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ORIGINAL ARTICLE

Diversity, migration routes, and worldwide population genetic structure of *Lecanosticta acicola*, the causal agent of brown spot needle blight

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Abstract

Lecanosticta acicola is a pine needle pathogen causing brown spot needle blight that results in premature needle shedding with considerable damage described in North America, Europe, and Asia. Microsatellite and mating type markers were used to study the population genetics, migration history, and reproduction mode of the pathogen,

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based on a collection of 650 isolates from 27 countries and 26 hosts across the range of *L. acicola*. The presence of *L. acicola* in Georgia was confirmed in this study. Migration analyses indicate there have been several introduction events from North America into Europe. However, some of the source populations still appear to remain unknown. The populations in Croatia and western Asia appear to originate from genetically similar populations in North America. Intercontinental movement of the pathogen was reflected in an identical haplotype occurring on two continents, in North America (Canada) and Europe (Germany). Several shared haplotypes between European populations further suggests more local pathogen movement between countries. Moreover, migration analyses indicate that the populations in northern Europe originate from more established populations in central Europe. Overall, the highest genetic diversity was observed in south-eastern USA. In Europe, the highest diversity was observed in France, where the presence of both known pathogen lineages was recorded. Less than half of the observed populations contained mating types in equal proportions. Although there is evidence of some sexual reproduction taking place, the pathogen spreads predominantly asexually and through anthropogenic activity.

KEYWORDS
forest pathology, introduction pathways, invasive pathogen, mating type, microsatellites, *Mycosphaerella dearnessii*, *Pinus*

1 | INTRODUCTION

The genus *Lecanosticta* includes nine species, among which *Lecanosticta acicola* is the oldest documented and most well known (van der Nest et al., 2019b). *L. acicola* (formerly *Mycosphaerella dearnessii*) is an important pathogen of *Pinus* spp. causing brown spot needle blight (BSNB) disease that results in premature needle cast, leading to growth reduction and possible death of the trees. Historically, the most prominent damage caused by *L. acicola* has been on *Pinus palustris* plantations in the south-eastern United States (Siggers, 1944; Sinclair & Lyon, 2005), where the pathogen was also first described (de Thümen, 1878). To date, *L. acicola* has been reported on 53 pine species and subspecies (van der Nest et al., 2019a) and on the non-pine host *Cedrus libani* (Oskay et al., 2020).

Due to the significant damage it causes, *L. acicola* is listed as a quarantine pathogen in numerous countries (EPPO, 2022) and extra measures for containment have been applied in the European Union, where the pathogen is classified as a regulated non-quarantine pest (European Commission, 2019). Overall, climate extremes, global trade, and failure to implement proper quarantine measures have been commonly considered as essential factors exacerbating the spread of invasive plant pathogens, including *L. acicola* (Adamson et al., 2018b, 2021; Drenkhan et al., 2014b, 2020; Fisher et al., 2012; Ghelardini et al., 2017; Hanso & Drenkhan, 2009; Jürisoo et al., 2021). Furthermore, climate change, especially warmer winters, has been thought to be one of the main reasons for northwards spread of several forest pathogens (Hanso & Drenkhan, 2013).

In the last decade, the distribution of *L. acicola* has increased, particularly in Europe, where the pathogen has been reported in numerous new countries (van der Nest et al., 2019a). Since 2008, the pathogen has spread into northern Europe and has been found in Estonia, Lithuania, Latvia, and Sweden (Adamson et al., 2015; Cleary et al., 2019; Markovskaja et al., 2011; Mullett et al., 2018). Several new reports of *L. acicola* have been documented in eastern Europe (EPPO, 2018; Georgieva, 2020; Golovchenko et al., 2020; Stamenova et al., 2018) and the pathogen has reached the British Isles (Mullett et al., 2018). *L. acicola* has also expanded its range in Asia and is now present in Turkey, western Asia (Oskay et al., 2020). To date, the pathogen has been documented in areas of North and South America, Europe, and West and East Asia (Oskay et al., 2020; van der Nest et al., 2019a) but is distinctly lacking in the Southern Hemisphere.

Initially, *L. acicola* was thought to originate from Central America (Evans, 1984). However, in a recent study by van der Nest et al. (2019b) using a large collection of isolates from Central America, several distinct *Lecanosticta* species were described, although *L. acicola* was not recovered. As a result, it was proposed that this fungus may originate from North America. Three lineages of *L. acicola* have been proposed. Kais (1972) and Huang et al. (1995) indicated the presence of two distinct lineages of the pathogen based on isolates originating from the northern and southern states of the United States. These two lineages were supported based on multigene sequence data, while a third lineage was also identified from Mexico (van der Nest et al., 2019b). Janoušek et al. (2016) suggested, based on evolutionary modelling, that there were separate introductions of the two lineages into Europe, one lineage introduced and spreading in south-western Europe and the other in central and northern Europe. In addition, Huang et al. (1995) proposed that the

pathogen populations in Asia originate from south-eastern United States. So far, the third lineage seems contained in Mexico (van der Nest et al., 2019b).

L. acicola is a heterothallic ascomycete (Janoušek et al., 2014), but the species predominantly reproduces asexually and spreads via conidia that are dispersed over short distances by rain splash and dew (Siggers, 1939; Skilling & Nicholls, 1974). Previous studies have indicated the presence of both mating types of *L. acicola* in several European countries (Janoušek et al., 2016; Laas et al., 2019; Ortíz de Urbina et al., 2017; Sadiković et al., 2019). Based on the proportion of mating types and genetic analyses, sexual reproduction probably takes place in Austria and Germany, and possibly also in Estonia (Janoušek et al., 2016; Laas et al., 2019). Sexual reproduction of the pathogen taking place in Europe was confirmed by a recent report by Mesanza et al. (2021) describing the presence of the sexual state on *Pinus radiata* in Spain. Genetic recombination by sexual reproduction is one of the main factors, along with mutations, migration, and genetic drift, that can increase genetic diversity, possibly changing its adaptive potential in new environments (McDonald & Linde, 2002). The occurrence of sexual reproduction would also produce airborne ascospores capable of long-distance spread (Kais, 1971), another potential reason for the fast and recent expansion of *L. acicola*.

Overall, a high number of clones and low genetic diversity of *L. acicola* have been registered in Europe in several country- or regional-based population studies (Adamčíková et al., 2021; Laas et al., 2019; Sadiković et al., 2019). There is, however, a lack of information regarding the genetic structure and origin of the recently recorded populations of *L. acicola* in northern Europe and western Asia. A combined population study incorporating all isolates from previous studies and including those from more recent outbreaks would shed light on the migration history of the pathogen in Europe and determine the distribution area of the lineages described by Janoušek et al. (2016). It would also provide information about the reproductive mode and genetic diversity in populations, attributes that are the basis for producing genetic variation and creating new genotypes of the pathogen, which are important for assessing evolutionary potential. Therefore, the objectives of this study were to (i) investigate the worldwide genetic diversity and population structure of *L. acicola* including recently found populations in northern Europe and western Asia, (ii) elucidate the possible migration history of the pathogen, and (iii) assess the possibility of sexual reproduction taking place in the studied populations.

2 | RESULTS

2.1 | Isolates and haplotype identification

In total, 650 isolates from 27 countries and 26 different host taxa were used in the study. With the exception of *Cedrus libani*, all isolates were obtained from *Pinus* hosts. Of the collected isolates, 524 originated from 19 countries in Europe, 103 from North America (Canada, Mexico, United States), 18 from West Asia (Turkey, Georgia), three from East Asia (China, Japan), and two from South America (Colombia) (Table S1, Figure 1). In all downstream analyses, the samples from West Asia were considered part of Europe due to their geographical closeness. The internal transcribed spacer (ITS) sequences obtained for isolates from Colombia matched 100% to the sequences of the epitype culture of *L. acicola* in GenBank (NR_120239), the sequences of the isolates from Mexico had 99.6%–99.8% identity match with NR_120239, and the isolate obtained from Georgia showed 100% similarity with NR_120239 based on ITS sequencing. This represents the first confirmed record of *L. acicola* in Georgia using molecular methods. A culture was deposited in the Fungal Culture Collection of the Estonian University of Life Sciences (culture collection number: TFC101254). The ITS sequence of the isolate was deposited in GenBank (MZ323309).

The obtained sequences of the translation elongation factor 1-α gene region (*TEF1*) were 501 bp in length. Of the 15 isolates sequenced, four different elongation factor (EF) haplotypes were obtained. The sequences for two isolates from France (original culture codes B1254 and B1599) were found to be identical with isolates from Germany, Lithuania, and Canada (codes 18313, 23677, 17787, and 23696, Table S1) and with a reference sequence obtained from GenBank (accession number KC013002.2) marked as the Northern lineage or lineage 1 in van der Nest et al. (2019a).

Isolates from Canada, Estonia, and the north-eastern United States (culture codes 16637, 17789, 15644, and 17853, Table S1) were described by another EF haplotype. Named isolates contained a unique single-nucleotide polymorphism at location 22 of the obtained alignment—adenine (A)—while all other observed sequences were characterized by thymine (T) at the position.

One EF haplotype was present in isolates from the south-eastern United States, Spain, and France (culture codes 18065, 14881, 17856, and 16628, Table S1) and it was identical to the reference sequence (GenBank accession number MK015399) marked as the Southern lineage or lineage 2 in van der Nest et al. (2019a). One haplotype was unique to the south-eastern United States (culture code 18071, Table S1). All obtained *TEF1* sequences were deposited in GenBank (MZ826765–MZ826779, Table S1).

