Patterns of genetic diversification in the invasive hybrid plant pathogen *Phytophthora* x*alni* and its parental species *P. uniformis*

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Abstract (150-250 words)

Interspecific hybridization is increasingly recognized to play an important role in the evolution of fungi and oomycetes. In pathogens, this process 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.* x*alni*, which originated in Europe from the hybridization of *P. uniformis* and *P.* x*multiformis*. Here, we investigated the population structure of *P.* x*alni* (158 isolates) and *P. uniformis* (87 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. xalni* populations dominated by MLG Pxa-1. Twenty-four out of the 36 *P.* x*alni* MLGs detected were new and restricted to specific countries. Most of them showed a loss of heterozygosity (LOH) at one or a few microsatellite loci compared to other MLGs. This LOH may allow a stabilization within the *P.* x*alni* genome or a rapid adaptation to stress situations. Alternatively, alleles may be lost because of random genetic drift in small, isolated populations. The genetic structure of *P.* x*alni* populations in the Czech Republic, Hungary and Sweden seem to be shaped by river systems. Additional studies would be necessary to confirm these patterns of population diversification and to better understand the factors driving it.

Key-words: oomycetes, hybridization, biological invasion, loss of heterozygosity, river systems, genetic differentiation

Introduction

In fungi (kingdom Mycota) and fungus-like oomycetes (kingdom Stramenopila), hybridization can be defined as the process of genome fusion between non-conspecific individuals, which occurs both sexually and asexually and generates offspring of mixed ancestry [1–3]. 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 [4, 5]. Hybridization plays an important role in the evolution of plant pathogenic fungi and oomycetes [6–8], 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) [7, 9–11].

Until the 1990s, only a few hybrid plant pathogens, mainly causing diseases to crops, were known [12], such as the *Tilletia caries* x *T. leavis* hybrid [13] or the *Ustilago hordei* x *U. nuda* hybrid [14]. At that time it was mainly thought that fungal hybrids were relatively rare in nature [15]. Successively, thanks to the development and implementation of new DNA-based techniques [16], it has become evident that hybridization events and hybrid species formation are more frequent than previously supposed [2, 7]. To date, various molecular markers are used to characterize hybrids and their populations, including simple sequence repeats (SSRs) [17], single nucleotide polymorphism (SNPs), and mitochondrial DNA (mtDNA) [18].

Hybridization success strongly depends on the type of isolation (i.e. geographical or reproductive) between the involved species. In sympatric fungal species (i.e. co-existing in the same geographic area), interspecific hybridization is extremely difficult because of the presence of reinforced reproductive barriers [3]. Hence, hybridization is more likely to occur between geographically isolated (i.e. allopatric), but not necessarily reproductively isolated, species [5, 11]. In recent years, international plant trade has led to numerous introductions of

microorganisms to new geographic areas [19–21], thereby considerably increasing the opportunities for new interspecific hybridization events to occur. Because of this ample movement of species around the globe, it is not uncommon that for hybrids no information exists about their parental species. A well-known example is the oilseed rape pathogen *Verticillium longisporum,* whose parental species are of unknown origin [22, 23].

Phytophthora is a cosmopolitan genus of oomycetes, mainly containing obligate plant pathogens that cause damage in both forest and agricultural systems [24]. Recent studies showed that interspecific hybridization can occur between *Phytophthora* species [8, 25, 26]. 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* ITS-clades [18], including clades 1 (e.g [27, 28]), 6 (e.g. [18, 29]), 7 (e.g.[30]), and 8 (e.g. [25]). Thus, in the genus *Phytophthora* interspecific hybridization is increasingly considered as an important process for the generation of new species [18, 31].

In the early 1990s, a sudden alder (*Alnus* spp.) decline was observed in the UK [32] and later on in several other European regions [30]. The declining trees (mainly *A. incana* and *A. glutinosa*) showed a sparse crown and bleeding lesions on the root collar and stem [33]. This new lethal disease was attributed to *Phytophthora alni 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 [30]. Successively, the three variants were considered as three subspecies, i.e. *P. alni* ssp. *alni, P. alni* ssp. *multiformis*, and *P. alni* ssp. *uniformis* [30], and recently described as species [34]. Brasier *et al.* [35] 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.* x*alni* are *P.* x*multiformis* and *P. uniformis* [34, 36]. *P.* x*multiformis* is itself a tetraploid hybrid species which origin is still unknown [34, 37]. On the other side, *P.*

uniformis is a diploid species, which was possibly introduced to Europe from North America [38]. Because of its frost tolerance, this species is more frequently found in the northern parts of Europe [39], although it has been also reported in the south of the continent [40].

In a previous study, Aguayo *et al*. [37] evidenced a low polymorphism in *P.* x*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.* x*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.* x*alni* population.

In this study, we aimed at determining the genetic population structure of *P.* x*alni* and *P. uniformis* in four European countries that were not analyzed by Aguayo et al. [37], i.e. Austria, Czech Republic, Lithuania, and Switzerland, and in three previously analyzed countries (Hungary, Spain and Sweden). Specifically, we addressed the following questions: (1) Do *P.* x*alni* populations in Austria, Czech Republic, Lithuania, and Switzerland show the same diversity pattern reported by Aguayo et al. [37] for other European countries?; (2) Given that *P.* x*alni* spreads locally through riverine networks [41], are populations of this pathogen genetically structured by the river systems?; And (3) Is the population structure of the more frost tolerant parental species *P. uniformis* [39] in Sweden similar to that in central Europe?

MATERIALS AND METHODS

Phytophthora x*alni* and *P. uniformis* isolates

In this study, a total of 168 *P. x*alni and 90 *P. uniformis* isolates from 7 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 WSL, at Lithuanian State Research Institute Nature Research Centre (NRC) and from 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 as a quarantine organism subjects 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. x*alni and *P. uniformis* cultures: i) isolates from Austria, Czech Republic, Lithuania, Spain and Switzerland were grown on liquid-clarified V8 juice medium [42] for 5-7 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 [43]; ii) isolates from Sweden were grown on V8 agar for 7 days and DNA was extracted from mycelia harvested from the active growing margin of the cultures using NucleoSpin® Plant II 118 (Macherey-Nagel, Hoerdt, France)[39]; and iii) isolates from Hungary were grown in pea-broth [24] for 7–10 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 [44].

Phytophthora species identification

Prior to genotyping, all isolates were identified to species. Samples from Austria, Czech Republic [45], Hungary [46] and Sweden [39] have been identified to species before this study. *P. alni* s.l. isolates obtained from Lithuania, Spain and Switzerland were identified using the set-specific primers described by Ioos *et al*. [47], followed by sequencing of the ribosomal internal transcribed spacer (ITS) using ITS-6 [16] and ITS-4 [48] primer set. PCR amplification and sequencing were done as previously described by Schoebel *et al*. [43]. 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. x*alni* and *P. uniformis* isolates were genotyped at the ten microsatellite loci PA17, PA23 [49], PAU3, PAU9, PAU32 [38], PAU11, PAU14, PAU15, PAU56, and PAU72 [37] using the single-tube nested PCR method developed by Schuelke [50]. This method implied the labeling of the forward primers with a M13-F700 tag (5'-CACGACGTTGTAAAACGAC-3'). PCR reactions were conducted using the Type-it Microsatellite PCR Kit (Qiagen, Valencia, CA, USA), following a modified manufacturer's protocol. Modifications included the use (i) of less forward primer than reverse primer (0.1 µM and 0.2 µM, respectively; Schuelke [50]), and (ii) of only 3 µl of Master Mix per reaction [43, 51]. 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 [43]. PCR amplifications were performed on Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA). PCR products were run on ABI 3130 DNA Analyser with the GeneScan™ 500 LIZ® Size Standard for fragment analysis. All alleles were scored using the software GeneMapper v. 3.7 (Applied Biosystems).

