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Genetic diversity of the pine pathogen *Lecanosticta acicola* in Slovenia and Croatia

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Brown spot needle blight (BSNB), a disease of pine trees caused by the fungus *Lecanosticta acicola*, has been known in Slovenia since 2008 and in Croatia since 1975. Recent outbreaks in Slovenia prompted this study to compare *L. acicola* populations in these two neighbouring European countries. Sixty-nine isolates collected from three pine species (*Pinus mugo*, *P. halepensis* and *P. nigra*) were used to determine the phylogenetic relationships, genetic structure, and reproductive strategy of the pathogen. *EF1-α* sequences showed that Slovenian and Croatian isolates share a common ancestry with individuals from central and northern Europe. Population structure analysis revealed four distinct population clusters of *L. acicola* in these two countries, generally corresponding to their respective geographic location and host. An unequal ratio of mating types and a low overall genetic diversity in the population indicated a strong influence of asexual reproduction. Although some of the oldest recorded European occurrences of BSNB are from Croatia, this study provided no evidence that the population studied in Croatia was the source of the sampled outbreaks in Slovenia. Recent outbreaks of *L. acicola* in Slovenia are most likely due to introductions from other, yet to be identified, sources.

Keywords: brown spot needle blight, elongation factor, host preference, mating type, population genetics, *Pinus*

Introduction

Lecanosticta acicola (formerly *Mycosphaerella dearnessii*; *Scirrhia acicola*) is a foliar pathogen that causes brown spot needle blight (BSNB) of pine trees. Infected needles show yellow to brown spots or bands of discolouration, become necrotic, and are heavily shed. Severe infection can lead to tree death (Anonymous, 2015). *Lecanosticta acicola* has been reported in over 30 countries on a wide range of pine species (CABI, 2016) and more recently, there have been increasing numbers of reports from Europe, indicating its spread in this region (Adamson *et al.*, 2018; Mullett *et al.*, 2018).

It was previously hypothesized that Central America could be the centre of origin of *L. acicola* (Evans, 1984). Of the nine species that have been described in *Lecanosticta*, eight are known only from Mesoamerica, suggesting that this region is a centre of diversity for the genus (van der Nest *et al.*, 2019). The latter study using DNA sequence data also failed to confirm the presence of *L. acicola* in Central America. The pathogen is believed to be native to North America (van der Nest *et al.*, 2019) from where it has spread to other continents, such as Europe (Janoušek *et al.*, 2016).

Three lineages of *L. acicola* have been identified based on pathogenicity, morphology and RAPD markers, as well as phylogenetic and microsatellite analyses of isolates from a global collection (Kais, 1972; Huang *et al.*, 1995; Janoušek *et al.*, 2016; van der Nest *et al.*, 2019). The first of these lineages (or 'northern lineage') is represented by isolates collected in the northern USA, Canada, and central and northern Europe (Huang *et al.*, 1995; Janoušek *et al.*, 2016; van der Nest *et al.*, 2019). The second lineage (or 'southern lineage') comprises isolates from the southern USA, Spain, France, Colombia and Asia (Huang *et al.*, 1995; Janoušek *et al.*, 2016; van der Nest *et al.*, 2019). These two lineages represent the source of all European *L. acicola* populations (Janoušek *et al.*, 2016). The third lineage is represented by isolates only from Mexico (van der Nest *et al.*, 2019).

The first record of *L. acicola* in southeast Europe was in 1975 in Crvena Luka, Croatia, on 15-year-old *Pinus halepensis* trees (Milatović, 1976). The severely affected plantation, covering an area of 1.5 ha, was treated by

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applying silvicultural measures in combination with multiple fungicide applications, resulting in the suppression of the pathogen spread but not its full eradication (Glavaš, 1979). The pathogen has subsequently been found throughout this same area, with varying levels of virulence (Glavaš, 1979; Glavaš & Margaletić, 2001). According to Glavaš & Margaletić (2001), the largest infected area in Croatia was around Kožino (36 km from Crvena Luka), with over 500 ha of *P. halepensis* heavily affected by BSNB.

In Slovenia, BSNB was first detected in 2008 in Bled and in 2009 in Ljubljana on *P. sylvestris* and *P. mugo* (Jurc & Jurc, 2010). Both findings appeared to be isolated incidents and infected trees were removed, with no subsequent new infections reported from those locations. During 2014–2016, the disease was found at several locations throughout Slovenia on *P. nigra*, *P. sylvestris* and *P. mugo* (Hauptman & Sadiković, 2015; authors' personal observations).

In the bordering countries to the west and north of Slovenia, BSNB has been reported in Italy (La Porta & Capretti, 2000) and Austria (Kirisits & Cech, 2012). Other reports of the disease from nearby countries in this part of Europe, such as from Bulgaria and Greece, are now considered to be caused by *Dothistroma* species (Mullett *et al.*, 2018).