Analyses across 11 microsatellite markers resulted in a total of 172 alleles (Table S2). All observed loci were polymorphic, with the number of alleles ranging from four at loci MD5 and MD11 to 54 at locus MD8. Locus MD1 was monomorphic in north-eastern United States, in western Asia, and all over Europe, except in south-western Europe. Locus MD11 was monomorphic in north-eastern United States, western Asia and all over Europe, including south-western Europe, showing polymorphism only in the south-eastern United States. Microsatellite marker MD6 did not amplify isolates from Mexico, Slovakia, Japan, and China. In the following analyses, missing microsatellite data were treated as unknown data according to instructions given for each software program used. In total, 284 different multilocus haplotypes (MLHs) were found in the collection of isolates. All populations, except Belarus ($N = 3$), Ireland ($N = 3$), Mexico ($N = 4$), and Japan ($N = 2$), contained clones. In total,

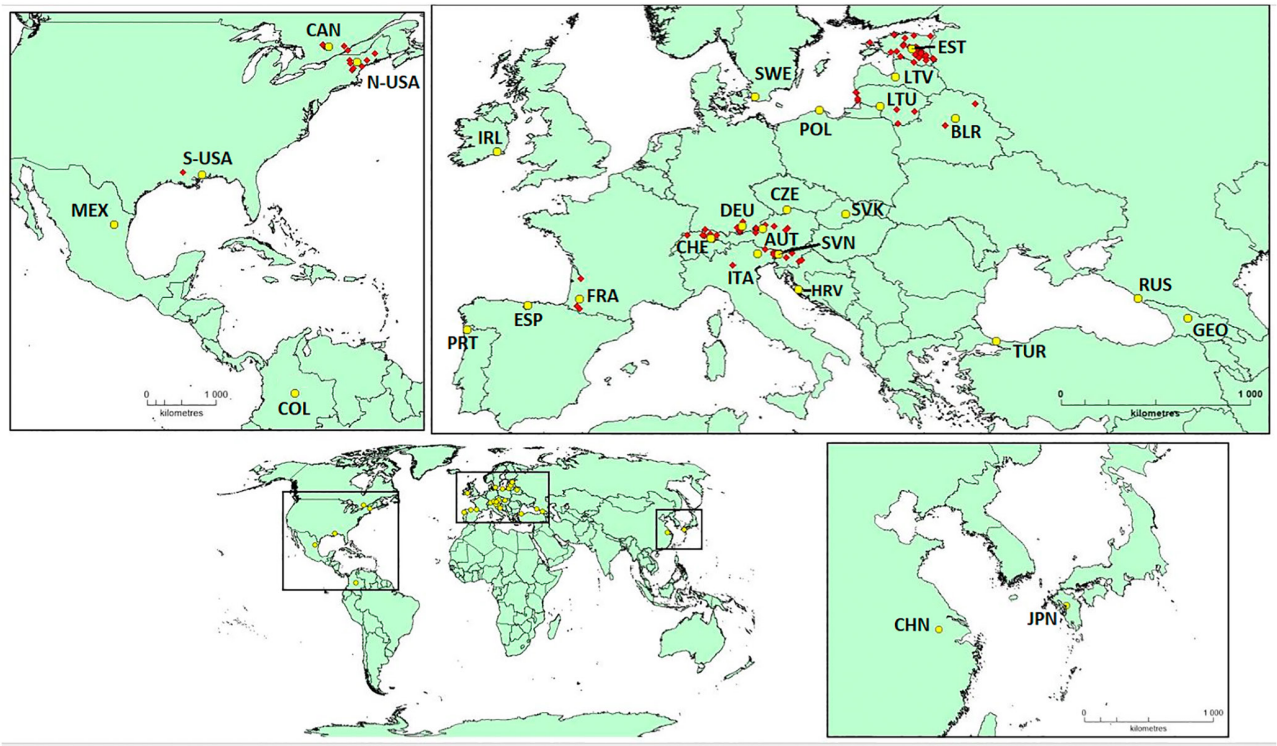

FIGURE 1 Map of the sampling locations (red dots) of *Lecanosticta acicola*. Yellow points indicate the weighted geographical midpoint of a particular sampling area and the representative population (Table S1). Definition of population codes: AUT, Austria; BLR, Belarus; CAN, Canada; CHE, Switzerland; CHN, China; COL, Colombia; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; GEO, Georgia; HRV, Croatia; IRL, Ireland; ITA, Italy; JPN, Japan; LTU, Lithuania; LTV, Latvia; N-USA, north-eastern United States; MEX, Mexico; POL, Poland; PRT, Portugal; RUS, Russia; S-USA, south-eastern United States; SVK, Slovakia; SVN, Slovenia; SWE, Sweden; TUR, Turkey.

100 haplotypes appeared more than once, and 16 haplotypes were found in more than one population (Table S3). The most common haplotype (MLH 196) appeared 45 times in four different populations (Turkey, Belarus, Lithuania, and Estonia). The second most frequent haplotype (MLH 125) was identified 23 times in two populations (Austria and Switzerland) and the third most frequent one (MLH 83) was identified 19 times in Slovenia. One haplotype (MLH 225) was found to be present on two continents, in Canada (North America) and Germany (Europe). One haplotype (MLH 257) was shared between China and Japan.

The global clonal fraction of *L. acicola* was 0.563. Overall, the clonal fraction index for Europe (0.600) was higher than for America (0.371) (Table 1), although for north-eastern America (CAN and N-USA combined) the clonal fraction was considerably higher (0.566, data not shown) and closer to the value found for Europe. In Europe, the clonal fraction ranged from 0.333 (ESP) to 0.818 (SVK). The population S-USA had a notably smaller clonal fraction (0.174) than other populations.

2.2 | Genetic diversity

Isolates from S-USA contained the highest number of unique alleles (36), followed by MEX (8) (Table 1, Table S2). Unique alleles were not found in N-USA and CAN. In Europe, unique alleles were found in eight of the 21 populations, with the highest number in AUT (six), followed by EST, HRV, and TUR (three). One allele was unique to East Asia, being present only in CHN and JPN.

In the East Asian populations of CHN and JPN, a total of 11 alleles was found. Eight of these alleles were also only found in S-USA, south-western Europe, or COL (Table S2).

The highest allelic richness (A_R) was recorded in S-USA (3.570). For Europe, the highest allelic richness was found in FRA (2.364), followed by SVN (2.290) and EST (2.233). The highest private allelic richness (PA_R) was also recorded in S-USA (1.968). In Europe, the highest private allelic richness was observed in TUR (0.442), followed by SVN (0.331) and RUS (0.302).

Likewise, the highest mean number of different alleles (Na), mean unbiased diversity (uh), and mean haploid genetic diversity (*h*) were observed in S-USA (Table 1). In Europe, the highest mean number of different alleles was recorded in LTU (4.545), EST (4.273), and SVN (3.909). Both the highest mean unbiased diversity and the highest mean haploid genetic diversity in Europe were observed in FRA, EST, and LTU.

2.3 | Population differentiation and genetic distance

According to the analysis of molecular variance (AMOVA), no significant differences were found between population pairs of

TABLE 1 Diversity statistics of *Lecanosticta acicola* populations based on 11 microsatellite markers

Region/ population code	*N*[a]	No. of haplotypes[b]	No. of alleles	Unique alleles	Allelic richness A_R (*SE*[c]) cc[d]	Private allelic richness PA_R (*SE*[c]) cc[d]	Mean number of different alleles Na (*SE*[c]) cc[d]	Mean unbiased diversity uh (*SE*[c]) cc[d]	Mean haploid genetic diversity *h* (*SE*[c]) cc[d]	Clonal fraction
America	**105**	**66**	**125**	**61**	**3.827 (0.337)**	**2.718 (0.353)**	**11.364 (2.028)**	**0.775 (0.046)**	**0.762 (0.045)**	**0.371**
CAN	19	12	31	0	2.248 (0.412)	0.161 (0.126)	2.818 (0.672)	0.407 (0.102)	0.372 (0.093)	0.368
COL[e]	2	1	11	3	–	–	–	–	–	–
MEX[e]	4	4	17	8	–	–	–	–	–	–
N-USA	34	11	21	0	1.730 (0.339)	0.134 (0.118)	1.909 (0.436)	0.251 (0.108)	0.223 (0.095)	0.676
S-USA	46	38	98	36	3.570 (0.469)	1.968 (0.365)	8.909 (1.856)	0.672 (0.096)	0.653 (0.093)	0.174
East Asia	**3**	**2**	**11**	**1**	–	–	–	–	–	–
CHN[e]	1	1	10	0	–	–	–	–	–	–
JPN[e]	2	2	11	0	–	–	–	–	–	–
Europe	**542**	**217**	**103**	**46**	**2.549 (0.434)**	**1.440 (0.461)**	**9.455 (3.864)**	**0.460 (0.097)**	**0.458 (0.097)**	**0.600**
AUT	31	15	30	6	1.902 (0.432)	0.253 (0.253)	2.727 (1.054)	0.263 (0.097)	0.245 (0.097)	0.516
BLR[e]	3	3	17	0	–	–	–	–	–	–
CHE	50	12	22	0	1.766 (0.350)	0.050 (0.034)	2.000 (0.467)	0.258 (0.110)	0.234 (0.100)	0.760
CZE	16	6	19	0	1.727 (0.384)	0.068 (0.061)	1.727 (0.384)	0.242 (0.111)	0.199 (0.091)	0.625
DEU	33	19	33	1	1.918 (0.410)	0.188 (0.128)	3.000 (0.982)	0.267 (0.099)	0.251 (0.093)	0.424
ESP	9	6	15	0	1.364 (0.152)	0.044 (0.044)	1.364 (0.152)	0.176 (0.077)	0.145 (0.064)	0.333
EST	127	62	47	3	2.233 (0.355)	0.172 (0.111)	4.273 (1.294)	0.421 (0.092)	0.414 (0.090)	0.512
FRA	10	6	26	0	2.364 (0.244)	0.028 (0.028)	2.364 (0.244)	0.524 (0.064)	0.431 (0.053)	0.400
GEO[e]	1	1	11	0	–	–	–	–	–	–
HRV	24	8	17	3	1.477 (0.319)	0.271 (0.194)	1.545 (0.455)	0.126 (0.091)	0.108 (0.079)	0.667
IRL[e]	3	3	15	0	–	–	–	–	–	–
ITA[e]	4	2	15	0	–	–	–	–	–	–
LTU	106	52	49	1	2.164 (0.388)	0.115 (0.072)	4.545 (1.734)	0.377 (0.102)	0.369 (0.100)	0.509
LTV[e]	1	1	11	0	–	–	–	–	–	–
POL	16	6	16	0	1.455 (0.207)	0.009 (0.009)	1.455 (0.207)	0.200 (0.084)	0.167 (0.070)	0.625
PRT[e]	2	1	11	0	–	–	–	–	–	–
RUS	17	8	18	2	1.455 (0.455)	0.302 (0.302)	1.636 (0.636)	0.091 (0.091)	0.080 (0.080)	0.529
SVK	11	2	10	0	–	–	–	–	–	0.818
SVN	58	16	43	1	2.290 (0.530)	0.331 (0.202)	3.909 (1.289)	0.331 (0.117)	0.309 (0.110)	0.724
SWE[e]	3	1	11	0	–	–	–	–	–	–

TABLE 1 (Continued)

Region/ population code	N^a	No. of haplotypes[b]	No. of alleles	Unique alleles	Allelic richness A_R (SE^c) cc[d]	Private allelic richness PA_R (SE^c) cc[d]	Mean number of different alleles Na (SE^c) cc[d]	Mean unbiased diversity uh (SE^c) cc[d]	Mean haploid genetic diversity *h* (SE^c) cc[d]	Clonal fraction
TUR	17	6	24	3	2.182 (0.464)	0.442 (0.219)	2.182 (0.464)	0.315 (0.105)	0.263 (0.087)	0.647

Notes: Definition of population codes: AUT, Austria; BLR, Belarus; CAN, Canada; CHE, Switzerland; CHN, China; COL, Colombia; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; GEO, Georgia; HRV, Croatia; IRL, Ireland; ITA, Italy; JPN, Japan; LTU, Lithuania; LTV, Latvia; N-USA, north-eastern United States; MEX, Mexico; POL, Poland; PRT, Portugal; RUS, Russia; S-USA, south-eastern United States; SVK, Slovakia; SVN, Slovenia; SWE, Sweden; TUR, Turkey.