Population diversity analyses

P. xalni and *P. uniformis* isolates were assigned to multilocus genotypes (MLGs) with the software GENODIVE v. 2.0b27, using the Stepwise Mutation Model (SMM) as distance index under the assumption that allele repeat length differ by ancestry. The distance threshold that can assign individuals to the same MLG was set at zero (0) to differentiate closely related MLGs [52]. Genotypic richness, diversity and evenness of *P. xalni* populations were calculated using POPPR v. 2.6.1 [53]. Genotypic richness was estimated by the number of observed (MLGs) and expected, i.e. corrected by sample size based on a rarefaction procedure (eMLGs), multilocus genotypes. To avoid distorted genotypic diversity values due to uneven sample sizes, a corrected Simpson's index (D) was calculated as $D = ((N/N-1)) * \lambda$, where N was the sample size and λ the Simpson's index [53]. Genotype evenness was evaluated using the E_5 index. This measure indicate the distribution of genotype abundance across population, where 1 shows that genotypes are equally abundant in population and 0 – population is dominated by one genotype. E_5 is less susceptible to the effect of sample size [54]. To roughly assess the evolution over time of the genotypic diversity in *P. xalni* populations, the number of eMLGs and the incidence of the in Europe prevalent MLG Pxa-1 [37] were plotted against the maximal number of years between the first official isolation of *P. xalni* in a country and the sampling for this study.

To evaluate the genetic relationships among the *P.* x*alni* MLGs, a Minimum Spanning Network (MSN) was constructed using P$_{OPP}$R and visualized using igraph ver. 1.1.2 [55]. The MSN was based on Bruvo's genetic distance [56] 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.* x*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 [41], for analyses isolates were assigned to corresponding river basins and systems (Figure 1). Discriminant analysis of principal components (DAPC) [57] was performed to describe clusters of genetically related *P.* x*alni* MLGs across Czech, Hungarian and Swedish river systems. DAPC was conducted using adegenet v.2.1.1 [58] with prior known *P.* x*alni* population information. To evaluate population differentiation in all river systems and rivers, an Analysis of Molecular Variance (AMOVA) was conducted [59] using P$_{OPP}$R v. 2.6.1. In most studies, population differentiation is measured using Wright's F-statistics [60]. However, due to its sensitivity to population size, migration and mutation rate estimates [61, 62], this statistic is not the best suited for polyploid organisms. In fact, compared to diploid organisms, polyploid organisms have higher total number of chromosome copies and thus different migration and mutation measures [63]. The Φ (phi) statistic [64] implemented in AMOVA was suggested in evaluation of polyploid organism population structure studies as a good alternative to Wright's F-statistics [63]. The total *P.* x*alni* genetic variation (in Czech Republic, Hungary and Sweden) was partitioned at three levels: i) within river basins, ii)

among river basins within one river system, iii) among river systems. Both full dataset and clone corrected data were used for calculations.

RESULTS

Population diversity

A total of 258 *P. alni* s.l. isolates (168 *P.* x*alni* isolates and 90 isolates of *P. uniformis*) from seven different European countries were genotyped at 10 microsatellite loci (Supplementary Table S1). For 245 isolates (158 isolates of *P.* x*alni* and 87 *P. uniformis* isolates) alleles were successfully amplified at all loci. In total, 23 alleles were identified in the analyzed *P.* x*alni* and *P. uniformis* populations, with one (*P.* x*alni* and *P. uniformis*) to three (*P.* x*alni*) alleles per locus. Compared to previous studies [37, 38, 49], no new alleles were found.

P. x*alni*. The 158 *P.* x*alni* isolates that were successfully genotyped at all loci belonged to 36 different MLGs, 24 of which had never been found before. The most common MLG was Pxa-1[38], 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 [37], which was found in the Czech Republic, Hungary, Spain and Sweden. All other MLGs were rare (1-9 isolates each) and mainly restricted to a specific country (Table 2, Supplementary Table S1). The number of MLGs observed in a country ranged from one (Switzerland) to 21 (Czech Republic). After a rarefaction procedure (N = 4, corresponding to the smallest population being analyzed), the highest genotypic richness was observed in Sweden (eMLG = 5.6 ± 1.0). Genotypic diversity assessed with the corrected Simpson's index showed the highest values in Austria, Spain and Sweden and the lowest in Switzerland and Lithuania (Table 1). Overall evenness of *P.* x*alni* MLGs across the seven European countries was low (E_5 = 0.3). However, considerable differences in the E_5-values were observed among countries (Table 1). MLGs in Austria, Spain and Sweden were distributed more evenly than in other countries,

whereas the most uneven distribution of MLGs was observed in the population in the Czech Republic (E_5 = 0.33). Overall, genotypic diversity in *P.* x*alni* populations showed a positive trend with increasing age of the population, whereas the incidence of Pxa-1 considerably decreased (Figure 2).

The MSN based on Bruvo's genetic distance discriminated three different groups of *P.* x*alni* MLGs (Figure 3). The first group included MLGs closely related to the widespread MLG Pxa-1. The second group was connected to Pxa-1 and consisted mainly of MLGs from Sweden. The third group also originated from Pxa-1 and included MLGs mostly from the Czech Republic and Austria.

P. uniformis. The 87 *P. uniformis* isolates belonged to the MLGs Pu-E1 (66 isolates) and Pu-E2 (21 isolates) that were previously described by Aguayo et al. [38]. 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, Lyckebyån and Nykopingsån (Figure 1) with an overall incidence of 31.3% (Table 1, Appendix xx). No significant differences (chi-square test: X^2 = 0.03; P = 0.86) were observed in the incidence of Pu-E1 and Pu-E2 in Western (i.e. river system North Sea) and Eastern (i.e. river system Baltic Sea) Sweden.

Allelic patterns in rare *Phytophthora* x*alni* MLGs

Twenty-four out of the 36 MLGs of *P.* x*alni* detected in this study were represented by 1-2 isolates (Table 2). The highest incidence of these rare genotypes was observed in the Czech Republic (16 MLGs), whereas in the other six countries they were rather scarce. Allele patterns at the 10 microsatellite loci showed that the rare MLGs were characterized by a loss of alleles at 1-3 loci compared to their genetically closest MLG (Table 2). Totally, 35 alleles were lost at 8 loci, with most MLGs (19 out of 24) lacking one allele at one locus. The

highest number of missing alleles (7) was observed at locus M-Pau 56, whereas at loci M-Pau3 and PA17 no alleles were lost. At the other loci, allele loss varied from 2 to 7.

Since Aguayo *et al*. [46] previously assigned *P*. x*alni* alleles to the corresponding *P*. x*multiformis* (*P. xm*) or *P. uniformis* (*P. u*) subgenomes, we could link allele loss to the specific parental subgenome. Eighteen rare MLGs formed after allele loss in the *P*. x*multiformis* subgenome, 4 MLGs after allele loss in the *P. uniformis* subgenome and two MLGs after allele loss in both subgenomes (Table 2). In Hungarian, Lithuanian, Spanish and Swedish *P*. x*alni* populations, alleles were lost only in the *P*. x*multiformis* subgenome. MLGs with allele loss in the *P. uniformis* subgenome were found in Austria and the Czech Republic, whereas the two MLGs 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*. x*multiformis* subgenome and one MLG after allele loss in its *P. uniformis* subgenome. Nine rare MLGs most likely originated from an allele loss in other rare MLGs.