Lecanosticta acicola is a heterothallic ascomycete (Janoušek *et al.*, 2014). Sexually produced ascospores are responsible for long-range dispersal, while the conidia provide more localized infection and spread such as via rain splash (Skilling & Nicholls, 1974). The sexual state has been recorded only in Colombia, Central America and southern USA (Kais, 1971; Evans, 1984). However, in light of the new evidence regarding the global distribution of *L. acicola* (van der Nest *et al.*, 2019), all previous findings based exclusively on morphological studies, especially in Central America, must be confirmed with DNA sequence data. Although evidence for sexual recombination has been alluded to in certain European *L. acicola* populations based on microsatellite data (Janoušek *et al.*, 2016), all reported BSNB infections in Europe have been linked only to conidial dispersal.

Reports of *L. acicola* populations possibly undergoing sexual recombination in central Europe (Janoušek *et al.*, 2016), and the increase in reports of BSNB during the last 3 years in Slovenia, have raised questions regarding the reproductive mode and population structure of *L. acicola* in this region. The aims of this study were thus (i) to confirm the identity of the *Lecanosticta* species in Slovenia and Croatia; (ii) to investigate the genetic diversity and population structure of the pathogen in Slovenia and Croatia; (iii) to determine whether the populations are undergoing sexual recombination; and (iv) to determine if the current population in Croatia, where the pathogen was first reported in the region, could be the source of recent outbreaks in Slovenia.

Materials and methods

Sample collection, isolation and identification

During 2014–2016, needles were collected from infected *P. mugo, P. nigra* and *P. halepensis* trees from four locations in Slovenia and Croatia where BSNB was reported to be present (Table 1; Fig. 1). Needles collected from each tree were sealed in paper envelopes and placed in cold storage at 4 °C. Only needles with symptoms and visible conidiomata were chosen for isolation, up to three isolations per tree, and up to 10 trees per location, were selected using the single spore isolation method described by Barnes *et al.* (2004). Cultures were maintained on dothistroma sporulation medium (DSM: 2% malt extract, 1.5% Difco agar (Becton Dickinson), 0.5% yeast extract (Sigma-Aldrich) and 1% streptomycin solution (Sigma-Aldrich)) at 21 °C. In addition, *L. acicola* cultures collected previously in 2009 from Bled, and in 2009 and 2013 from Ljubljana (Jurc & Jurc, 2010), were purified to single-spore isolates and included in this study. Representative isolates are maintained at the culture collection of the laboratory of Forest Protection at the Slovenian Forestry Institute (ZLVG) and at the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA was extracted from pure fungal cultures using the technique described by Goodwin *et al.* (1992). The concentration of DNA extracts and their purity was measured using a ND-1000 spectrophotometer (NanoDrop Technologies) and diluted to approximately 30 ng μL^{-1} working stock. Prescreening for *L. acicola* isolates was carried out using the conventional PCR primers designed by Ioos *et al.* (2010). Amplification of a *c.* 237 bp fragment of the elongation factor 1-α (*EF1-α*) gene confirmed the presence of *L. acicola*, and these isolates were used in further analyses for species confirmation using sequencing and microsatellite analyses.

Table 1 Information on *Lecanosticta acicola* isolates used in this study.

Country	Locality	Cluster[a]	Host	*N*[b]	Date collected	Collectors
Slovenia	Tolmin	A	*Pinus nigra*	12	2016	N. Ogris, D. Jurc
	Trenta	B, C	*Pinus mugo*	18	2014, 2015	D. Sadiković, T. Hauptman
	Bled	C	*P. mugo*	4	2009	D. Jurc
	Čatež	C	*P. mugo*	5	2015	T. Hauptman
	Ljubljana	C	*P. mugo*	4	2009, 2013	D. Jurc
Croatia	Kožino	D	*Pinus halepensis*	26	2015	D. Sadiković, D. Diminić

[a]Assignment of clusters based on DAPC analysis (see Fig. 4).
[b]Number of isolates obtained from each site.

Figure 1 Six sampling locations in Slovenia and Croatia from where *Lecanosticta acicola* isolates were obtained.

Phylogenetic analyses

The *EF1*-α gene was amplified for all putative *L. acicola* isolates using the same reactions and conditions as described by Barnes *et al.* (2004). Amplified products were cleaned with the Wizard SV gel and PCR clean-up system (Promega) and sequenced in both directions at the GATC Biotech Sanger Sequencing Facility in Germany using the same primers as in the PCRs. Sequences were edited in BioEdit v. 7.2.0. (Hall, 1999) and deposited in GenBank (MF872253–MF872269). The sequence dataset was aligned using the muscle algorithm in mega v. 6.0 (Tamura *et al.*, 2013).

Maximum parsimony (MP) analysis was conducted using paup v. 4.0b10 (Swofford, 2003). Phylogenies were constructed using the heuristic search option based on parsimony, with a random stepwise addition of 1000 replicates with tree bisection reconnection (TBR) selected as the branch swapping algorithm. Gaps were considered as an additional character with equal value. Branching point confidence levels were ascertained using 1000 bootstrap replicates.