[a]Number of isolates.

[b]Equivalent to the number of isolates in the clone-corrected dataset (*N* [cc]).

[c]*SE*, standard error.

[d]cc, based on a clone-corrected dataset.

[e]Due to small sample size (*N* [cc] < 6), these populations were excluded from population genetic analyses.

neighbouring European countries FRA–ESP, AUT–CZE, and CZE–DEU ($p > 0.05$) (Table 2). All other populations were significantly differentiated from each other.

Nei's genetic distance indicated that the populations N-USA and CAN are genetically rather similar to most populations in Europe (Table S4, Figure 2). The population S-USA is genetically similar only to ESP and genetically distant from all other populations. Similarly, ESP is distinct from all other European populations included in the analysis of Nei's genetic distance, except for FRA.

2.4 | Isolation by distance, phylogenetic tree, and population structure

The Mantel test for isolation by distance among 16 American and European populations represented by at least six isolates revealed significant correlation between geographical distance and Nei's genetic distance ($R^2 = 0.1679$, $p = 0.030$, Figure 3a). Isolation by distance was also supported in Europe ($R^2 = 0.2292$, $p = 0.010$, Figure 3b), but rejected in North America ($R^2 = 0.9975$, $p = 0.166$, Figure 3c).

The $\ln(\Pr(X|K))$ method of choosing the best number of STRUCTURE clusters indicated that seven clusters describe the dataset best (Figure S1), whereas the ΔK statistic indicated that two clusters explained the data best (Figure S2). At $K = 2$ one of the clusters (indicated in red) dominates not only in S-USA, MEX, COL, and the East Asian populations JPN and CHN, but also in the south-western European populations FRA, ESP, and PRT (Figure 4). The other cluster (indicated in light blue) dominates in N-USA, CAN, western Asia, and most of Europe, whilst also occurring in the south-western European population FRA. At $K = 4$ a clear central European cluster is differentiated (indicated in green) and from $K = 5$ up to $K = 7$ a single cluster (brown) dominates in HRV, W-ASIA, and part of CAN.

In populations POL, BLR, IRL, CZE, HRV, ESP, RUS, S-USA, MEX, and JPN, all isolates were dominated by a single cluster, whereas other populations contained isolates belonging to multiple different clusters ($K = 7$, Figure 4). The proportion of the STRUCTURE clusters in populations indicates differences between geographical regions (Figure 5). The northern European populations EST, LTV, LTU, POL, and BLR shared a roughly similar structure, with the light blue cluster dominating. Isolates from the Curonian Spit region in LTU belonged to the same cluster (green, $K = 4$–7) as those in central Europe. In central Europe, CHE and SVN show a more diverse structure with all the previously mentioned clusters represented in small proportions without a single dominating cluster. Populations from HRV, RUS, and TUR belong primarily to the brown cluster, which also occurs in CAN and N-USA. Up to $K = 5$, isolates from ESP, FRA, and PRT were mostly placed into the red cluster that is also dominating in S-USA; however, at $K = 6$ and $K = 7$ isolates from south-western Europe were mostly placed into a cluster (pink) that has only a marginal proportion in S-USA.

The neighbour-joining (NJ) dendrogram covering 28 populations indicates the presence of four groups: the first group

TABLE 2 Population differentiation according to analysis of molecular variance (AMOVA) between 16 populations of *Lecanosticta acicola*

Population code	AUT	CAN	CZE	DEU	ESP	EST	FRA	HRV	LTU	S-USA	POL	RUS	CHE	SVN	TUR
AUT	0.000														
CAN	0.001	0.000													
CZE	**0.335**	0.002	0.000												
DEU	0.003	0.001	**0.136**	0.000											
ESP	0.001	0.001	0.003	0.001	0.000										
EST	0.001	0.001	0.002	0.001	0.001	0.000									
FRA	0.001	0.001	0.013	0.001	**0.240**	0.001	0.000								
HRV	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000							
LTU	0.001	0.001	0.031	0.001	0.001	0.001	0.001	0.001	0.000						
S-USA	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000					
POL	0.001	0.001	0.005	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.000				
RUS	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000			
CHE	0.036	0.001	0.044	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000		
SVN	0.001	0.002	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.011	0.000	
TUR	0.001	0.001	0.006	0.001	0.004	0.001	0.011	0.001	0.001	0.001	0.006	0.001	0.001	0.001	0.000
N-USA	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Notes: In the table the AMOVA *p*-values are shown. The populations that are similar according to AMOVA ($p > 0.05$) are indicated in bold. Definition of population codes: AUT, Austria; CAN, Canada; CHE, Switzerland; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; HRV, Croatia; LTU, Lithuania; N-USA, north-eastern United States; POL, Poland; RUS, Russia; S-USA, south-eastern United States; SVN, Slovenia; TUR, Turkey.

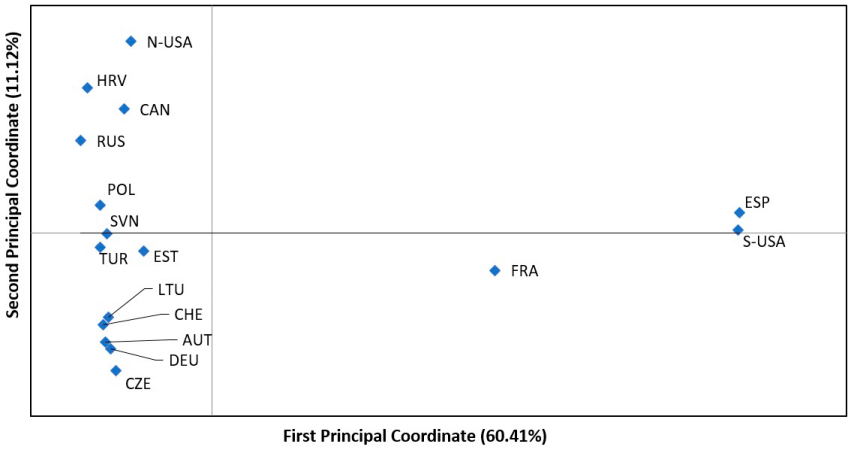

FIGURE 2 Principal coordinate analysis of Nei's genetic distance of 16 populations of *Lecanosticta acicola*. Definition of population codes: AUT, Austria; CAN, Canada; CHE, Switzerland; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; HRV, Croatia; LTU, Lithuania; N-USA, north-eastern United States; POL, Poland; RUS, Russia; S-USA, south-eastern United States; SVN, Slovenia; TUR, Turkey.

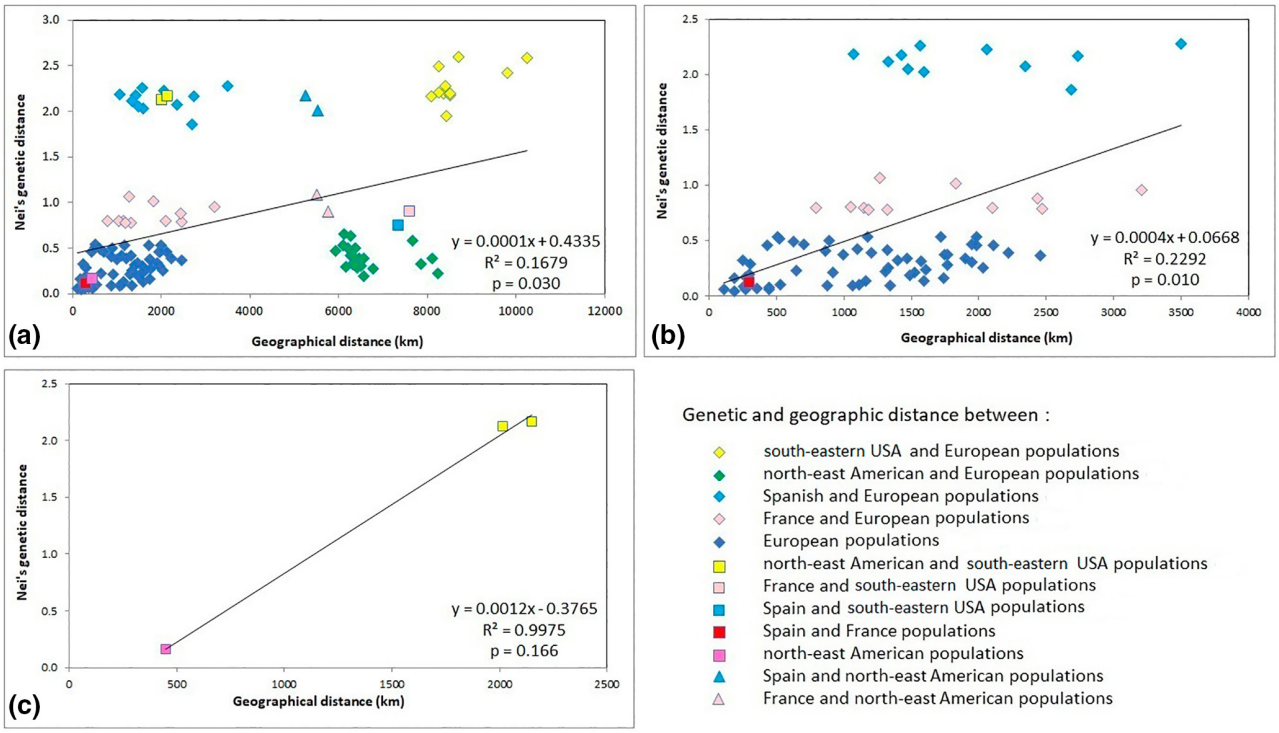

FIGURE 3 Results of the Mantel test on geographical and Nei's genetic distances of populations with at least six samples (*N* [cc] ≥ 6). Each point represents the combination of geographical and genetic distance values for each pair of populations compared. Legend (bottom right corner) explains symbols and colours representing the population pairs on the figures. (a) All 16 populations from North America and Europe (including western Asia). (b) Thirteen populations from Europe (including western Asia). (c) Three populations from North America.

includes populations from East Asia (CHN and JPN), S-USA, and COL; the second group includes populations from north-eastern America (N-USA and CAN) and most of the populations from Europe (Figure 6); MEX stands out as an independent clade (indicated as group 3, Figure 6); and the last group includes populations from south-western Europe (FRA, ESP, and PRT). However, the bootstrap support for most of the specific nodes is weak. Close genetic relationships, supported by the high bootstrap values, were observed between CHN and JPN and between FRA, ESP, and PRT in the dendrogram.

