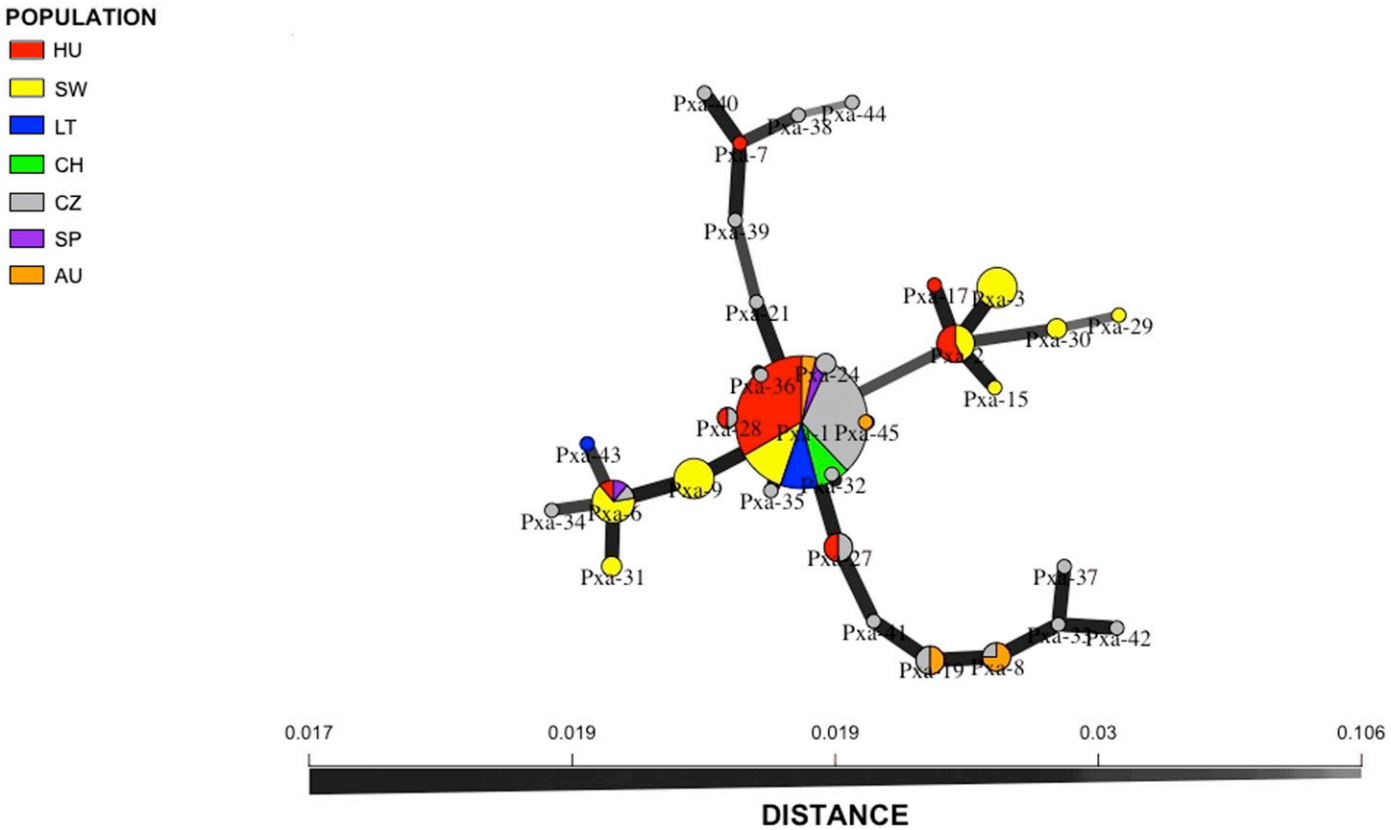

Fig. 3. Minimum spanning network based on Bruvo's genetic distance (Bruvo et al. 2004) of the 31 multilocus genotypes (MLGs) of *Phytophthora* × *alni* detected in this study. Each node represents a different MLG, and node size is proportional to the abundance of the specific MLG. HU, Hungary; SW, Sweden; LT, Lithuania; CH, Switzerland; CZ, Czech Republic; SP, Spain; and AU, Austria.