For maximum likelihood (ML) analysis, PhyML v. 3.0 (Guindon *et al.*, 2010) was used. Generalized time reversible (GTR) substitution model was selected as the most appropriate substitutional model using PhyML smart model selection (Lefort *et al.*, 2017). Confidence levels for nodes were assessed with 1000 bootstrap replicates.

Bayesian inference (BI) analysis was performed using MrBayes v. 3.2 (Ronquist *et al.*, 2012). The GTR substitution model with gamma-distributed rate variation across sites, and the proportion of invariable sites was selected. Four Markov chain Monte Carlo (MCMC) runs were initiated with 3 000 000 generations and six independent runs. Trace analysis was performed using tracer v. 1.6 (Rambaut & Drummond, 2013) and 15% of the initial steps were discarded as burn-in. For all analyses, *Dothistroma septosporum* and *D. pini* were used as the out-group taxa.

The data underpinning the above-mentioned phylogenetic analyses are deposited in Zenodo (https://doi.org/10.5281/zenodo.2554827).

Microsatellite amplification and dataset arrangement

All isolates were screened with 11 previously developed microsatellite markers for *L. acicola* (Janoušek *et al.*, 2014). Reaction conditions and PCR amplification protocols were the same as those described by Janoušek *et al.* (2014), with the exception that the primer annealing temperature for MD6 was reduced to 60 °C, and increased to 60 °C for MD11.

Based on the lengths and colours of the fluorescent dyes, the PCR amplicons were pooled into two panels for fragment analysis: MD1, MD2, MD6, MD7 and MD9 in panel 1; and MD4, MD5, MD8, MD10, MD11 and MD12 in panel 2. Pooled PCR amplicons were run on the Applied Biosystems 3730xl DNA Analyzer and sized with GeneScan 500 LIZ size standard. Allele sizes were determined using the GeneMapper v. 4.1 software (Applied Biosystems).

Two datasets, based on microsatellite allele sizes, were generated for the analyses; a clone-corrected and non-clone-

corrected dataset. The clone-corrected dataset, which comprised populations consisting of a single individual representing each unique multilocus haplotype (MLH), was used to analyse population structure and to calculate gene diversity, allelic richness and private allele richness. The non-clone-corrected dataset was used to calculate measures of haplotypic diversity. Both datasets were used to test for recombination in the populations.

Population structure

STRUCTURE v. 2.3.4 was used to detect and depict population clustering, based on microsatellite allele sizes (Falush *et al.*, 2003). The software applies a Bayesian model-based clustering algorithm (MCMC) to assign individuals to a specific number of clusters (K), using multilocus haplotype data generated with unlinked genetic markers. Thirty independent runs of $K = 1$–10 were performed to estimate the optimal number of clusters. Each run consisted of 100 000 burn-in iterations, followed by 500 000 MCMC iterations. The optimal number of clusters was estimated using STRUCTURESELECTOR (Li & Liu, 2018), which implements the Evanno method (Evanno *et al.*, 2005), as well as four alternative statistical measures (MEDMEDK, MEDMEAK, MAXMEDK and MAXMEAK) that have shown improved accuracy when analysing populations with an uneven sample size (Puechmaille, 2016). The convergence of all 30 STRUCTURE runs was performed in CLUMPP (Jakobsson & Rosenberg, 2007) with the 'Greedy' algorithm set to calculate pairwise symmetric similarity coefficient (SSC) for the identification of similar runs (SSC > 0.9) in 10 000 random input orders of runs. The final output was visualized in DISTRUCT (Rosenberg, 2004).

In addition to the Bayesian approach, exploratory discriminant analysis of principal components (DAPC) was implemented in the ADEGENET package in R STUDIO v. 3.3.2 (Jombart *et al.*, 2010) to visualize the population genetic structure. DAPC minimizes the differences within groups and maximizes the differences between groups, using discriminant analysis subsequent to principal components analysis, and without assumptions of Hardy–Weinberg and linkage equilibrium within the populations (Jombart *et al.*, 2010). The optimal number of clusters was determined using the 'find.clusters' function, with Bayesian information criterion (BIC) applied for assessing the best supported model. The optimal number of retained principal components was assessed using function 'xvalDapc', according to cross-validation metric (Jombart *et al.*, 2010).

Hierarchical analysis of molecular variance (AMOVA), using GENALEX v. 6.5 (Peakall & Smouse, 2012), was performed to evaluate the extent of population differentiation and structure among populations, hosts species groups, and within these groups.

Genetic diversity

Gene diversity per population was calculated using FSTAT v. 2.9.3 (Goudet, 2002). The clonal fraction (CF), defined by Zhan *et al.* (2003) as the proportion of fungal individuals derived as a result of asexual reproduction, was calculated as 1 − [(number of unique haplotypes)/(total number of isolates)]. Haplotypic diversity, defined as the likelihood that two individuals sampled at random have different haplotypes (with values ranging from 0 to 1, where the latter represents the maximum diversity of individuals) was calculated using MULTILOCUS v. 1.3b (Agapow & Burt, 2001). Allelic richness (A_R), defined as the number of distinct alleles in a population and private allelic richness (PA_R), defined as the number of private (not found in other populations) alleles in the population, were estimated using ADZE (Szpiech *et al.*, 2008).