The NJ tree based on Nei's genetic distance between isolates showed an overall similar clustering into clades as defined by the STRUCTURE analyses (Figure S3). From the isolate-based figure it is evident that most isolates from south-western Europe (FRA, ESP, PRT) are genetically close but two isolates from France cluster together with samples from central Europe. Isolates from central and northern Europe indicated a mixed migration history with isolates from the same country being distributed among several clades.

2.5 | Modelling of population history

The first set of approximate Bayesian computation (ABC) scenarios was used to investigate the demographic history between

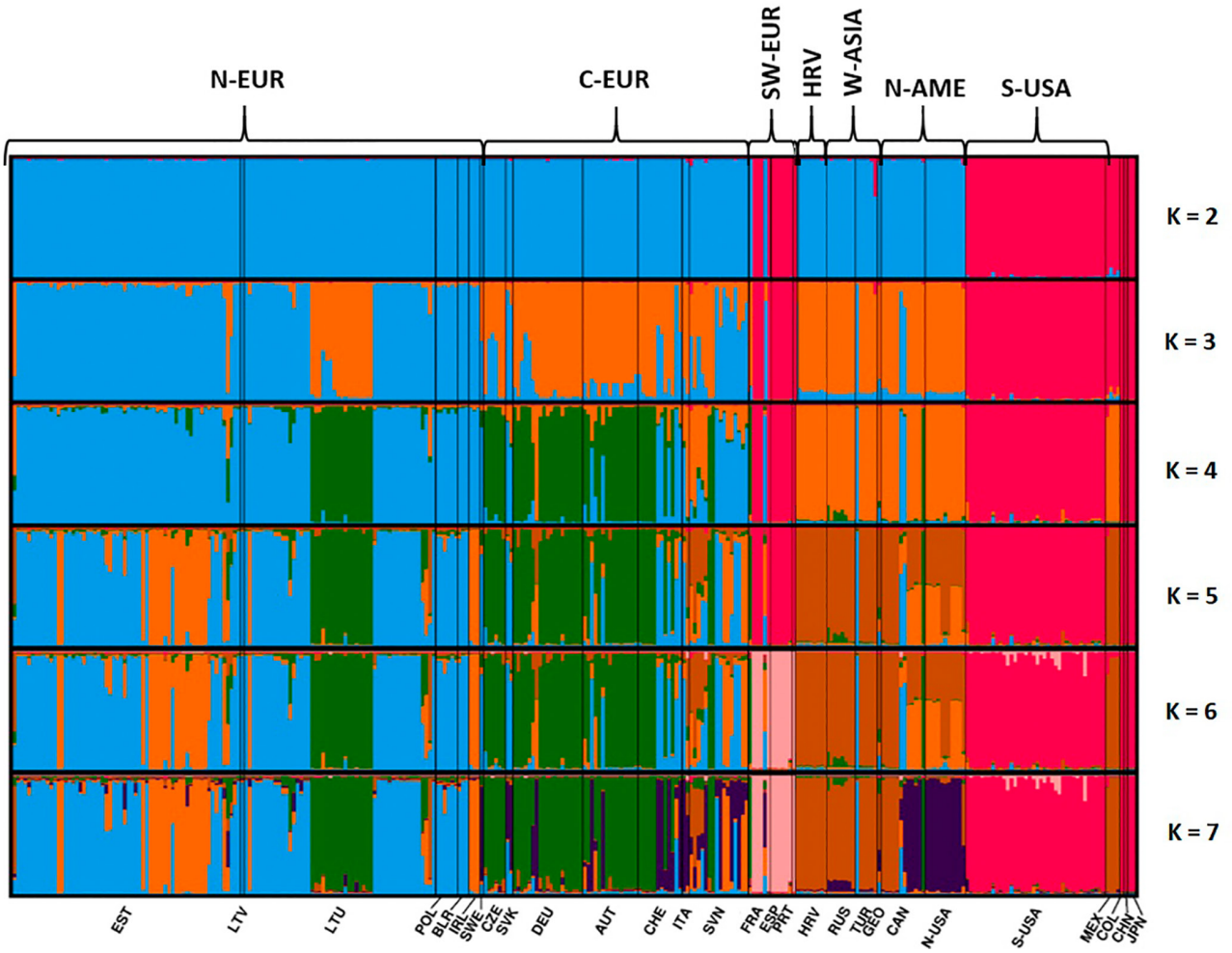

FIGURE 4 STRUCTURE clustering of the *Lecanosticta acicola* clone-corrected dataset, representing $K = 2$–7. Optimal number of clusters ($K = 2$) by ΔK and ($K = 7$) by ln(Pr(X|K)). Population codes are displayed under the figure; above the figure division into regions as analysed in the migration analyses is displayed. Definition of population codes: AUT, Austria; BLR, Belarus; CAN, Canada; CHE, Switzerland; CHN, China; COL, Colombia; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; GEO, Georgia; HRV, Croatia; IRL, Ireland; ITA, Italy; JPN, Japan; LTU, Lithuania; LTV, Latvia; N-USA, north-eastern United States; MEX, Mexico; POL, Poland; PRT, Portugal; RUS, Russia; S-USA, south-eastern United States; SVK, Slovakia; SVN, Slovenia; SWE, Sweden; TUR, Turkey.

the three main clusters indicated in America and Europe (Figure 4, $K = 3$). In Analysis 1, the posterior probabilities were highest for Scenario 17 ($p = 0.3760$, Table S5), where SW-EUR originated from N-AME and S-USA, while the merged population EUR originated from an admixture event between N-AME and an unsampled population. According to the estimated parameters N-AME was derived from the ancestral population a median of 30,600 and a mode of 35,200 generations ago and S-USA was derived from N-AME a median of 6880 and a mode of 4970 generations ago (Table S6). From the European populations, SW-EUR was derived from S-USA and N-AME a median of 512 and a mode of 628 generations ago and EUR was derived from N-AME a median of 99 and a mode of 51 generations ago. The scenario suggesting that the American populations originate from Europe was not supported (S1.20, $p = 0.000$), neither was the scenario suggesting that all named regions were derived separately from the ancestral population (S1.1, $p = 0.000$).

The most supported scenario in Analysis 2 (S2.3) suggested that N-EUR was derived from EUR ($p = 0.5026$, Table S5) a median of 40 and a mode of 32 generations ago (Table S6) with a weak bottleneck occurring (short duration and high number of founders).

The scenario with the highest support in Analysis 3 revealed that the populations in Croatia and western Asia (HRV and W-ASIA) originated from N-AME (S3.5, $p = 0.7077$, Table S5) a median of 205 and a mode of 235 generations ago (Table S6). The population of C-EUR originated from an admixture event between N-AME and an unsampled population a median of 115 and a mode of 77 generations ago.

A graphical representation of the winning historical scenarios showing the most supported historical events in the demographic history of *L. acicola* based on the observed populations is presented in Figure 7. Confidence in scenario choice with 95% credibility intervals for each analysis is presented in Table S5 and posterior distributions of parameters are presented in Table S6. Figures of the model checking results are presented in Figure S4.

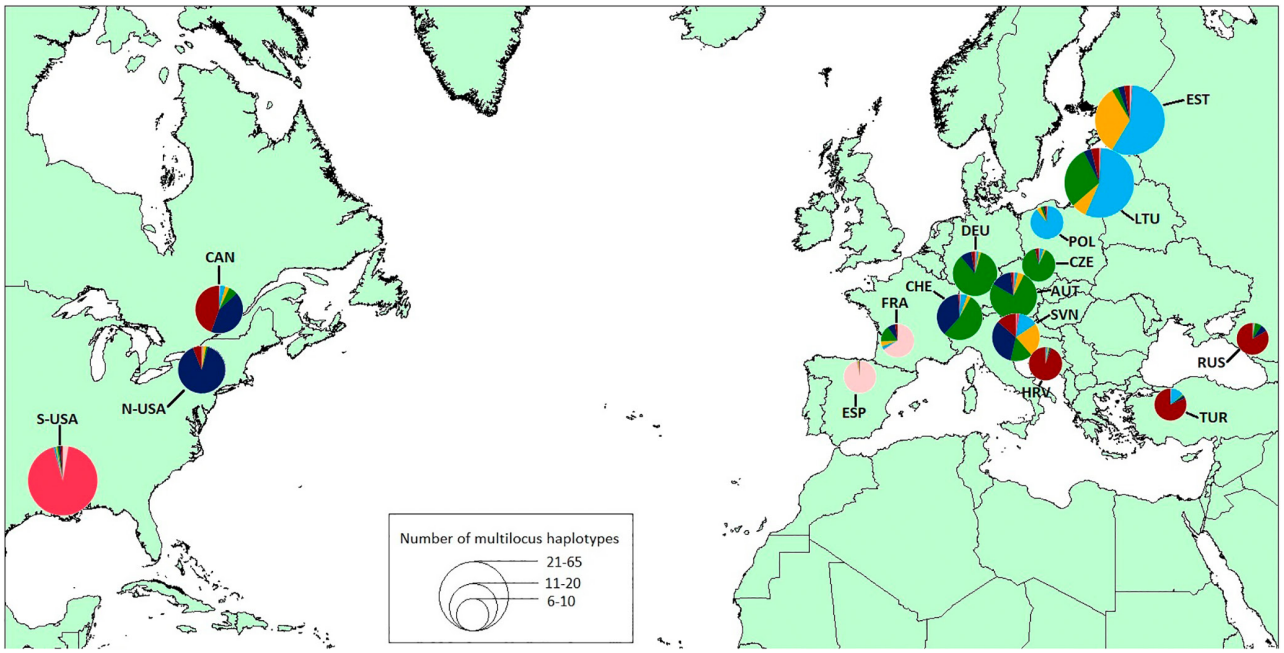

FIGURE 5 The proportion of STRUCTURE clusters ($K = 7$) in *Lecanosticta acicola* populations with at least six samples (N [cc] ≥ 6). Definition of population codes: AUT, Austria; CAN, Canada; CHE, Switzerland; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; HRV, Croatia; LTU, Lithuania; N-USA, north-eastern United States; POL, Poland; RUS, Russia; S-USA, south-eastern United States; SVN, Slovenia; TUR, Turkey.