with one single MLG (Pxa-1) including more than half of the isolates. This MLG was particularly frequent in Lithuania and Switzerland (88.9 and 100% of the isolates, respectively). Since the *P.* × *alni* isolates from these two countries originated from three distinct and spatially well delimited populations (one in Switzerland and two in Lithuania), Pxa-1 was most likely the founder MLG. A strong dominance (in total 80% of the isolates) of Pxa-1 was previously reported by Aguayo et al. (2016) in mainly French and Hungarian populations. The same study also revealed a decline of clonal richness over time with an increased frequency of the dominant MLG. Our analyses show rather an increase in the number of expected (i.e., assuming equal population sizes) MLGs and at the same time a decrease in the incidence of Pxa-1 over time. Indeed, the highest genotypic diversity was detected in Sweden and Austria, the two countries among those included in our study with the longest time period between the first official isolation of *P.* × *alni* (1996 in both countries) (Brasier et al. 2004; Redondo et al. 2015) and the year when sampling was conducted. However, this result should be considered with caution: First, a variable time lag may exist between the year of official detection of an invasive pathogen and the year of introduction of this pathogen. Second, the sampling design varied considerably among countries (e.g., in some countries samples originated from a few small populations and in other countries from a widespread monitoring). And third, the structure of local *P.* × *alni* populations may be influenced by several factors, including landscape structure (in particular, connectivity between river systems) and type and intensity of management of the infected stands. In the future, it would be interesting to determine whether the pattern of decreasing incidence of the initial founder MLG persists or it may be reversed by, e.g., a reduced fitness of the new clonal MLGs.

TABLE 3. Incidence of previously described and new multilocus genotypes (MLGs) of *Phytophthora* × *alni* in the different river systems and river basins in the Czech Republic, Hungary, and Sweden

Country	River system	River basin	N	MLGs			Pxa-1 (%)
				Previously described[a]	New	Total	
Czech Republic	Danube	Dyje	6	2	3	5	2 (33.3)
		Morava	2	2	0	2	2 (66.6)
	Elbe-Vltava	Elbe	4	2	2	4	1 (25.0)
		Ploučnice	3	2	1	3	1 (33.3)
		Ohře	4	1	2	3	2 (50.0)
		Sázava	5	3	1	4	1 (20.0)
		Berounka	11	2	3	5	7 (63.6)
		Vltava	13	1	3	4	10 (76.9)
Hungary	Danube	Repce	16	4	1	5	10 (62.5)
	Balaton	Zala	23	3	2	5	19 (82.6)
Sweden	Flowing to Baltic Sea	Alsteran	1	1	0	1	1 (100)
		Helgeå	6	2	0	2	1 (16.0)
		Lyckebyån	3	1	1	2	1 (33.3)
		Ronnebyån	6	1	0	1	0
	Flowing to North Sea	Kävlingeå	8	3	1	4	0
		Mölndalsån	7	1	0	1	7 (100)
		Ronneå	8	1	0	1	0
		Viskan	2	0	2	2	0

[a] MLGs previously described by Aguayo et al. (2016).