Population differentiation

The total number of *P*. x*alni* MLGs detected in single river basins in the Czech Republic, Hungary and Sweden ranged from one to 5 (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 but Viskan, harbored one to 4 already described MLGs. Moreover, in 13 out of 17 basins one to 3 new MLGs were identified. These were particularly frequent in Czech river basins, whereas they were more rare in Hungarian and Swedish basins (Table 3).

Discriminant analysis of principal components (DAPC) revealed the presence of three distinct clusters of *P.* x*alni* MLGs across the three countries (Figure 4). The first cluster contained the majority of MLGs from all countries. The second cluster included MLGs found in the Czech river system Elbe-Vtlava and in river basins of the Baltic Sea river system (Eastern Sweden). Finally, the third cluster harbored MLGs of the North Sea river system (Western Sweden) and Hungary.

Analysis of molecular variance (AMOVA) showed that most of the genetic variation associated with microsatellites was accounted for by the within river basin component (Table 4). With the full data set this component explained 56.9% ($\sigma = 0.074$) of the variation, whereas if only one representative isolate of each MLG was considered (clone-corrected data set), up to 86.98% ($\sigma = 0.174$) of the variation. Noteworthy, the component among river basins within river systems showed a significant contribution (33%, $\sigma = 0.042$) to the total variation with non-clone-corrected data, but not with clone-corrected data (Table 4). The same situation was observed with the component among river systems which was insignificant (13.37%, $\sigma = 0.026$) with clone-corrected data (Table 4).

DISCUSSION

In this study we investigated the genetic population structure of *P.* x*alni* and *P. uniformis* in seven (*P.* x*alni*) and five (*P. uniformis*) different European countries using microsatellite markers. Our analyses revealed an overall higher genotypic diversity in *P.* x*alni* than in *P. uniformis* confirming previous results by Aguayo *et al.* [38, 46]. The 87 genotyped *P. uniformis* isolates belonged to two MLGs (Pu-E1 and Pu-E2), one of which (Pu-E2) was only found in Sweden with an incidence of 31.4%. In the previous study by Aguayo *et al.* [38], 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

conclude about a possible increase in the incidence of Pu-E2 in Sweden. In any case, our study confirms the low genetic diversity of *P. uniformis* in Europe that was previously reported by Aguayo *et al.* [46].

The overall *P. xalni* population across the seven European countries considered in this study showed a low genotypic diversity, with one single MLG (Pxa-1) including more than half (about 54%) of the isolates. This MLG was particularly frequent in Lithuania and Switzerland (88.9 and 100% of the isolates, respectively). Since the *P. xalni* 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.* [46] 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. xalni* (1996 in both countries) [30, 39] 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, and 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).

Out of the 36 MLGs of *P. xalni* detected in our study, 24 were new, i.e. previously undescribed. These were found in all countries except Switzerland and only one new MLG occurred in more than one country (Pxa-28 in Czech Republic and Hungary). Unlike MLGs,

no new alleles were detected at the microsatellite loci compared to the previous genotyping conducted by Aguayo *et al.* [46]. All new MLGs seem to have arisen from another MLG through allele loss at one or more loci. In 24 cases, the allele loss resulted in a homozygous microsatellite locus, i.e. in a so-called loss of heterozygosity (LOH). Twenty-four MLGs formed after allele loss in the *P.* x*multiformis* subgenome, four MLGs in the *P. uniformis* subgenome, and two MLGs in both subgenomes. Given that *P.* x*multiformis* is itself a tetraploid hybrid [34], its subgenome in *P.* x*alni* may be less stable than the subgenome of the pure 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 (i.e. paralogous chromosomes merged within a single nucleus) chromosomes [65, 66]. Since high levels of heterozygosity may have negative effects on cell functioning, following hybrid formation natural selection will foster mechanisms like LOH to promote genome stabilization [67]. However, alleles can also be lost because of random evolutionary forces (e.g. genetic drift) influencing genetic variation in populations. Usually, consequences of such forces are more pronounced in small, isolated populations than in large, interconnected ones.

LOH is not a peculiarity of hybrids, but occurs also in pure species. In pure *Phytophthora* species it was already observed using microsatellite or SNP markers in *P. cinnamomi* [68]*, P. capsici* [69, 70], and *P. ramorum* [71, 72]. Dobrowolski *et al.* [68] suggested that LOH is a consequence of mitotic crossing over (or recombination), i.e. a reciprocal crossover between homologue chromosomes with heterozygous alleles for a specific marker that can produce daughter cells that are homozygous for the same marker [73]. 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 [69]. Kasuga *et al*. [71] showed that atypical *P. ramorum* phenotypes recovered from trunk cankers on oaks (*Quercus* sp.) in California are 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. In asexually reproducing *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 [72]. The high incidence of LOH in specific *P. x*alni* populations might thus suggest the local presence of stress factors for this species. Since *P. x*alni* is a pathogen with optimal growth temperatures above 22 °C [35, 74], one of these factors may be the winter temperature in soil, water and bark. In this regard, a study conducted by Černý and Strnadová [75] in the Czech Republic, the country considered in this study with the highest incidence of LOH events, showed that the winter survival of *P. x*alni* in necrotic bark tissue of black alder (*Alnus glutinosa*) is very limited. Soil properties, in particular pH [74, 76], might also be potential sources of stress. As observed by Kasuga *et al.* [71] in *P. ramorum* and oaks, the chemical composition of alder bark may also potentially induce genomic changes in *P. x*alni*. However, to definitively conclude that LOH in *P. x*alni* is due to selective evolutionary forces, we should determine if 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 the small, fragmented populations. This fragmentation in *P. x*alni* 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. Noteworthy, although resulting in a reduced genetic variation in the population (i.e. less allelic variants), LOH produces new MLGs.

Population differentiation analyses revealed some geographic structure within the overall *P.* x*alni* population in the Czech Republic, Hungary and Sweden. In particular, the pathogen population seems to be shaped to a certain degree by the river systems. Indeed, both with the complete and the clone-corrected data set, based on AMOVA analysis most of the genetic variation resides within river systems. Aguayo *et al.* [46] showed how the population structure of *P.* x*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). This differentiation might be promoted by the lack of connections between the different populations which would considerably reduce gene flow. Particularly intriguing is the clear separation visible in the DAPC between *P.* x*alni* MLGs from Eastern (Baltic Sea river system - rivers flowing into the Baltic Sea) and Western (Skagerrak and Kattegat river system - rivers flowing into the North Sea) Sweden. An alder decline survey conducted in Bavaria (Germany) revealed that the main pathway for *P. alni* s.l. to reach new sites was through infected alder seedlings from nurseries [41]. Thus, it may be possible that different founder MLGs originating from different nurseries were initially introduced into East and West Sweden, from which the current populations have originated.

In conclusion, our study indicates that the *P.* x*alni* populations in Austria, Czech Republic, Lithuania, Sweden and Switzerland show basically the same diversity pattern as that reported by Aguayo et al. [46] 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 a LOH at one or a few microsatellite loci compared to other MLGs. This LOH may allow a stabilization within the subgenome of the

hybrid parental species *P.* x*multiformis* or a rapid adaptation to stress situations. Alternatively, alleles may be lost because of random genetic drift in isolated populations. The genetic structure of local populations seem to be shaped by river systems. Additional studies would be necessary to confirm this pattern of population diversification and to better understand the factors driving it.

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AUTHOR' CONTRIBUTIONS

SP and GN conceived the study. GN conducted the genetic and statistical analyses. GN and SP wrote the first draft of the manuscript. VZ produced the maps shown in the manuscript. All authors provided isolates and contributed to the final version of the manuscript.