2.6 | Mating type distribution and haploid linkage disequilibrium

The mating type idiomorphs were successfully identified for 629 isolates. Both mating type idiomorphs were present in 14 populations out of 28 (Table 3). In both East Asian populations (CHN and JPN) only *MAT1-2* was identified. The exact binomial test on the mating type ratios indicated that in six populations (ESP, FRA, IRL, MEX, SVN, and S-USA) equal ratios of the mating type idiomorphs ($p > 0.05$) were found based on the non-cc dataset and in 10 populations (CHE, DEU, ESP, FRA, IRL, MEX, N-USA, POL, SVN, and S-USA) based on the cc dataset. Therefore, in these populations, sexual reproduction is possible.

The index of association indicated that random mating occurred only in ESP and S-USA populations based on the non-cc dataset and additionally in SVN, HRV, and CZE populations based on the cc dataset, the last two being unexpected because only one mating type was identified in those populations (Table 3). The calculation of the index of association and the standardized index of association was not successful for the RUS population.

3 | DISCUSSION

This study, which includes 650 isolates from 27 countries, represents the most comprehensive population genetics analysis of *L. acicola* to date. The objective was to combine previously studied populations with newly collected data, particularly from northern Europe and western Asia, to determine the global diversity and pathways of movement of the pathogen. STRUCTURE clustering roughly corresponded with the geographic distribution of the isolates and revealed a subdivided population structure in several regions. The results provide evidence for several separate pathogen introductions from America into Europe and suggest that the recently discovered populations in northern Europe originate from previously described *L. acicola* populations in Europe. However, the populations in western Asia and Croatia appear to originate from a separate introduction event from North America. Despite quarantine efforts, *L. acicola* is now widespread in Europe and seems to be spreading via anthropogenic activity and both asexual and sexual natural dispersal.

The global set of *L. acicola* isolates can be divided into two main groups supported by STRUCTURE and genetic distance analyses. The distribution areas of those groups correspond with the results of previous studies describing lineages within the species (Huang et al., 1995; Janoušek et al., 2016; Kais, 1972; van der Nest et al., 2019a). Most of Europe, western Asia, and north-eastern North America comprise one genetically similar group, while south-western Europe, southern USA, and East Asia comprise the second group. Finer levels of substructure could be observed in northern and central Europe, while populations in Croatia, Russia, and Turkey stand out with rather homogeneous structure.

Overall, in central Europe the populations were genetically similar. Results suggest genetic exchange between countries with several shared haplotypes being found. The modelled population history suggests that the central European populations originate from north-eastern North America and an unsampled population. The fact that numerous alleles that were documented in several

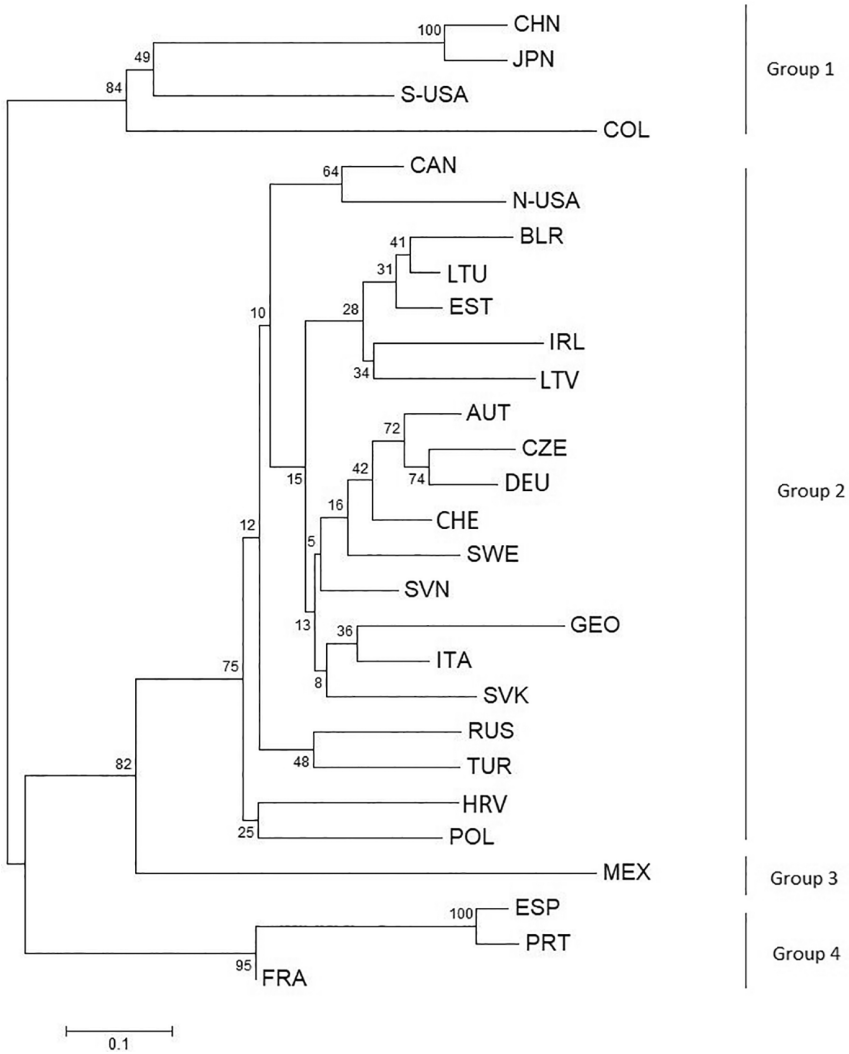

FIGURE 6 Neighbour-joining tree of genetic distances (Da, Nei, 1972) for 28 populations as implemented in POPTREE v. 2 with 10,000 bootstraps used to generate confidence at branch points. Definition of population codes: AUT, Austria; BLR, Belarus; CAN, Canada; CHE, Switzerland; CHN, China; COL, Colombia; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; GEO, Georgia; HRV, Croatia; IRL, Ireland; ITA, Italy; JPN, Japan; LTU, Lithuania; LTV, Latvia; N-USA, north-eastern United States; MEX, Mexico; POL, Poland; PRT, Portugal; RUS, Russia; S-USA, south-eastern United States; SVK, Slovakia; SVN, Slovenia; SWE, Sweden; TUR, Turkey.

European populations were not found in America (Table S2) highlights that some of the populations in Europe historically originate from unsampled populations. Sampling additional populations in North America could bring a better understanding of the origin of the European populations and about how the American populations themselves might have emerged.

This study revealed that the highest allelic diversity occurs in the south-eastern United States, where sexual reproduction takes place, supporting the findings of Janoušek et al. (2016). However, in most of the European populations, the genetic diversity was not much lower, and in some cases even higher, than in north-eastern American populations (N-USA and CAN), which probably represent the native range of the pathogen, and which are possibly the source of most of the European populations. In general, species are thought to have higher diversity in their native area when compared to regions where they were recently introduced (McDonald & McDermott, 1993). Additional sampling in northern America would, however, almost certainly reveal more diversity, filling the gap due to the currently more thorough sampling in Europe.

Up to $K = 5$, the populations of south-western Europe were dominated by the same cluster as were those of south-eastern United States, which is in accordance with the previous study by Janoušek et al. (2016). In south-western Europe, one haplotype was found to be shared between France, Spain, and Portugal, indicating shared origin or close connections between the populations. Surprisingly, the microsatellite data and the EF sequences revealed that both described lineages of the pathogen are present in France. Overall, the analyses of population history gave most support to the scenario where the south-western European populations were formed through an admixture event between south-eastern United States and north-eastern North America and are older than other populations in Europe. However, the NJ tree based on genetic distance placed isolates from France closer to samples originating from Germany and Switzerland. The presence of both N-AME and S-USA STRUCTURE clusters (at $K = 2$) and both North and South lineages in south-western Europe strongly suggest at least two independent introductions to the region, either directly from North America, or via spread from European countries. The high levels of genetic diversity found in France, indicated by high allelic richness, mean unbiased diversity, and mean haploid genetic diversity, are most probably due to isolates from genetically different populations being present there.