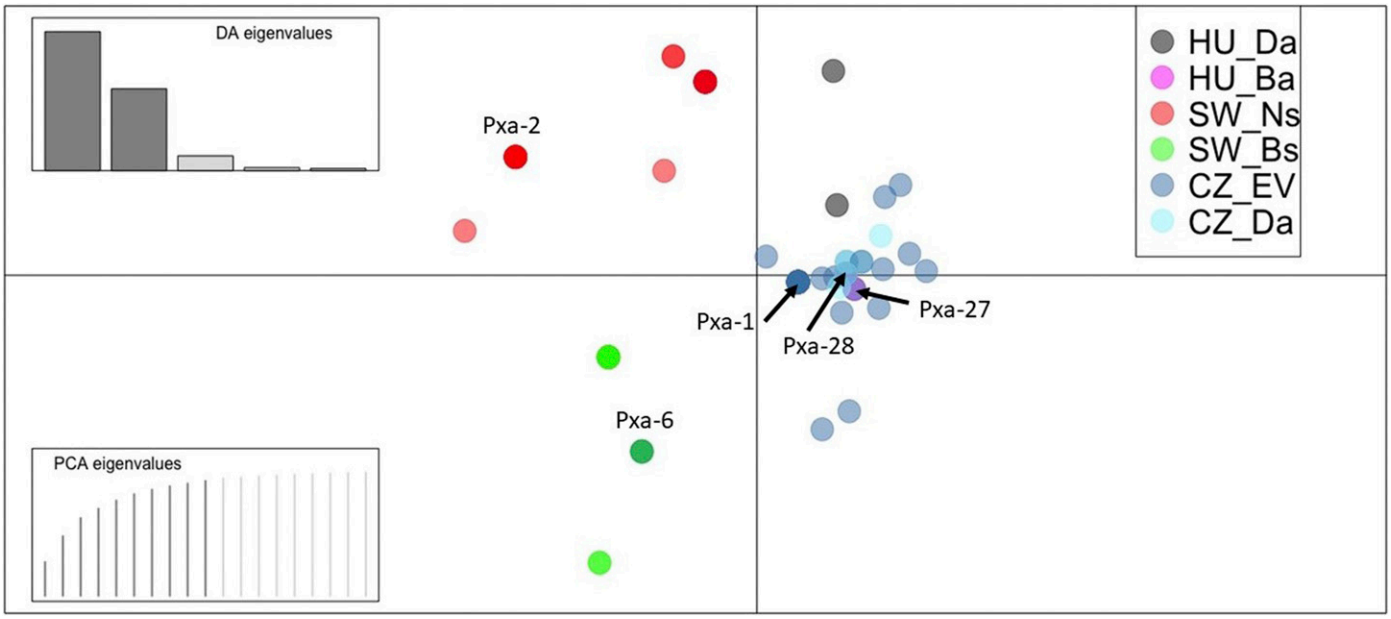

Fig. 4. Discriminant analysis of principal components (DAPC) of *Phytophthora* × *alni* in river systems in the Czech Republic (CZ), Hungary (HU), and Sweden (SW). Each circle represents a multilocus genotype (MLG). HU_Da, Hungary Danube; HU_Ba, Hungary Balaton; SW_Ns, Sweden North Sea; SW_Bs, Sweden Baltic Sea, CZ_EV, Czech Republic Elbe-Vltava; and CZ_Da, Czech Republic Danube. The names of the five MLGs present in more than one country included in the analyses are given (the particular colors of the corresponding dots result from the overlap of different country colors).

Out of the 31 MLGs of *P.* × *alni* detected in our study, 19 were new, i.e., previously undescribed. These were found in all countries except Switzerland and Spain, and only two of them occurred in more than one country (Pxa-27 and Pxa-28 in the Czech Republic and Hungary). Unlike MLGs, no new alleles were detected at the microsatellite loci compared with the previous genotyping conducted by Aguayo et al. (2016). This suggests that mutations leading to new allelic variants in *P.* × *alni* occur rarely. All new MLGs seem to have arisen from another MLG through allele loss at one or more loci. In 18 cases, the allele loss resulted in a homozygous microsatellite locus, i.e., in a loss of heterozygosity (LOH). Fifteen MLGs formed after allele loss in the *P.* × *multiformis* subgenome, three MLGs in the *P. uniformis* subgenome, and one MLG in both subgenomes. Given that *P.* × *multiformis* is itself a tetraploid hybrid (Husson et al. 2015), its subgenome in *P.* × *alni* may be less stable than the subgenome of the nonhybrid species *P. uniformis*, which could explain the more frequent loss of alleles. Hybrid organisms combine genetic material of the two parents, which implies a certain degree of genetic divergence between homeologous chromosomes (i.e., paralogous chromosomes merged within a single nucleus) (Albertin and Marullo 2012; Mixão and Gabaldón 2018). Since high levels of heterozygosity may have negative effects on cell functioning, natural selection following hybrid formation will foster mechanisms such as LOH to promote genome stabilization (Louis et al. 2012). However, alleles can also be lost because of random evolutionary processes (e.g., genetic drift) influencing genetic variation in populations. Usually, consequences of such processes are more pronounced in small, isolated populations than in large, interconnected ones.