REFERENCES

1. Abbott R, Albach D, Ansell S, et al (2013) Hybridization and speciation. J Evol Biol 26:229–246. https://doi.org/10.1111/j.1420-9101.2012.02599.x

2. Schardl CL, Craven KD (2003) Interspecific hybridization in plant-associated fungi and oomycetes: A review. Mol Ecol 12:2861–2873. https://doi.org/10.1046/j.1365-294X.2003.01965.x

3. Stukenbrock EH (2016) The role of hybridization in the evolution and emergence of new fungal plant pathogens. Phytopathology 106:104–112. https://doi.org/10.1094/PHYTO-08-15-0184-RVW

4. Giraud T, Refrégier G, Le Gac M, et al (2008) Speciation in fungi. Fungal Genet Biol 45:791–802. https://doi.org/10.1016/j.fgb.2008.02.001

5. Restrepo S, Tabima JF, Mideros MF, et al (2014) Speciation in fungal and oomycete plant pathogens. Annu Rev Phytopathol 52:289–316. https://doi.org/10.1146/annurev-phyto-102313-050056

6. Arnold ML (2004) Natural hybridization and the evolution of domesticated, pest and disease organisms. Mol Ecol 13:997–1007. https://doi.org/10.1111/j.1365-294X.2004.02145.x

7. Gibson AK, Refrégier G, Hood ME, Giraud T (2014) Performance of a hybrid fungal pathogen on pure-species and hybrid host plants. Int J Plant Sci 175:724–730. https://doi.org/10.1086/676621

8. Kroon LPNM, Brouwer H, de Cock AWAM, Govers F (2012) The genus *Phytophthora* anno 2012. Phytopathology 102:348–364. https://doi.org/10.1094/PHYTO-01-11-0025

9. Arnold ML, Martin NH (2010) Hybrid fitness across time and habitats. Trends Ecol Evol 25:530–536. https://doi.org/10.1016/j.tree.2010.06.005

10. Brasier CM (2000) The rise of the hybrid fungi. Nature 405:134–135. https://doi.org/10.1038/35012193

11. Stukenbrock EH, McDonald BA (2008) The origins of plant pathogens in agro-ecosystems. Annu Rev Phytopathol 46:75–100. https://doi.org/10.1146/annurev.phyto.010708.154114

12. Olson Å, Stenlid J (2002) Pathogenic fungal species hybrids infecting plants. Microb Infect 4:1353–1359. https://doi.org/10.1016/S1286-4579(02)00005-9

13. Flor HH (1932) Heterothallism and hybridization in *Tilletia tritici* and *T. levis*. J Agric Res 44:49–58

14. Fullerton RA, Nielsen I (1974) Identical genes for virulence in the smuts *Ustilago hordei* and *U. nigra*, and inheritance of virulence on the barley cultivars keystone and conquest. Can J Plant Sci 54:253–257

15. Barton NH (2001) The role of hybridization in evolution. Mol Ecol 10:551–568

16. Cooke DEL, Drenth A, Duncan JM, et al (2000) A molecular phylogeny of *Phytophthora* and related oomycetes. Fungal Genet Biol 30:17–32. https://doi.org/10.1006/fgbi.2000.1202

17. Hegarty MJ, Hiscock SJ (2005) Hybrid speciation in plants: new insights from molecular studies. New Phytol 165:411–423. https://doi.org/10.1111/j.1469-8137.2004.01253.x

18. Burgess TI (2015) Molecular characterization of natural hybrids formed between five related indigenous clade 6 *Phytophthora* species. PLoS ONE 10:1–23. https://doi.org/10.1371/journal.pone.0134225

19. Fisher MC, Henk DA, Briggs CJ, et al (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature 484:186–194. https://doi.org/10.1038/nature10947

20. Garbelotto M, Gonthier P (2013) Biology, epidemiology, and control of *Heterobasidion* species worldwide. Annu Rev Phytopathol 51:39–59. https://doi.org/10.1146/annurev-phyto-082712-102225

21. Callaghan S, Guest D (2015) Globalisation, the founder effect, hybrid *Phytophthora* species and rapid evolution: new headaches for biosecurity. Australas Plant Path 44:255–262. https://doi.org/10.1007/s13313-015-0348-5

22. Inderbitzin P, Davis RM, Bostock RM, Subbarao K V. (2011) The ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. PLoS ONE 6:564–574. https://doi.org/10.1371/journal.pone.0018260

23. Depotter JRL, Seidl MF, Wood TA, Thomma BPHJ (2016) Interspecific hybridization impacts host range and pathogenicity of filamentous microbes. Curr Opin Microbiol 32:7–13. https://doi.org/10.1016/j.mib.2016.04.005

24. Erwin DC, Ribeiro OK (1996) *Phytophthora* Disease Worldwide. APS Press, St. Paul, MN

25. Bertier L, Leus L, D'hondt L, et al (2013) Host adaptation and speciation through hybridization and polyploidy in *Phytophthora*. PLoS ONE 8:e85385. https://doi.org/10.1371/journal.pone.0085385

26. Brasier CM, Cooke DEL, Duncan JM (1999) Origin of a new *Phytophthora* pathogen through interspecific hybridization. PNAS 96:5878–5883. https://doi.org/10.1073/pnas.96.10.5878

27. Man In 'T Veld WA, De Cock AWAM, Summerbell RC (2007) Natural hybrids of resident and introduced *Phytophthora* species proliferating on multiple new hosts. Eur J Plant Pathol 117:25–33. https://doi.org/10.1007/s10658-006-9065-9

28. Goss EM, Cardenas ME, Myers K, et al (2011) The plant pathogen *Phytophthora andina* emerged via hybridization of an unknown *Phytophthora* species and the Irish Potato Famine pathogen, *P. infestans*. PLoS ONE 6:. https://doi.org/10.1371/journal.pone.0024543

29. Nagel JH, Gryzenhout M, Slippers B, et al (2013) Characterization of *Phytophthora* hybrids from ITS clade 6 associated with riparian ecosystems in South Africa and Australia. Fungal Biol 117:329–347. https://doi.org/10.1016/j.funbio.2013.03.004

30. Brasier CM, Kirk S a, Delcan J, et al (2004) *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. Mycol Res 108:1172–1184. https://doi.org/10.1017/S0953756204001005

31. Bertier L, Leus L, D'hondt L, et al (2013) Host adaptation and speciation through hybridization and polyploidy in *Phytophthora*. PLoS ONE 8:1–14. https://doi.org/10.1371/journal.pone.0085385

32. Gibbs JN (1995) *Phytophthora* root disease of alder in Britain. EPPO Bulletin 25:661–664. https://doi.org/10.1111/j.1365-2338.1995.tb01118.x

33. Gibbs JN (2003) Management and control of *Phytophthora* disease of alder. In: Gibbs JN, Van Dijk C, Webber JF (eds) Phytophthora disease of alder in Europe. Forestry Commission, Edinburgh, pp 73–78

34. Husson C, Aguayo J, Revellin C, et al (2015) Evidence for homoploid speciation in *Phytophthora alni* supports taxonomic reclassification in this species complex. Fungal Genet Biol 77:12–21. https://doi.org/10.1016/j.fgb.2015.02.013

35. Brasier CM, Rose J, Gibbs JN (1995) An unusual *Phytophthora* associated with widespread alder mortality in Britain. Plant Pathol 44:999–1007. https://doi.org/10.1111/j.1365-3059.1995.tb02658.x

36. Ioos R, Barrès B, Andrieux A, Frey P (2006) Characterization of microsatellite markers in the interspecific hybrid *Phytophthora alni* ssp. *alni*, and cross-amplification with related taxa. Mol Ecol Notes 7:133–137. https://doi.org/10.1111/j.1471-8286.2006.01554.x