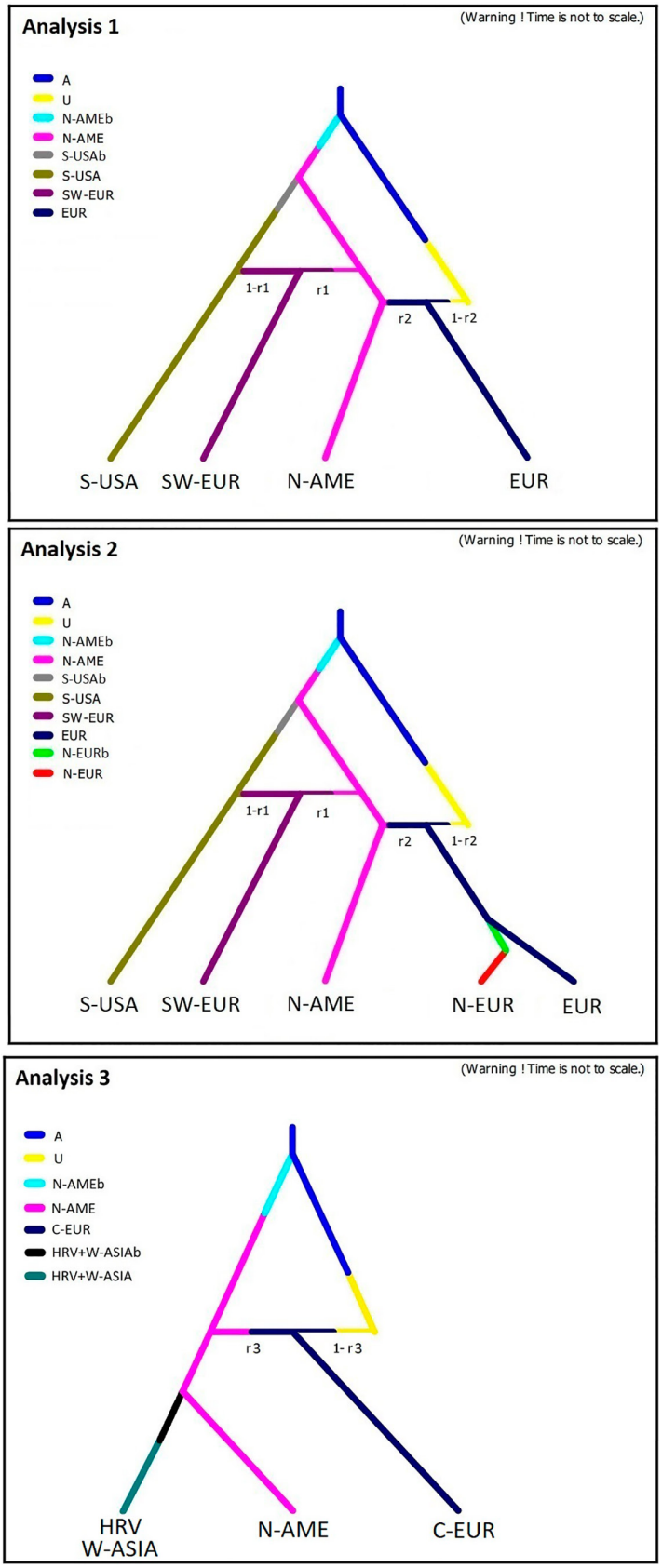

FIGURE 7 A graphical representation of the historical scenarios, most supported by the approximate Bayesian computation (ABC) analyses. A, ancestral population; U, unsampled population; N-AME, north-eastern America (N-USA + CAN); S-USA, south-eastern United States; SW-EUR, south-western Europe; EUR, combined population of C-EUR, HRV, and W-ASIA; C-EUR, central Europe; N-EUR, northern Europe; HRV, Croatia; W-ASIA, western Asia; b, bottleneck event; r1, r2, and r3, rates of admixture; thickness of line indicates the contribution from populations (r and r − 1).

Microsatellite diversity was surprisingly high in the northern European populations of Estonia and Lithuania, considering that *L. acicola* has been known to be present there for only little longer than a decade. Based on the results of the current analyses, modelling of population history suggests that *L. acicola* in northern Europe originates from other populations in Europe and not from a separate

TABLE 3 A summary of the mating type distribution and index of association results

Population	*MAT1-1-1* non-cc[a]	*MAT1-2* non-cc[a]	*p*-value of exact binomial test non-cc[a]	*MAT1-1-1* cc[b]	*MAT1-2* cc[b]	*p*-value of exact binomial test cc[b]	I_A[c] non-cc[a]	$\bar{r}_d$[e] non-cc[a]	*p*-value of I_A[c] and $\bar{r}_d$[e] non-cc[a]	I_A cc[b]	$\bar{r}_d$ cc[b]	*p*-value of I_A and $\bar{r}_d$ cc[b]
AUT	2	29	0.000	2	13	0.007	2.582	0.543	0.001	1.901	0.386	0.001
BLR	3	0	–	3	0	–	–	–	–	–	–	–
CAN	17	2	0.001	10	2	0.039	1.708	0.250	0.001	1.053	0.157	0.001
CHE	3	47	0.000	3	9	0.146	2.025	0.678	0.001	0.558	0.186	0.001
CHN	0	1	–	0	1	–	–	–	–	–	–	–
COL	0	2	–	0	1	–	–	–	–	–	–	–
CZE	0	16	–	0	6	–	1.173	0.403	0.001	0.109	0.036	0.509
DEU	24	9	0.014	13	6	0.167	0.987	0.177	0.001	0.921	0.157	0.003
ESP	3	6	0.508	2	4	0.688	−0.173	−0.058	0.848	−0.376	−0.126	1.000
EST	102	24	0.000	44	17	0.001	1.251	0.163	0.001	0.309	0.039	0.003
FRA	7	2	0.180	4	1	0.375	6.516	0.731	0.001	5.440	0.609	0.001
GEO	1	0	–	1	0	–	–	–	–	–	–	–
HRV	0	24	–	0	8	–	0.409	0.410	0.004	0.106	0.106	0.543
IRL	2	1	1.000	2	1	1.000	–	–	–	–	–	–
ITA	4	0	–	2	0	–	–	–	–	–	–	–
JPN	0	2	–	0	2	–	–	–	–	–	–	–
LTV	1	0	–	1	0	–	–	–	–	–	–	–
LTU	63	29	0.001	30	13	0.014	0.673	0.104	0.001	0.516	0.078	0.001
MEX	2	2	1.000	2	2	1.000	–	–	–	–	–	–
N-USA	31	3	0.000	8	3	0.227	2.041	0.689	0.001	0.968	0.324	0.001
POL	13	3	0.021	4	2	0.688	1.317	0.439	0.001	1.189	0.396	0.014
PRT	1	0	–	1	0	–	–	–	–	–	–	–
RUS	0	17	–	0	8	–	–	–	–	–	–	–
S-USA	26	20	0.461	21	17	0.627	0.090	0.010	0.223	−0.068	−0.007	0.809
SVK	11	0	–	2	0	–	–	–	–	–	–	–
SVN	26	28	0.892	4	9	0.267	2.001	0.404	0.001	0.139	0.030	0.260
SWE[d]	0	3	–	0	1	–	–	–	–	–	–	–
TUR	17	0	–	6	0	–	2.956	0.602	0.001	2.534	0.634	0.001

Notes: Definition of population codes: AUT, Austria; BLR, Belarus; CAN, Canada; CHE, Switzerland; CHN, China; COL, Colombia; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; GEO, Georgia; HRV, Croatia; IRL, Ireland; ITA, Italy; JPN, Japan; LTU, Lithuania; LTV, Latvia; N-USA, north-eastern United States; MEX, Mexico; POL, Poland; PRT, Portugal; RUS, Russia; S-USA, south-eastern United States; SVK, Slovakia; SVN, Slovenia; SWE, Sweden; TUR, Turkey.

[a]non-cc, nonclone-corrected dataset.

[b]cc, clone-corrected dataset.

[c]I_A, index of association.

[d]Due to small sample size (N [cc] < 6), these populations were excluded from population genetic analyses.

[e]$\bar{r}_d$, standardized index of association.

introduction event from America. Strong support is given for several introductions of the pathogen. Based on the STRUCTURE results, isolates with different genetic origins are present in Lithuania, with samples from the Curonian Spit belonging to a different cluster than isolates from other sites in the country. Two haplotypes were found to be shared between Germany (Bavaria region) and Lithuania (Palanga Botanical Garden and Curonian Spit region), supporting introduction of the pathogen from central Europe. Plant trade has also probably contributed to the spread the pathogen throughout northern Europe, as populations shared similar structure and haplotypes between the Baltic countries. In addition, one haplotype identified from the first *L. acicola* report in Belarus was also present in Estonia and Lithuania, and another in Estonia and Ireland. It is assumed that there have been several introduction events into Estonia, because for several years after the first report of the pathogen, only isolates with the MAT1-1-1 mating type were found in the country before those with MAT1-2 appeared (Adamson et al., 2015). Therefore, introduction of genetically different strains of the pathogen is probably the reason for the higher diversity observed in northern Europe.

In this paper the presence of *L. acicola* in Georgia was confirmed with sequence data, demonstrating the pathogen's continuing range expansion in western Asia. A previous report from Georgia (Kizikelashvili, 1987) was considered to be due to taxonomic confusion with the red band needle blight pathogen *Dothistroma* (Barnes et al., 2016; Matsiakh et al., 2018; Oskay et al., 2020). A unique allele shared only between Turkey and Georgia (allele 159, locus MD6, see Table S2) indicates a connection between these geographically close populations. The results of the STRUCTURE analyses show that isolates from the Black Sea coast of Russia, Turkey, and Georgia belong to the same cluster that also predominates in Croatia, Canada, and Mexico. Also, the isolate-based NJ tree indicates that strains from the western Asian populations are genetically close to the Mexican and north-east American populations. The ABC analyses supported the scenario where populations from western Asia and Croatia originated from northern America.

Croatia is known to host one of the oldest populations of *L. acicola* in Europe, with the first description dating from 1975 (Milatović, 1976). The results of the ABC analyses suggested that the cluster containing Croatia and western Asia is older than the central European one. It was unexpected that the Croatian and western Asian populations clustered together because of the distance between them. It is possible that there has been natural spread of the pathogen across the Balkan peninsula to Turkey and the Black Sea coast of Russia from Croatia. However, the spread of the pathogen is limited when reproducing asexually, and even if sexual recombination takes place there should be records of diseased pine stands from Croatia through to Turkey. Recently, *L. acicola* has been reported from Romania and Bulgaria (EPPO, 2018; Georgieva, 2020; Stamenova et al., 2018) but to date, there have not been any reports from Balkan countries that would indicate the overland dispersal of the pathogen. Possibly only one of the populations in the western Asian region could have a link with Croatia. In Croatia, as in Russia, only the *MAT1-2* idiomorph has been found. However, in Turkey and Georgia only the *MAT1-1-1* idiomorph was found. Therefore, it is possible that the Croatian and western Asian populations originated from separate introduction events and the similarities between these populations are due to their origin being genetically similar populations in America.