Wright's fixation index (F_{ST}), and Nei's unbiased genetic distance (D_a) (Nei, 1978), used for assessment of genetic differences (and relationships) between populations, were estimated in GENALEX v. 6.5.

Mating type determination and mode of reproduction

The mating types of isolates were determined using the primer pairs MdMAT1-1F, MdMAT1-1R and MdMAT1-2F, MdMAT1-2R, described by Janoušek *et al.* (2014). Amplifications were achieved in 12.5 μL volume reactions, using 5 μL AmpliTaq Gold 360 PCR Master Mix (Applied Biosystems), 0.2 μL 360 GC Enhancer (Applied Biosystems), 0.25 μL of each 10 mM primer pair, 3.3 μL H_2O and 3 μL of working DNA stock. Touchdown PCR was used with an initial cycle set of 95 °C for 10 min, nine cycles of 95 °C for 30 s, 67 °C for 45 s with a temperature drop of 1 °C after each cycle, and 72 °C for 60 s. This was followed by a second cycle set of 29 cycles of 95 °C for 30 s, 57 °C for 45 s with a time increment of 5 s after each cycle, 72 °C for 60 s, ending with a final elongation at 72 °C for 10 min. Mating types were determined using gel electrophoresis based on the expected size of the PCR products (560 bp for MAT1-1; 288 bp for MAT1-2).

To determine the extent of recombination or clonality in populations, tests for linkage disequilibrium (LD) were calculated based on the index of association (I_A) and $\bar{r}_d$ in MULTILOCUS v. 1.3b using clone-corrected and non-clone-corrected datasets. The $\bar{r}_d$ is a modification of I_A that removes the dependency on the number of loci and is considered a more reliable measure of association (Agapow & Burt, 2001). The observed values of indices of LD were compared with the data produced by 1000 randomizations to simulate random mating. The hypothesis of non-random mating is significant where $P \leq 0.05$.

Parsimony tree-length permutation test (PTLPT) builds trees from the MLH data using parsimony in PAUP v. 4.0b10 (Swofford, 2003) and compares the observed tree lengths to the lengths of a randomized dataset that simulates sexually reproducing populations. The test relies on the fact that all regions of the genome are similarly inherited under clonality. Thus, asexually reproducing populations would have fewer, shorter, well-resolved trees, unlike sexually reproducing populations, which would display longer tree lengths and more complex trees (Burt *et al.*, 1996). The null hypothesis of sexual reproduction is supported when $P \geq 0.05$. The randomized dataset was created in MULTILOCUS v. 1.3 using 1000 permutations.

Results

Isolates

From the six sampling locations (Fig. 1), 69 isolates were obtained and confirmed to be *Lecanosticta* using the conventional PCR primers. Isolates were obtained from three different pine tree species (*P. mugo*, *P. nigra* and *P. halepensis*) found in a national park in Trenta, tree groves in Tolmin, trees planted as ornamentals in Čatež and Ljubljana (all Slovenia) and in old tree plantations in Kožino (Croatia; Fig. 2).

Phylogenetic analysis

Seventeen selected isolates, representing all sampling locations, were confirmed as *L. acicola* based on phylogenetic analysis using the *EF1-α* sequences (*c.* 490 bp).

In the MP analysis, of the 226 aligned nucleotides, 144 characters were constant, 61 were parsimony-informative, and 21 parsimony-uninformative characters. Tree length, consistency index, homoplasy index, retention index and rescaled consistency index were 93, 1.0, 0, 1.0 and 1.0, respectively.

Phylogenies generated in ML, MP and BI analyses all had the same topology, and the MP tree was chosen for representation (Fig. 3). Isolates from Slovenia and Croatia all grouped in lineage 1 or the northern lineage of *L. acicola* (Janoušek *et al.*, 2016; van der Nest *et al.*, 2019). Isolates from Slovenia were identical in sequence to the ex-type isolate (KC013002) of *L. acicola*, while those from Croatia all contained a unique fixed single base polymorphism that formed a subclade within lineage 1 (Fig. 3).