37. Aguayo J, Halkett F, Husson C, Frey P (2016) Genetic diversity and origins of the homoploid-type hybrid *Phytophthora* x *alni*. Appl Environ Microbiol 82:7142–7153. https://doi.org/10.1128/AEM.02221-16.Editor

38. Aguayo J, Adams GC, Halkett F, et al (2013) Strong genetic differentiation between North American and European populations of *Phytophthora alni* subsp. *uniformis*. Phytopathology 103:190–199. https://doi.org/10.1094/PHYTO-05-12-0116-R

39. Redondo MA, Boberg J, Olsson CHB, Oliva J (2015) Winter conditions correlate with *Phytophthora alni* subspecies distribution in Southern Sweden. Phytopathology 105:1191–1197. https://doi.org/10.1094/PHYTO-01-15-0020-R

40. Varela CP, Martínez CR, Aguín Casal O, et al (2012) First Report of *Phytophthora alni* subsp. *uniformis* on Black Alder in Spain. Plant Dis 96:589–589. https://doi.org/10.1094/PDIS-10-11-0891-PDN

41. Jung T, Blaschke M (2004) *Phytophthora* root and collar rot of alders in Bavaria: Distribution, modes of spread and possible management strategies. Plant Pathol 53:197–208. https://doi.org/10.1111/j.0032-0862.2004.00957.x

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

43. Schoebel CN, Jung E, Prospero S (2013) Development of new polymorphic microsatellite markers for three closely related plant-pathogenic *Phytophthora* species using 454-pyrosequencing and their potential applications. Phytopathology 103:1020–7. https://doi.org/10.1094/PHYTO-01-13-0026-R

44. Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

45. Štěpánková P, Černý K, Strnadová V, et al (2013) Identification of *Phytophthora alni* subspecies in riparian stands in the Czech Republic. Plant Protect Sci 49:S3–S10. https://doi.org/10.17221/41/2013-PPS

46. Aguayo J, Halkett F, Husson C, et al (2016) Genetic diversity and origins of the homoploid-type hybrid *Phytophthora ×alni*. Appl Environ Microbiol 82:7142–7153. https://doi.org/10.1128/AEM.02221-16

47. Ioos R, Andrieux A, Marçais B, Frey P (2006) Genetic characterization of the natural hybrid species *Phytophthora alni* as inferred from nuclear and mitochondrial DNA analyses. Fungal Genet Biol 43:511–529. https://doi.org/10.1016/j.fgb.2006.02.006

48. White T, Burns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc., New York, pp 315–322

49. Ioos R, Barrès B, Andrieux A, Frey P (2007) Characterization of microsatellite markers in the interspecific hybrid *Phytophthora alni* ssp. *alni,* and cross-amplification with related taxa. Mol Ecol Notes 7:133–137. https://doi.org/10.1111/j.1471-8286.2006.01554.x

50. Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234. https://doi.org/10.1038/72708

51. Burokienė D, Prospero S, Jung E, et al (2015) Genetic population structure of the invasive ash dieback pathogen *Hymenoscyphus fraxineus* in its expanding range. Biol Invasions 17:2743–2756. https://doi.org/10.1007/s10530-015-0911-6

52. Meirmans PG, Van Tienderen PH (2004) genotype and genodive: two programs for the analysis of genetic diversity of asexual organisms. Mol Ecol Notes 4:792–794. https://doi.org/10.1111/j.1471-8286.2004.00770.x

53. Kamvar ZN, Tabima JF, Grünwald NJ (2014) Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2:e281. https://doi.org/10.7717/peerj.281

54. Grünwald NJ, Goodwin SB, Milgroom MG, Fry WE (2003) Analysis of genotypic diversity data for populations of microorganisms. Phytopathology 93:738–746. https://doi.org/10.1094/PHYTO.2003.93.6.738

55. Csardi G, Nepusz T (2006) The igraph software package for complex network research. InterJournal, Complex Systems 1695:9

56. Bruvo R, Michiels NK, D'Souza TG, Schulenburg H (2004) A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. Mol Ecol 13:2101–2106. https://doi.org/10.1111/j.1365-294X.2004.02209.x

57. Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet 11:94

58. Jombart T (2008) Adegenet: A R package for the multivariate analysis of genetic markers. Bioinformatics 24:1403–1405. https://doi.org/10.1093/bioinformatics/btn129

59. Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics 131:479–491. https://doi.org/10.1007/s00424-009-0730-7

60. Holsinger KE, Weir BS (2009) Genetics in geographically structured populations: defining, estimating and interpreting Fst. Nature Rev Genet 10:639–650. https://doi.org/10.1038/nrg2611.Genetics

61. Fu R, Gelfand AE, Holsinger KE (2003) Exact moment calculations for genetic models with migration, mutation, and drift. Theor Popul Biol 63:231–243. https://doi.org/10.1016/S0040-5809(03)00003-0

62. Meirmans PG, Hedrick PW (2011) Assessing population structure: FSTand related measures. Mol Ecol Resour 11:5–18. https://doi.org/10.1111/j.1755-0998.2010.02927.x

63. Meirmans PG, Liu S, Tienderen PH Van (2018) The analysis of polyploid genetic data. J Hered 283–296. https://doi.org/10.1093/jhered/esy006

64. Ronfort J, Jenczewski E, Bataillon T, Rousset F (1998) Analysis of population structure in autotetraploid species. Genetics 150:921–930

65. Albertin W, Marullo P (2012) Polyploidy in fungi: evolution after whole-genome duplication. P Roy Soc B-Biol Sci 279:2497–2509. https://doi.org/10.1098/rspb.2012.0434

66. Mixão V, Gabaldón T (2018) Hybridization and emergence of virulence in opportunistic human yeast pathogens. Yeast 35:5–20. https://doi.org/10.1002/yea.3242

67. Louis VL, Despons L, Friedrich A, et al (2012) *Pichia sorbitophila*, an interspecies yeast hybrid, reveals early steps of genome resolution after polyploidization. G3 (Bethesda) 2:299–311. https://doi.org/10.1534/g3.111.000745

68. Dobrowolski MP, Tommerup IC, Shearer BL, O'Brien P a (2003) Three clonal lineages of *Phytophthora cinnamomi* in Australia revealed by microsatellites. Phytopathology 93:695–704. https://doi.org/10.1094/PHYTO.2003.93.6.695

69. Lamour KH, Mudge J, Gobena D, et al (2012) Genome sequencing and mapping reveal loss of heterozygosity as a mechanism for rapid adaptation in the vegetable pathogen *Phytophthora capsici*. MPMI 25:1350–1360. https://doi.org/10.1094/MPMI-02-12-0028-R

70. Hu J, Diao Y, Zhou Y, et al (2013) Loss of heterozygosity drives clonal diversity of *Phytophthora capsici* in China. PLoS ONE 8:e82691. https://doi.org/10.1371/journal.pone.0082691

71. Kasuga T, Bui M, Bernhardt E, et al (2016) Host-induced aneuploidy and phenotypic diversification in the Sudden Oak Death pathogen *Phytophthora ramorum*. BMC Genomics 17:385. https://doi.org/10.1186/s12864-016-2717-z

72. Dale AL, Feau N, Everhart SE, et al (2019) Mitotic recombination and rapid genome evolution in the invasive forest pathogen *Phytophthora ramorum*. mBio 10:e02452-18. https://doi.org/10.1128/mBio.02452-18

73. Lee PS, Greenwell PW, Dominska M, et al (2009) A fine-structure map of spontaneous mitotic crossovers in the yeast *Saccharomyces cerevisiae*. PLOS Genetics 5:e1000410. https://doi.org/10.1371/journal.pgen.1000410

74. Haque MMU, Hidalgo E, Martín-García J, et al (2015) Morphological, physiological and molecular characterization of *Phytophthora alni* isolates from Western Spain. Eur J Plant Pathol 142:731–745. https://doi.org/10.1007/s10658-015-0647-2

75. Černý K, Strnadová V (2012) Winter survival of *Phytophthora alni* subsp. *alni* in aerial tissues of black alder. J For Sci 58:328–336. https://doi.org/10.17221/11/2012-JFS

76. Aguayo J, Elegbede F, Husson C, et al (2014) Modeling climate impact on an emerging disease, the *Phytophthora alni* -induced alder decline. Glob Change Biol 20:3209–3221. https://doi.org/10.1111/gcb.12601

77. Cerny K, Gregorova B, Holub V, Strnadova V (2003) First finds of "alder-*Phytophtora*" in the Czech Republic. Czech Mycology 55:291–296.