LOH is not a peculiarity of hybrids, but occurs also in nonhybrid species. In nonhybrid *Phytophthora* species, it was already observed using microsatellite or SNP markers in *P. cinnamomi* (Dobrowolski et al. 2003), *P. capsici* (Hu et al. 2013; Lamour et al. 2012), and *P. ramorum* (Dale et al. 2019; Kasuga et al. 2016). Dobrowolski et al. (2003) suggested that LOH is a consequence of mitotic crossing over (or recombination), i.e., a reciprocal crossover between homolog chromosomes with heterozygous alleles for a specific marker that can produce daughter cells that are homozygous for the same marker (Lee et al. 2009). According to the same authors, mitotic recombination may have some evolutionary advantage, including purging deleterious mutations and generating genetic variation within asexual lineages. In *P. capsici*, indirect evidence indicated that LOH may be associated with changes in two phenotypic traits, namely virulence and mating type (Lamour et al. 2012). Kasuga et al. (2016) showed that atypical *P. ramorum* phenotypes recovered from trunk cankers on oaks (*Quercus* sp.) in California were characterized by genomic alterations including partial aneuploidy and copy-neutral LOH. The authors hypothesized that the specific chemical environment of the bark of oaks (particularly the presence of phenolic compounds) may account for these abnormalities in the genome of the exposed *P. ramorum* strains. A change from triploidy to diploidy was also observed in *P. infestans* after exposure to artificial stress conditions (Li et al. 2017). In *Phytophthora* species, LOH may, thus, be an important mechanism driving clonal diversification and promoting rapid adaptation to stress conditions given by, e.g., changing environmental conditions, resistant host genotypes, or fungicides, by reducing the time to fix beneficial recessive alleles in a population (Dale et al. 2019). The high incidence of LOH in specific *P.* × *alni* populations might thus suggest the local presence of stress factors for this species. Since *P.* × *alni* is a pathogen with optimal growth temperatures above 22°C (Brasier et al. 1995; Haque et al. 2015), one of these factors may be the winter temperature in soil, water, and bark. In this regard, a study conducted by Černý and Strnadová (2012) in the Czech Republic, the country in this study with the highest incidence of LOH events, showed that the winter survival of *P.* × *alni* in necrotic bark tissue of black alder (*Alnus glutinosa*) is very limited. Soil properties, in particular pH (Aguayo et al. 2014; Haque et al. 2015), might also be potential sources of stress. As observed by Kasuga et al. (2016) in *P. ramorum* and oaks, the chemical composition of alder bark may also potentially induce genomic changes in *P.* × *alni*. However, to definitively conclude that LOH in *P.* × *alni* is due to selective evolutionary forces, we should determine whether the microsatellite loci considered in this study are linked with specific loci under selection. Indeed, alleles could also disappear because of random genetic drift in small, fragmented populations (see for plants, Young et al. 1996), with no effect on fitness of *P.* × *alni*. This fragmentation in the *P.* × *alni* population may be a consequence of the naturally scattered distribution of the host trees (*Alnus* sp.). The pathogen itself may exacerbate this situation by killing the infected alders, thereby increasing host patchiness. The same effect can result from an intensive management of the damaged stands, especially when alders are replaced with other tree species.

Population differentiation analyses revealed some geographic structure within the overall *P.* × *alni* population in the Czech Republic, Hungary, and Sweden. In particular, the patterns of observed genetic diversity seem to reflect the physical and, thus, genetic isolation of populations in discrete river systems. Aguayo et al. (2016) showed how the population structure of *P.* × *alni* was shaped by different hybridization events that took place in several European areas and produced the dominant MLG Pxa-1. Our analyses suggest a river-specific population differentiation after colonization by the initial MLG(s). Particularly intriguing is the clear separation visible in the DAPC between *P.* × *alni* MLGs from Eastern Sweden (Baltic Sea river system—rivers flowing into the Baltic Sea), Western Sweden (Skagerrak and Kattegat river system—rivers flowing into the North Sea), and central Europe (Czech Republic and Hungary). This might suggest that different MLGs founded the *P.* × *alni* populations in central Europe (most likely Pxa-1) and Eastern and Western Sweden.

In conclusion, our study indicates that the *P.* × *alni* populations in Austria, the Czech Republic, Lithuania, Sweden, and Switzerland show basically the same diversity pattern as that reported by Aguayo et al. (2016) for other European populations. This pattern consists in the predominance, at different frequencies, of MLG Pxa-1 and the occurrence with a variable number of rare MLGs. Most of the rare MLGs show an LOH at one or a few microsatellite loci compared with other closely related MLGs. This LOH may allow a stabilization within the subgenome of the hybrid parental species

TABLE 4. Analysis of molecular variance assessing the relative contribution of within river basins, among river basins within one river system, and among river systems components to the observed genetic variability in the overall *Phytophthora* × *alni* population in the Czech Republic, Hungary, and Sweden

Source of variation	df[a]	SSD[b]	MSD[c]	Sigma[d]	%	*P* value[e]
Within river basins	36	6.207	0.172	0.172	86.60	0.064
Among river basins within one river system	12	2.019	0.168	–0.001	–0.71	0.644
Among river systems	4	1.741	0.435	0.028	11.12	0.029

[a] df, degree of freedom.
[b] SSD, sum of squared deviations.
[c] MSD, mean squared deviations.
[d] Sigma, variance component estimate.
[e] *P* value, probability value of significance based on 999 permutations.

P. × *multiformis* or a rapid adaptation to stress situations. Alternatively, alleles may be lost because of random genetic drift in isolated populations, without effect on the fitness of *P.* × *alni*. In any case, in *P.* × *alni* degradation of the triploid genome generates new MLGs. This gives the false impression of increased genetic diversity, although in reality, allelic variants in the population get lost. The spatial genetic structure of local *P.* × *alni* populations seems to reflect the physical isolation of river systems. Additional studies will be necessary to confirm this pattern of population diversification and to better understand the factors driving it.

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