Microsatellite amplification

Of the 11 microsatellite markers amplified, MD2, MD1, MD9 and MD11 were monomorphic in all the isolates. One marker, MD8, was discarded because it produced stutter in the fragment analysis and the allele sizes could not be scored with confidence. In total, 684 alleles were

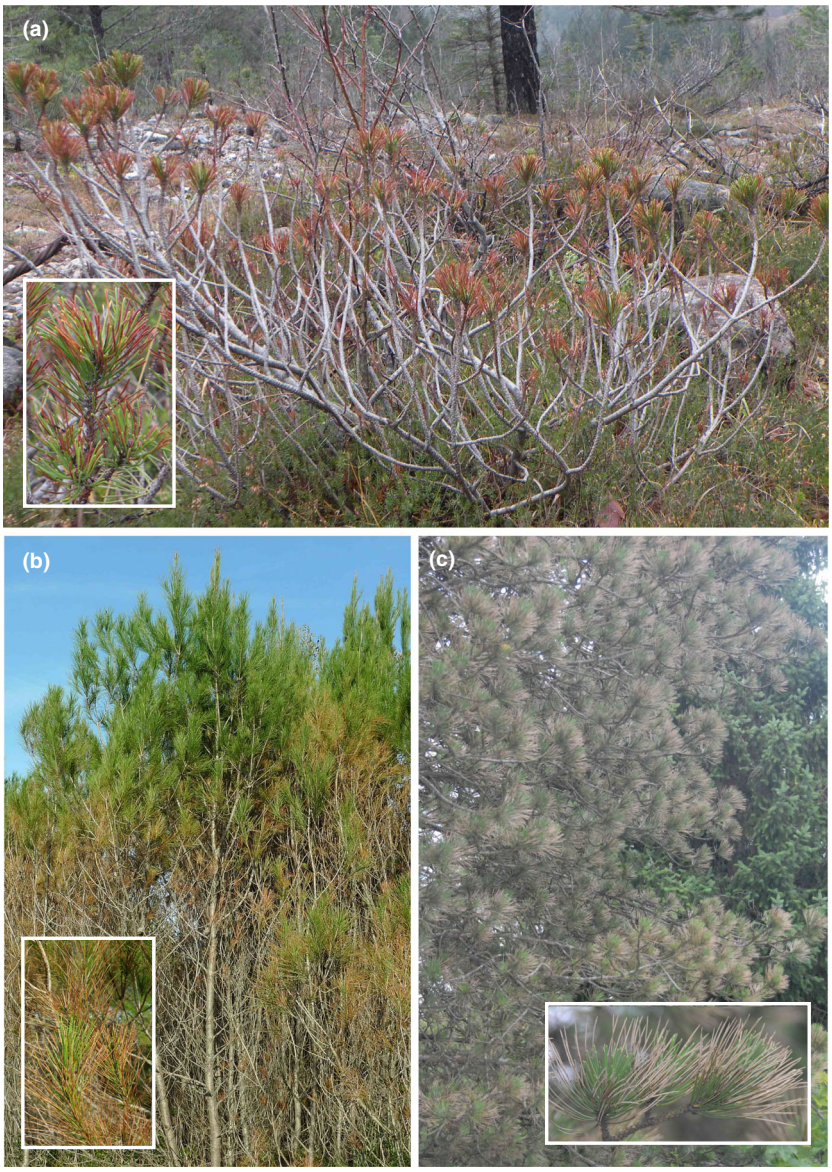

Figure 2 Disease symptoms caused by *Lecanosticta acicola* on three different *Pinus* hosts. (a) *Pinus mugo* in Trenta, Slovenia (photo: T. Hauptman); (b) *P. halepensis* in Kožino, Croatia (photo: D. Diminić); (c) *P. nigra* in Tolmin, Slovenia (photo: D. Jurc).