78. Szabó I, Nagy Z, Bakonyi J, Érsek T (2000) First report of *Phytophthora* root and collar rot of alder in Hungary. Plant Dis 84:1251–1251. https://doi.org/10.1094/PDIS.2000.84.11.1251A

79. Jovaišienė Z (2002) A new *Phytophthora* associated with alder mortality in Lithuania. In: Proceedings of the scientific international conference. Plant protection in the Baltic region in the context of integration to EU. Kaunas, Lithuania

80. Solla A, Pérez-Sierra A, Corcobado T, et al (2010) *Phytophthora alni* on *Alnus glutinosa* reported for the first time in Spain. Plant Pathol 59:798–798. https://doi.org/10.1111/j.1365-3059.2009.02254.x

81. Meier F, Engesser R, Forster B, et al (2009) Forstschutz-Überblick 2008. Eidgenössische Forschungsanstalt WSL, Birmensdorf, Schweiz (in German)

Table 1. Diversity of the *Phytophthora* x*alni* and *P. uniformis* populations in the seven European countries included in this study. N, sample size; MLG, number of observed multilocus genotypes; eMLG, expected number of multilocus genotypes in a population of N = 4 (i.e. smallest population being analyzed); Pxa-1, incidence of the most common MLG Pxa-1 [37]; D, modified Simpson's index; E_5, evenness index; Pu-1, MLG Pu-1 [37]; Pu-2, MLG Pu-2 [37].

					Phytophthora x*alni*				*Phytophthora uniformis*		
Country	First detection of *P. alni s.l.*[1]	Sampling year(s)	N	MLG	eMLG (± SE)	Pxa-1 (%)	D	E_5	N	Pu-1	Pu-2
Austria (AU)	1996 [30]	2007-2014	9	5	5.0 (± 0.0)	33.3	0.861	0.91	2	2	0
Czech Republic (CZ)	2001 [77]	2006-2014	49	21	5.4 (± 1.4)	54.0	0.699	0.33	7	7	0
Hungary (HU)	1999 [78]	2001-2009	39	7	3.2 (± 1.0)	74.4	0.442	0.45	0	n.a.[2]	n.a.
Lithuania (LT)	1999 [79]	2014	9	2	2.0 (± 0.0)	88.9	0.222	0.59	0	n.a.	n.a.
Spain (SP)	2009 [80]	2010-2012	4	3	3.0 (± 0.0)	50.0	0.833	0.91	12	12	0
Sweden (SW)	1996 [39]	2013-2015	41	9	5.6 (± 1.0)	24.4	0.852	0.89	67	46	21
Switzerland (CH)	2008 [81]	2015	7	1	1.0 (± 0.0)	100.0	0.00	n.a.	0	n.a.	n.a.
Total	-	-	*158*	*36*	*5.2 (± 1.4)*	*54.1*	*0.695*	*0.3*	*87*	*66*	*21*

[1]According to [30, 39, 77, 78, 79, 80, 81].

[2]n.a., non applicable.

Table 2. Allelic patterns at the 10 microsatellite loci of the 24 rare (i.e. including only 1-2 isolates) *Phytophthora* x*alni* MLGs detected in this study. For the locus/loci where the rare MLG differ from the genetically closest MLG, alleles are given (underscored: allele(s) lost in the rare MLG).

Rare MLG	Country[1]	Closest MLG	N	Microsatellite loci										Allele loss[2]	Subgenome[3]
				M-Pau3	M-PAU9	M-PAU11	M-PAU14	M-PAU15	PA17	PA23	M-PAU32	M-PAU56	M-PAU72		
Pxa-27	HU	Pxa-1	2	-	-	-	-	95 (~~92~~/95)	-	-	-	-	-	1	*P.* x*m*
Pxa-28	HU, CZ	Pxa-1	2	-	-	-	-	-	-	157 (157/~~171~~)	-	-	-	1	*P.* x*m*
Pxa-29	SW	Pxa-30	1	-	-	-	84/89 (84/89/~~99~~)	-	-	-	-	-	-	1	*P.* x*m*
Pxa-30	SW	Pxa-2	2	-	-	-	-	-	-	-	-	91/98 (91/~~94~~/98)	162/174 (162/174/~~177~~)	2	*P.* x*m*
Pxa-31	SW	Pxa-6	2	-	-	-	89 (~~84~~/89)	-	-	-	-	-	-	1	*P.* x*m*
Pxa-32	CZ	Pxa-1	1	-	-	-	-	92 (92/~~95~~)	-	-	-	-	-	1	*P. u*
Pxa-33	CZ	Pxa-8	1	-	-	-	-	-	-	-	107 (107/~~113~~)	-	-	1	*P. u*
Pxa-34	CZ	Pxa-6	1	-	-	209 (~~173~~/209)	-	-	-	-	-	-	-	1	*P. u*
Pxa-35	CZ	Pxa-1	1	-	-	-	-	-	-	171 (~~157~~/171)	-	-	-	1	*P.* x*m*
Pxa-36	CZ	Pxa-1	1	-	-	-	-	-	-	-	113 (~~107~~/113)	-	-	1	*P.* x*m*

Pxa-37	CZ	Pxa-33 / Pxa-39	1	-	-	-	-	95 (~~92~~/95), (95)	-	-	107 (107), (107/~~113~~)	-	-	1	*P.* x*m* and *P. u*
Pxa-38	CZ	Pxa-19	1	-	-	-	-	-	-	-	-	-	162 (162/~~174~~)	1	*P.* x*m*
Pxa-39	CZ	Pxa-8	1	-	-	-	-	95 (~~92~~/95)	-	-	-	-	-	1	*P.* x*m*
Pxa-40	CZ	Pxa-24	1	-	100 (100/~~109~~)	-	-	-	-	-	-	-	162/177 (162/~~174~~/177)	2	*P.* x*m* and *P. u*
Pxa-41	CZ	Pxa-38	1	-	-	173 (173/~~209~~)	-	-	-	-	-	91/94 (91/94/~~98~~)	162 (162/~~174~~)	3	*P.* x*m*
Pxa-42	CZ	Pxa-27	1	-	-	-	-	-	-	-	-	91/94 (91/94/~~98~~)	-	1	*P.* x*m*
Pxa-43	CZ	Pxa-38	2	-	-	-	-	95 (~~92~~/95)	-	-	-	-	-	1	*P.* x*m*
Pxa-44	CZ	Pxa-27	1	-	-	-	-	-	-	-	-	-	162/177 (162/~~174~~/177)	1	*P.* x*m*
Pxa-45	CZ	Pxa-43	1	-	109 (~~100~~/109)	-	89 (~~84~~/89/~~99~~)	-	-	-	113 (~~107~~/113)	91 (91/~~94~~/~~98~~)	-	6	*P.* x*m*
Pxa-46	CZ	Pxa-33	1	-	-	173 (173/~~209~~)	-	-	-	-	-	-	-	1	*P.* x*m*
Pxa-47	LT	Pxa-42	1	-	-	173 (173/~~209~~)	84/89 (84/89/~~99~~)	-	-	-	-	91 (91/~~94~~)	-	3	*P.* x*m*
Pxa-48	SP	Pxa-8	1	-	-	-	-	-	-	-	-	91 (91/~~98~~)	-	1	*P.* x*m*
Pxa-49	AU	Pxa-8	2	-	-	-	-	-	-	-	113 (~~107~~/113)	-	-	1	*P.* x*m*
Pxa-50	AU	Pxa-19	1	-	-	209 (~~173~~/209)	-	-	-	-	-	-	-	1	*P. u*
Total Allele Loss			-	0	2	5	5	4	0	2	4	7	5	35	-