The presence of only one mating type (MAT1-2) and a shared haplotype between China and Japan from isolates obtained decades apart suggests that the spread of the pathogen in Asia is strongly affected by human activity and by the introduction of a limited number of strains. However, a larger sample size of the Asian populations is needed to confirm this. Huang et al. (1995) suggested that the East Asian populations have a south-east American origin, but Janoušek et al. (2016) found that the isolates from East Asia formed a unique group that is not part of the Southern lineage based on EF sequences. However, a more recent global phylogenetic study also placed East Asian *L. acicola* isolates (China, Japan, and Korea) together with isolates originating from South America, southern United States, and south-western Europe (van der Nest et al., 2019b). The microsatellite data obtained in the current study indicated similarities between samples from East Asia and Southern lineage populations (south-eastern USA and south-western Europe) as several alleles were present only in the named regions (see Table S2). Although the number of isolates from East Asia was too low to use in the ABC analyses, STRUCTURE placed East Asian and south-east United States samples into the same cluster, and both NJ trees, based on genetic distances between populations and individual isolates, indicated a close connection between East Asian and south-east United States isolates.

Only half of the observed populations contained both mating types and an even smaller number of populations contained mating types in equal proportions (see Table 3). Additionally, all populations with more than four isolates contained clones, highlighting the predominantly asexual reproductive mode of the pathogen. As is expected after an initial introduction event, in most cases the new, recently introduced populations were found to contain only one mating type. However, in Ireland and Poland, both mating types were found, although the pathogen was only recently found in these countries. Several studies have concluded that sexual recombination could take place in some European populations based on the occurrence of both mating types and in some cases supported by microsatellite analyses (Janoušek et al., 2016; Laas et al., 2019; Sadiković et al., 2019). Based on the isolates used in this study, random mating was indicated in Spain, south-eastern United States, Slovenia, and, interestingly, also in Croatia and the Czech Republic, although only the *MAT1-2* idiomorph was documented there. Recently, the sexual state of *L. acicola* was found in *P. radiata* in Spain (Mesanza et al., 2021), proving that sexual reproduction of the pathogen takes place in Europe. The occurrence of sexual reproduction, together with the presence of both pathogen lineages, in south-western Europe raises concerns about whether sexual recombination between the two lineages could take place, particularly as it has been suggested that the lineages could represent distinct species (Janoušek et al., 2016).

The occurrence of shared haplotypes between countries illustrates the importance of human activity in the spread of *L. acicola* in Europe and between continents. Identical haplotypes being found in Canada and the Munich Botanical Garden in Germany provide evidence for direct anthropogenic transmission of the pathogen across the Atlantic Ocean. Overall, most European populations demonstrated a subdivided structure, the cause of which could be multiple introduction events. In cases where the pathogen is already widespread in the country, it is increasingly important to avoid new introductions and mixing of pathogen strains that originate from genetically different populations. Repeated introductions from genetically different populations could have serious outcomes as they increase the genetic diversity of the pathogen populations and create possibilities for the emergence of new haplotypes that may be more virulent or adapted to certain climate conditions (McDonald & Linde, 2002; Molofsky et al., 2014). Therefore, proper phytosanitary control measures, particularly effective quarantine rules and diagnostic methods, are needed to avoid pathogen introductions via plant trade.

In some cases, phytosanitary measures have proved to be effective in controlling pathogen spread, although it is difficult to eradicate the pathogen completely. In the Tallinn Botanic Garden, northern Estonia, fungicides were regularly used after the first detection of *L. acicola* (Kaur & Hermann, 2021), and although the pathogen is now widespread across Estonia, the haplotypes found in the botanical garden have not been detected in other Estonian locations (Laas et al., 2019). In Lithuania, after the first identification of the disease on the Curonian Spit, all heavily infected trees were felled and burned (Markovskaja et al., 2011). From the samples included in this study, the cluster present in the Curonian Spit region is contained there, although two isolates in the Palanga Botanical Garden, on the Lithuanian mainland, showed similar STRUCTURE clustering.

In numerous countries, the first records of *L. acicola* originate from non-native host species in city greeneries, botanical gardens, and arboreta (for example, see Cleary et al., 2019; Drenkhan & Hanso, 2009; Golovchenko et al., 2020; Mullett et al., 2018; Oskay et al., 2020). However, recently there have been a growing number of records of *L. acicola* from native *Pinus sylvestris* stands across Europe (Adamson et al., 2018a; Cech & Krehan, 2008; EPPO, 2012, 2015; Georgieva, 2020). In Bulgaria severe damage with defoliation from 50% to 100% has been reported from several *P. sylvestris* and *Pinus nigra* stands near the initial outbreak site, despite control measures being implemented after the initial discovery of the disease (Georgieva, 2020). A population study carried out by Laas et al. (2019) recorded the presence of potentially more pathogenic haplotypes in Estonia infecting both non-native *Pinus mugo* and native *P. sylvestris*. The results of the current analyses show that one of the potentially more aggressive haplotypes (MLH 196) is present in Estonia, Lithuania, Belarus, and Turkey. In Turkey, it was found on a *P. sylvestris* tree suffering high infection severity, with up to 80% of the canopy affected (Oskay et al., 2020). However, although this haplotype may have the potential to be a threat to the extensive natural stands of *P. sylvestris* in northern Europe, it may not demonstrate the same effect in southern regions because several reports and inoculation tests have indicated that southern and northern provenances of *P. sylvestris* have differing susceptibility to *L. acicola* (Jankovský et al., 2009; Phelps et al., 1978; Skilling & Nicholls, 1974).

The results of this study have indicated that pathogen strains with different origins exist in proximity in Europe. In addition, strong support is given for human activity (i.e., plant transportation) supporting the range expansion of the pathogen and leading to the co-existence of genetically different strains. At some level, sexual reproduction also takes place in the European populations. Recombination of different strains could lead to further increases in genetic diversity and produce more virulent strains. Many populations in Europe still contain a single mating type, are structurally homogeneous, have low genetic diversity, and only comprise one lineage. Therefore, it is important not only to avoid further human-mediated spread of the pathogen, but also to avoid mixing of populations. Continual monitoring of *L. acicola* will be needed to follow developments in the geographic spread, host range expansions, and ongoing changes in the genetic diversity, with a special focus on maintaining the extensive stands of native pine species in Europe.

4 | EXPERIMENTAL PROCEDURES

4.1 | Sample collection, fungal isolation, DNA extraction, and isolate identification

Needle samples with typical symptoms of BSNB were collected from a variety of *Pinus* taxa and from *C. libani*. Samples were obtained from a maximum of 30 sampling sites per country from a total of 27 countries in North and South America, Europe, and Asia (Figure 1, Table S1). Sampling sites in the same country were merged and referred to as populations, except for the United States, where samples were divided into two populations—north-eastern United States (N-USA) and south-eastern United States (S-USA).

Samples were collected from the lower parts of the tree canopy, placed in paper or plastic bags, and kept dry or stored at −20°C until pathogen isolation. Some isolates were obtained from culture collections (see Table S1). In most cases, one fungal isolate per sampled tree was obtained, except for Croatia, Munich Botanical Garden in Germany, Italy, Russia, Slovenia, Sweden, Turkey, Curonian Spit sampling sites in Lithuania, and the Mississippi sampling site in the United States, where up to six isolates per tree were obtained. Some of the data of isolates used in this study have been previously published in the context of country-specific population studies or new country and host records (Table S1), and DNA or mycelium for these isolates was received to be included in this study.

Isolations to pure culture were made according to Mullett and Barnes (2012). Isolates were grown in the dark at room temperature (21°C) on pine needle agar medium. The medium consisted

of 1 L filtered Scots pine needle extract (50 g fresh weight/L tap water boiled for 20 min), 15 g malt extract (Oxoid), and 15 g technical agar (Biolife) autoclaved at 106°C for 30 min (see Drenkhan et al., 2013). Approximately 0.04 g of mycelium from the colony edge was transferred to a 2-ml microcentrifuge tube and stored at −20°C until DNA extraction. Mycelium was homogenized with an MM400 homogenizer (Retsch GmbH) using metal beads (2.5 mm diameter). DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific) or as specified in Sadiković et al. (2019) for Croatian and Slovenian samples, in Raitelaitytė et al. (2020) for Polish samples, and in Mullett et al. (2018) for Portuguese samples.

L. acicola identity was confirmed by PCR with species-specific primers Latef-F and Latef-R (Ioos et al., 2010). The PCRs were performed in 20-μl reaction volumes. Cycling conditions were chosen according to Ioos et al. (2010) with modifications according to Drenkhan et al. (2014a). All PCRs were carried out using a Tprofessional thermocycler (Biometra). PCR products were visualized on a 1% agarose gel (SeaKem LE agarose) under UV light using a Quantum ST4-system (VilberLourmat SAS).

The ITS region of the *L. acicola* isolates obtained from Georgia, Mexico, and Colombia was sequenced in order to confirm the species identity and exclude the presence of other *Lecanosticta* species (Theron et al., 2022; van der Nest et al., 2019b). The ITS PCR was performed using the fungal-specific PCR primers ITS1-F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990). ITS PCR products were sequenced in a single direction using the primer ITS5 (White et al., 1990).

In addition, the *TEF1* region of a random selection of 15 isolates from Europe and North America was sequenced. PCR amplification and sequencing in both directions was done using the primers EF1-728F (Carbone & Kohn, 1999) and EF2 (O'Donnell et al., 1998). All PCR products were sequenced at the Estonian Biocentre in Tartu. Sequences were edited using BioEdit v. 7.2.5. and BLAST searches for the fungal taxa were performed in GenBank (NCBI).

4.2 | Haplotype identification

For multilocus haplotyping, 11 microsatellite markers were used: MD1, MD2, MD4, MD5, MD6, MD7, MD8, MD9, MD10, MD11, and MD12 (Janoušek et al., 2014). The PCR amplification conditions were as described in Janoušek et al. (2014, 2016). For fragment analysis, PCR products were pooled into two panels according to Janoušek et al. (2014) and run on a 3130XL genetic analyser (Applied Biosystems) with 500 LIZ Size Standard (Applied Biosystems) at the Estonian Biocentre in Tartu. Alleles were scored using GeneMapper v. 5.0 (Applied Biosystems).

Isolates with identical alleles at all microsatellite loci were considered clones. Two datasets were created: one containing all isolates (non-cc) and the other containing only one of each haplotype (cc) per population as defined in Table 1.