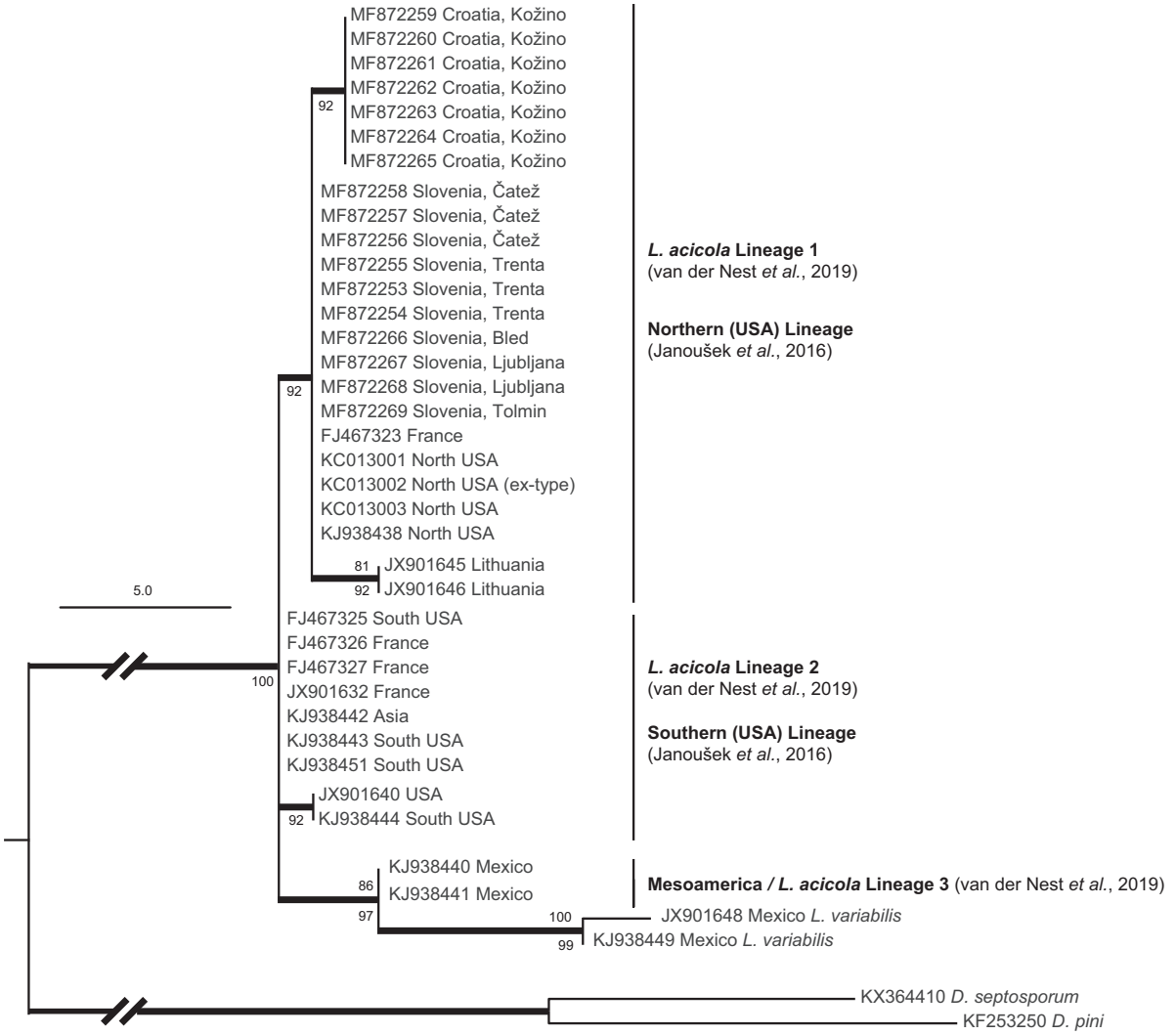

Figure 3 The most parsimonious tree based on maximum parsimony analyses of the *EF1-α* sequences of isolates of *Lecanosticta acicola*. Individuals from Slovenia and Croatia group in the northern lineage as defined by Janoušek *et al.* (2016) and lineage 1 (van der Nest *et al.*, 2019), with the isolates from Croatia representing a novel haplotype. Bold branches indicate Bayesian inference values >0.90. Maximum parsimony bootstrap support values (>75%) are indicated above branches, while maximum likelihood is below the branches. *Dothistroma septosporum* and *D. pini* were used as the outgroup taxa.

amplified in 69 individuals, using 10 microsatellite markers (Table S1). Thirty-one different alleles were detected across all populations. Twenty unique MLHs were obtained (Table 2). All 12 isolates from Tolmin (Slovenia) were clonal and were represented by a single MLH, while the Trenta (Slovenia) population ($N = 18$) had the highest number of MLHs at five.

Population structure

Both the Evanno and Puechmaille methods supported two ($\Delta K = 2$) as the optimal number of clusters (Fig. S1). However, STRUCTURE failed to reveal any differentiation between the analysed populations in the K scenarios tested (Fig. S2).

The discriminant analysis of principal components (DAPC), based on BIC K-means method, revealed that the optimal number of clusters for the analysed dataset was $K = 4$ (Fig. S3). Clusters generally correlated with geographic location and host (Fig. 4), and the composition of the inferred clusters is represented by Figure 4b. Isolates from Tolmin (Slovenia) isolated from *P. nigra* and isolates from Kožino (Croatia) collected from *P. halepensis*, formed separate clusters A and D, respectively (Fig. 4a). Cluster B was composed of the majority of individuals collected at Trenta from *P. mugo*, and cluster C contained two individuals from Trenta, and all the isolates from Bled, Ljubljana and Čatež, from *P. mugo*.

AMOVA revealed that a slightly higher percentage of variance resided within populations (51%) than among

Table 2 Number of haplotypes, genetic diversity values, allelic richness, private allelic richness and tests for random mating of *Lecanosticta acicola* population clusters in the studied region.