Table 3. Incidence of previously described and new MLGs of *Phytophthora* x*alni* in the different river systems and river basins in the Czech Republic, Hungary and Sweden.

| Country	River system	River basin	N	MLGs Previously described[1]	New	Total	Pxa-1 (%)
Czech Republic	Danube	Dyje	6	2	3	5	2 (33.3)
		Morava	3	1	1	2	2 (66.6)
	Elbe-Vltava	Elbe	4	1	3	4	1 (25.0)
		Ploučnice	3	2	1	3	1 (33.3)
		Ohře	4	2	2	4	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	Répce	16	4	1	5	10 (62.5)
	Balaton	Zala	23	3	2	5	19 (82.6)
Sweden	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
	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

[1]Multilocus genotypes previously described by Aguayo *et al.* [38].

Table 4. Analysis of Molecular Variance (AMOVA) 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* x*alni* population in the Czech Republic, Hungary and Sweden. d.f - degree of freedom, SSD - sum of squared deviations, MSD - mean squared deviations, Sigma (σ) - variance component estimate, P-value – probability value of significance based on 999 permutations.

| Source of variation	d.f.	SSD	MSD	Sigma	%	P-value
1) Complete data set						
- Within river basins	111	8.214	0.074	0.074	56.9	0.001
- Among river basins within one river system	13	4.08	0.313	0.042	33	0.001
- Among river systems	4	3.535	0.883	0.012	9.9	0.105
2) Clone-corrected data set						
- Within river basins	36	6.298	0.174	0.174	86.98	0.041