4.3 | Genetic data analyses

4.3.1 | Genetic diversity

The non-cc dataset was used to calculate the total number of haplotypes using GenAlEx v. 6.5 (Peakall & Smouse, 2012). The cc dataset was used to calculate the total number of alleles and unique alleles, the mean number of different alleles (Na), the mean haploid genetic diversity (*h*), and the mean unbiased diversity (uh) for each population using GenAlEx 6.5. The cc dataset was used to calculate the allelic richness (A_R, the number of distinct alleles in the population) and the private allelic richness (P_{AR}, the number of unique alleles in the population) in ADZE v. 1.0 (Szpiech et al., 2008). Because sample sizes across populations differed, a rarefaction approach was used with population sizes standardized to six (Szpiech et al., 2008). The clonal fraction was calculated for each population according to Zhan et al. (2003).

Due to low sample size (N [cc] < 6), 12 populations (BLR, CHN, COL, GEO, IRL, ITA, JPN, LTV, MEX, PRT, SVK, and SWE) were excluded from further population genetic analyses, unless otherwise stated.

An AMOVA was performed in GenAlEx v. 6.5 on the cc dataset to test for significant differentiation between populations.

4.3.2 | Isolation by distance

Mantel tests, conducted in GenAlEx v. 6.5, were used to test for isolation by distance on the cc dataset using Nei's genetic distance (Nei, 1972, 1978) and geographic distances. In total, three different analyses were performed for separate sampling regions. First, isolation by distance was tested among all populations. Next, isolation by distance was tested separately for the populations in North America and then for populations in Europe, in order to assess if the genetic distance between populations increases with distance. Generally, we would expect genetic differentiation to increase with distance in native populations. For introduced populations, we would expect a lack of isolation by distance.

For visualization of Nei's genetic distances and geographic distances, principal coordinates analysis (PCoA) was carried out in GenAlEx v. 6.5 using the covariance standardized method.

4.3.3 | Population clustering

The program STRUCTURE v. 2.3.4 (Falush et al., 2003) was used to estimate the most likely number of population clusters (*K*), assign isolates into genetically different groups, and thereby determine the structure within populations without any prior data on geographic location or host provided. For the STRUCTURE analysis the cc dataset was used. Each of 20 independent runs of $K = 1–25$ were carried out with 10,000 burn-in iterations followed by a run of 100,000. The most likely number of clusters (*K*) was determined using the

ln(Pr(X|*K*)) method (Pritchard et al., 2000, 2010) and the Δ*K* statistic (Evanno et al., 2005) in CLUMPAK (Kopelman et al., 2015). The final assignment of individuals to clusters was carried out on the optimum *K* by applying 100,000 burn-in iterations, followed by 1,000,000 runs. For each number of clusters (*K*), 20 independent runs were performed.

4.3.4 | Phylogenetic analysis

Phylogenetic relationships among the populations were inferred with POPTREE v. 2 (Takezaki et al., 2010) using the NJ method (Saitou & Nei, 1987) based on allele frequency of a cc dataset containing all 28 populations. A bootstrap test with 10,000 replications was run for the NJ tree to generate confidence at branch points.

Additionally, an NJ tree was constructed using all isolates to assess the genetic distance between isolates, independent of the geographic origin or population. For that purpose, the genetic distance between all isolates was calculated in POPULATIONS v. 1.2.31 (Langella, 2002) using Nei's standard distance D_A (Nei et al., 1983). The obtained distance matrix was used to construct an NJ tree in MEGA X (Kumar et al., 2018). Isolates were coloured as indicated by the population clustering results obtained with STRUCTURE according to $K = 7$ (Figure 4).

4.3.5 | Modelling of demographic history

To reconstruct the history of divergence among the observed *L. acicola* populations, ABC was performed on the cc dataset using DIYABC v. 2.1.0 (Cornuet et al., 2014). The STRUCTURE clusters (Figure 4) were considered to develop scenarios describing the demographic history between the main regions: north-eastern America (N-AME), south-eastern United States (S-USA), northern Europe (N-EUR), central Europe (C-EUR), south-western Europe (SW-EUR), Croatia (HRV), and western Asia (W-ASIA) (Figure 4). The populations from East Asia, Mexico, and Colombia were excluded from the analysis due to their small sample size.

As there are numerous possibilities for the origin of *L. acicola* in the observed regions, a stepwise procedure was used to address questions around historical scenarios (Konečný et al., 2013). The best scenario obtained in the first step was used to inform the scenarios of the next step. In total, three different analyses with various scenarios were performed. In Analysis 1 the relationship among the three main clusters in America and Europe was investigated, Analysis 2 was conducted to elucidate the origin of northern European populations, and Analysis 3 was conducted to investigate the origin of central Europe and the cluster dominating in Croatia and western Asia. In all scenarios, a bottleneck was modelled at the foundation of populations and the introduced populations were assumed to be isolated from each other and from source populations after the introduction events. A description of the analyses performed and scenarios is presented in Table S5.

Analysis 1: As Europe breaks into two clusters at $K = 3$, for the first analysis central Europe (C-EUR), Croatia (HRV), and western Asia (W-ASIA) were merged into EUR (Figure 4). The presence of an unsampled population was also considered as a possibility of populations originating from areas not represented in this study. Therefore, in Analysis 1 a set of 20 scenarios were included to test whether N-AME and S-USA were derived separately from an ancestral population (S1.1–S1.7, S1.19) or if one region was derived from the other (S1.8–S1.18). For the European populations the following were tested: if SW-EUR and EUR originated from an ancestral population (S1.1, S1.20), if SW-EUR originated from S-USA (S1.2, S1.5, S1.8, S1.9, S1.14, S1.15), from an admixture event between S-USA and N-AME (S1.3, S1.6, S1.10, S1.11, S1.16, S1.17), or from an admixture event between S-USA and EUR (S1.4, S1.7, S1.12, S1.13, S1.18, S1.19), and whether EUR was derived directly from N-AME (S1.2, S1.3, S1.4, S1.8, S1.10, S1.12, S1.14, S1.16, S1.18) or from an admixture event between N-AME and an unsampled population (S1.5, S1.6, S1.7, S1.9, S1.11, S1.13, S1.15, S1.17, S1.19) or if the American populations were derived from EUR and SW-EUR (S1.20).

Analysis 2: According to STRUCTURE clustering and their close geographical distance, populations EST, LTV, LTU, POL, BLR, IRL, and SWE were merged into the northern Europe (N-EUR) region for migration analyses. The question about the origin of *L. acicola* in N-EUR was tested in Analysis 2 via a set of six scenarios. The scenarios tested if N-EUR originated from N-AME (S2.1), EUR (S2.3), an unsampled population (S2.2), or an admixture event between these regions (S2.4, S2.5, S2.6).

Analysis 3: Subsequently, as the origin of the main clusters in Europe according to $K = 3$ was revealed, the demographic history between subclusters of central Europe (C-EUR), Croatia (HRV), and western Asia (W-ASIA) was explored based on seven scenarios in Analysis 3. The scenarios tested whether HRV and W-ASIA originated from N-AME (S3.5, S3.6), C-EUR (S3.2), an unsampled population (S3.1, S3.4), or an admixture event between these regions (S3.3, S3.7), and similarly whether C-EUR originated directly from N-AME (S3.1, S3.3), from an admixture event between N-AME and an unsampled population (S3.2, S3.4, S3.5, S3.7), or from an admixture event between HRV or W-ASIA with an unsampled population (S3.6).

Initially, the demographic priors of the tested scenarios were set with a broad range. After 100,000 preliminary runs, the prior checking option was used according to the DIYABC manual and prior distributions adjusted step by step and finally set up as mentioned in Table S6. The generalized stepwise model was followed for the microsatellite loci and default values for the mutation model parameters were used (Cornuet et al., 2014). Ten microsatellite markers (MD1, MD2, MD4, MD5, MD7, MD8, MD9, MD10, MD11, and MD12) were used in the ABC analyses.

For each simulation the commonly used genetic summary statistics were used (i.e., mean number of alleles and mean size variance for one sample and mean number of alleles, mean genetic diversity, F_{ST}, classification index, and $(d\mu)^2$ distance for two sample summary statistics). The obtained times of events are in generations and roughly one year represents one generation. Other statistics available in DIYABC were later used in model checking.

One million datasets were simulated for each scenario. The posterior probability of each scenario was estimated by polychotomous logistic regression on 1% of the simulated datasets closest to the observed dataset, transformed by linear discriminant analysis (Cornuet et al., 2014). Posterior distributions of parameters were estimated for the most supported scenario by the logit transformation of parameters and linear regression on 1% of the closest simulated datasets. Model checking was done using the summary statistics not used in model selection as recommended by Cornuet et al. (2010). Confidence in scenario choice was evaluated by analysing simulated pseudo-observed datasets with the same number of loci and individuals as a real dataset. One hundred pseudo-observed datasets were simulated for each scenario with parameter values taken from the same distributions as for previous ABC analyses.

4.4 | Mating type determination and mode of reproduction

Mating types of the isolates were determined using a set of primers developed by Janoušek et al. (2014). PCRs were carried out in 20-μl volumes according to Janoušek et al. (2014), with an initial denaturation step at 95°C for 12 min as described in Adamson et al. (2015). PCR products were visualized with gel electrophoresis. The presence of the *MAT1-1-1* and the *MAT1-2* idiomorph was indicated by an amplicon size of 560 bp and 288 bp, respectively.

To evaluate the possibility of sexual recombination in the populations, three tests were carried out on both the non-cc and cc datasets for populations with at least six isolates. In order to assess if the populations deviate significantly from the null hypothesis of a 1:1 ratio of mating types, the exact binomial test was used as described in Barnes et al. (2014). In addition, the index of association (I_A) and the standardized index of association ($\bar{r}_d$) were calculated in the R package poppr (Kamvar et al., 2014) to test for random mating in the populations. Both analyses involved comparing the values for the observed dataset with the values for 1,000 artificially recombined datasets.

AUTHOR CONTRIBUTIONS

Sample collection: M.L., K.A., I.B., J.J., M.S.M., K.A., M.A., L.B., H.B., T.S.B., P.C., T.C., M.C., L.G., L.J., S.M., I.M., J.B.M., F.O., B.P., K.R., D.S., and R.D. Laboratory and data analyses: M.L., K.A., and K.R. Manuscript preparation and writing: M.L., K.A., I.B., J.J., M.S.M., M.C., R.E, D.S., and R.D. All authors have read and agreed to the submitted version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

All the ITS and EF sequences are deposited into GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with accession numbers listed in Table S1. Isolates of *L. acicola* are stored in the Laboratory of Forest Pathology in the Estonian University of Life Sciences and in the Fungal Culture Collection, Estonian University of Life Sciences. All the relevant data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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