									Non-clone-corrected							Clone-corrected				
Cluster	*N*[a]	MLH[b]	Gene diversity[c]	Clonal fraction[d]	Haplotypic diversity[e]	A_R[f]	PA_R[g]	*MAT1-1* :*MAT1-2*[h]	I_A[i]	$\bar{r}_d$[i]	*P*	*L* obs.[j]	*L* rand.[k]	*P*	I_A[i]	$\bar{r}_d$[i]	*P*	*L* obs.[j]	*L* rand.[k]	*P*
A	12	1	0.000	0.917	0.000	NA	NA	12:0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
B	16	3	0.106	0.813	0.433	1.200 ± (0.13)	0.379 ± (0.19)	0:14 (2)	0.509	0.515	0.065	2	2	**0.03**	−0.500	−0.500	1.000	NA	NA	NA
C	15	12	0.428	0.200	0.967	1.485 ± (0.19)	0.523 ± (0.28)	5:2 (8)	0.228	0.059	0.096	19	18	0.09	0.134	0.035	0.273	18	17	0.08
D	26	4	0.101	0.846	0.545	1.300 ± (0.17)	0.541 ± (0.20)	0:23 (3)	−0.139	−0.140	1.000	4	4	**0.03**	−0.429	−0.447	1.000	NA	NA	NA

Statistically significant figures are highlighted in bold ($P < 0.05$). NA, not available for analysis because of a limited sample size.

[a]Number of individuals belonging to each cluster.

[b]Number of unique multilocus haplotypes.

[c]Calculated in FSTAT v. 2.9.3 (Goudet, 2002).

[d]Clonal fraction = 1 − [(number of unique haplotypes)/(number of isolates)].

[e]Calculated in MULTILOCUS v. 1.3b (Agapow & Burt, 2001).

[f]Allelic richness ± standard error (Szpiech *et al.*, 2008).

[g]Private allelic richness ± standard error (Szpiech *et al.*, 2008).

[h]Ratio of mating type idiomorphs; numeral in brackets indicates the number of isolates that failed during PCR amplifications.

[i]Values of index of association I_A and $\bar{r}_d$ obtained after 1000 randomizations in MULTILOCUS v. 1.3b.

[j]The observed length of the tree used in the parsimony tree-length permutation test, generated in PAUP v. 4.0b10 (Swofford, 2003)

[k]The most parsimonious PAUP tree found after 1000 randomizations in MULTILOCUS v. 1.3b.

L. acicola populations (49%). Similar results were observed when isolates were grouped based on host species, with 44% of total molecular variance residing among host species groups and 56% of variance residing within these groups (Table 3).

Genetic diversity

The population from Tolmin (Slovenia) making up cluster A displayed no diversity and had a clonal fraction of 0.917 (Table 2). Cluster C (Slovenia) had the highest gene (0.428) and haplotypic (0.967) diversity, contained the most haplotypes (12) and had the lowest clonal fraction (0.2). Clusters B (Slovenia) and D (Croatia) were similar in their diversity indices and had low gene diversity (0.106 and 0.101) and haplotypic diversity (0.433 and 0.545), respectively. Allelic richness, and private allelic richness ranged from $A_R = 1.2$, $PA_R = 0.379$ in cluster B (Slovenia), to $A_R = 1.485$ and $PA_R = 0.523$ in cluster C (Slovenia). F_{ST} and Nei's unbiased genetic distance (Table 4) showed moderate to high divergence among all clusters, and a low amount of gene flow between them. The highest divergence was observed between the two geographically closest genetic clusters A and B ($D_a = 0.660$, $F_{ST} = 0.888$).

Mating type determination and mode of reproduction

The mating type idiomorphs were successfully amplified for 55 of the 69 isolates (Table 2). Mating type MAT1-2 (288 bp) was detected in isolates from Trenta and Bled (Slovenia), and from Kožino (Croatia). MAT1-1 (560 bp) was present in isolates from Tolmin, Ljubljana and Čatež (Slovenia). There were consequently no locations where both mating types were present amongst the isolates studied.

The hypothesis for non-random mating could not be rejected when the clone-corrected and non-clone-corrected datasets, based on microsatellite alleles for clusters B, C and D, were analysed using the measure of I_A, $\bar{r}_d$

Table 3 Hierarchical analysis of molecular variance (AMOVA) of *Lecanosticta acicola* populations, grouped by host species and by populations.

	d.f.	Sum of squares	Mean squares	Estimate of variance	Total variation (%)	*P*
Among populations	5	20.088	4.018	0.897	49	0.001
Within populations	15	14.150	0.943	0.943	51	
Total	20	34.238		1.840	100	
Among host species groups	2	11.755	5.877	0.992	44	0.001
Within host species groups	18	22.483	1.249	1.249	56	
Total	20	34.238		2.241	100	

Table 4 Genetic distances (F_{ST}, D_a) between pairs of genetically defined clusters of *Lecanosticta acicola* populations estimated in GenAlEx v. 6.5.

	Cluster A	Cluster B	Cluster C	Cluster D
Cluster A	—	0.888	0.511	0.831
Cluster B	0.660	—	0.406	0.795
Cluster C	0.306	0.250	—	0.484
Cluster D	0.348	0.659	0.371	—

Values of D_a are positioned below the diagonal, while F_{ST} is above the diagonal.