- Among river basins within one river system	13	2.248	0.172	-0.0007	-0.35	0.492
- Among river systems	4	1.744	0.436	0.026	13.37	0.092
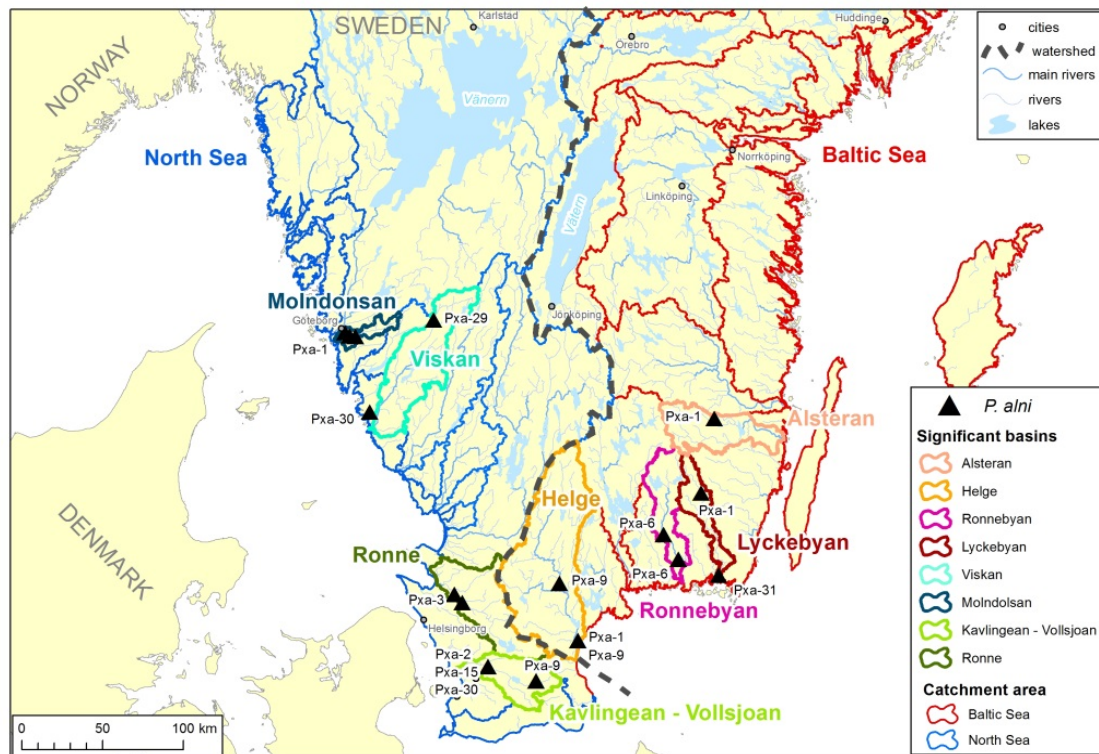
Figure 1. Main water systems in the Czech Republic (A), Hungary (B) and Sweden (C) from which *Phytophthora* x*alni* samples were genotyped in this study and geographic origin of the samples.
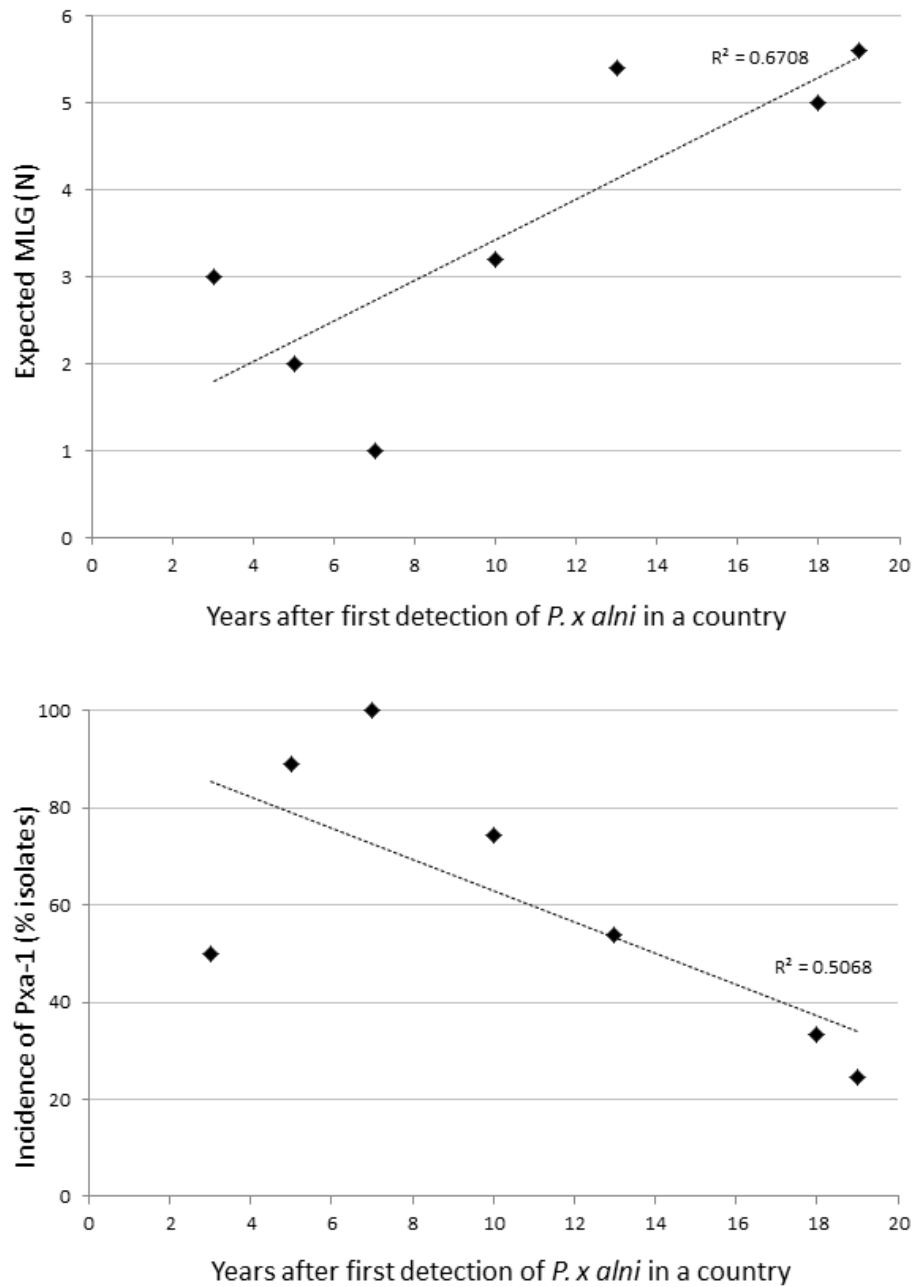
Figure 2. Relationship between the number of expected multilocus genotypes (eMLGs) (A) and the incidence (% isolates) of Pxa-1 in *P. xalni* populations (B) and the age of the populations (i.e. years between first isolation of the pathogen in a country and sampling for this study).
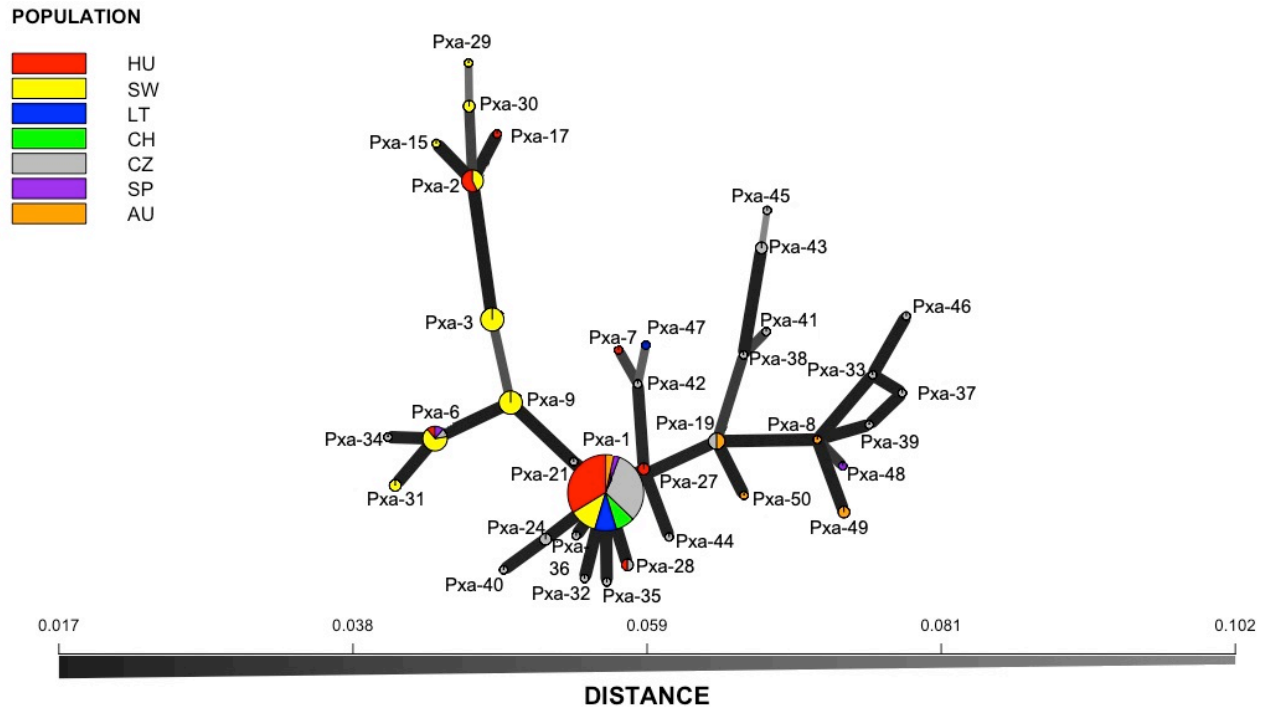
Figure 3. Minimum Spanning Network based on Bruvo's genetic distance [57] of the 36 multilocus genotypes (MLGs) of *Phytophthora* x*alni* detected in this study. Each node represents a different MLG and node size is proportional to the abundance of the specific MLG. HU, Hungary; SW, Sweden; LT, Lithuania; CH, Switzerland, CZ, Czech Republic; SP, Spain; AU, Austria.
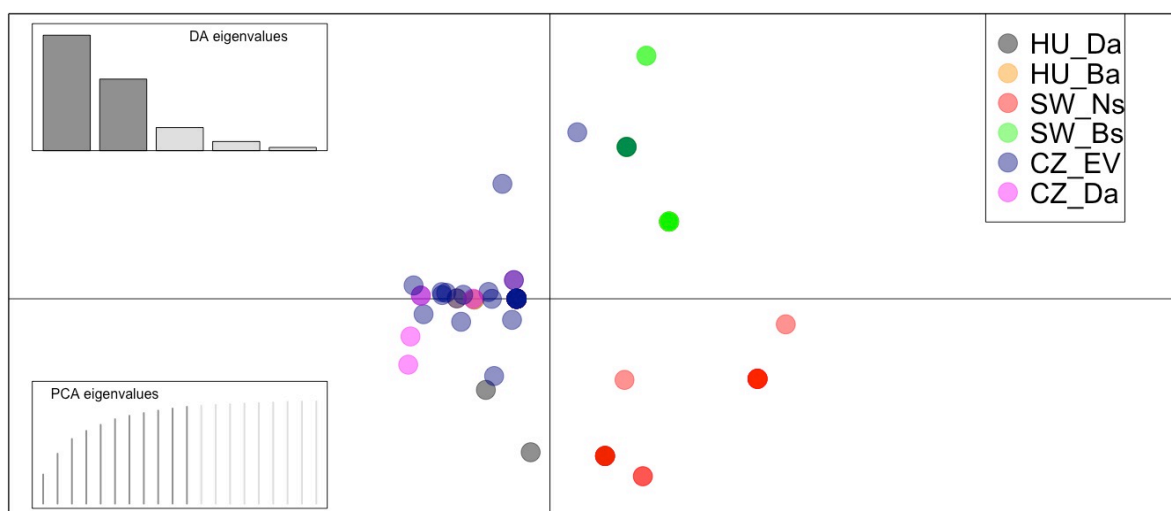
Figure 4. Discriminant Analysis of Principal Components (DAPC) of *Phytophthora xalni* in Czech Republic (CZ), Hungary (HU) and Sweden (SW). Each circle represents a MLG. HU_Da, Hungary Danube; HU_Ba, Hungary Balaton; SW_Ns, Sweden North Sea; SW_Bs, Sweden Baltic Sea, CZ_EV, Czech Republic Elbe-Vltava; CZ_Da, Czech Republic Danube.