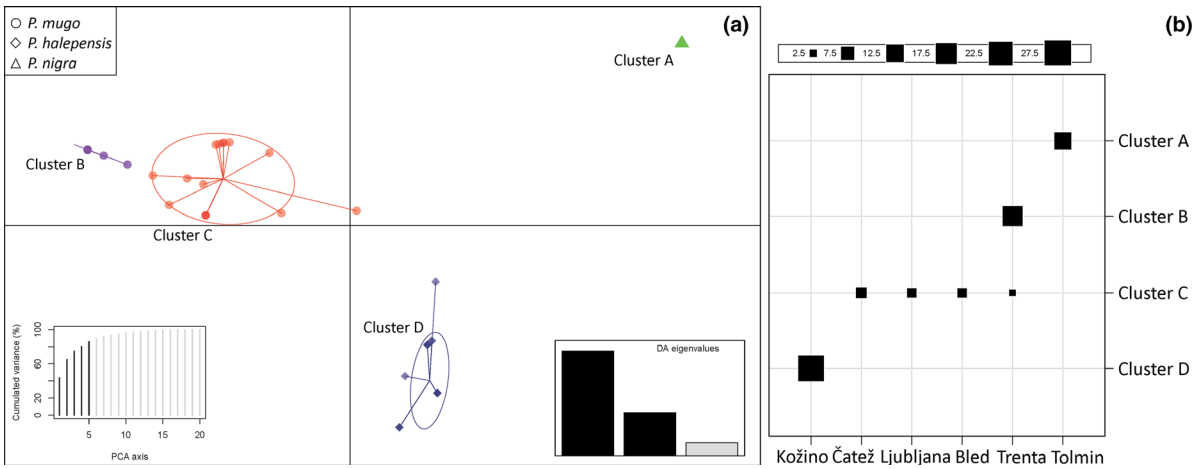

Figure 4 Population structure of the Slovenian and Croatian *Lecanosticta acicola* isolates. (a) Scatterplot of the discriminant analysis of principal components (DAPC); a total of five principal components (highlighted in the bottom left hand corner) were retained, comprising 85.7% of the conserved genetic variance. The first two discriminant analysis (DA) eigenvalues (highlighted in bottom right hand corner) correspond to the horizontal and vertical axes, respectively. Shapes (upper left hand corner) correspond to the host species from which an isolate was obtained. (b) Composition of the DAPC clusters with rows corresponding to inferred clusters, while columns correspond to sampled populations; size of squares in the legend above is proportional to the number of individuals comprising the clusters. [Colour figure can be viewed at wileyonlinelibrary.com].

and the PTLPT (Table 2). These measures could not be calculated for cluster A because this population represented only a single MLH. However, the sample sizes used in these calculations were small (only three and four isolates in two populations when clone-corrected), decreasing the probability of detecting significant linkage disequilibrium in the populations.

Discussion

Of the nine species described in *Lecanosticta*, only *L. acicola* was detected in this study. Comparisons of *EF1-α* sequences for *L. acicola* isolates from Slovenia and Croatia revealed two haplotypes present in isolates obtained from these two countries, one of which is unique to Croatia. These haplotypes showed close ancestral connections with isolates from northern and central Europe. DAPC partitioned isolates into four genetic clusters, roughly following their geographic distribution. Population structure and genetic diversity of these clusters reflected human-mediated dispersal. Although asexual reproduction has been the prevailing mode of propagation of the pathogen, there was evidence for sexual recombination in some of the populations in Slovenia.

It has previously been proposed that *L. acicola* populations in Europe originated from two different source populations in North America. These included a northern lineage and a southern lineage, emerging from studies based on morphology and pathogenicity (Kais, 1972), RAPD analyses (Huang *et al.*, 1995) as well as those based on phylogenetic inference and microsatellite analyses (Janoušek *et al.*, 2016; van der Nest *et al.*, 2019). In the present study, the *EF1-α* sequences of *L. acicola* from Croatia were different to those from Slovenia and to the ex-type isolate of the species by a one base-pair mutation at bp site 140. Consequently, the Croatian population represents a novel *EF1-α* haplotype of *L. acicola*. However, all isolates in this study from Slovenia and Croatia grouped together with the isolates from the northern lineage. This supports the hypothesis of Janoušek *et al.* (2016) that a common North American population was the original source of *L. acicola* infections in central and northern Europe.

The first outbreak of BSNB in Europe was in Spain (Martínez, 1942). Thirty years later, in 1975, *P. halepensis* trees severely infected with *L. acicola* were reported from Crvena Luka in Croatia (Milatović, 1976). Soon thereafter, BSNB infections were identified in Kožino in Croatia, as well as at several other locations throughout the Croatian coastal area (Glavaš & Margaletić, 2001). Croatia, therefore, could have been one of the entry points of *L. acicola* into Europe from where it could have subsequently spread to neighbouring countries, including Slovenia. However, the presence of a unique *EF1-α* haplotype of *L. acicola* found in the Kožino (Croatia) population, and evidence emerging from the microsatellite analyses, provides a strong indication that this particular population from Croatia was not the source of the current BSNB infections in Slovenia.

The population of *L. acicola* in Kožino (Croatia) displayed the lowest overall genetic diversity compared to other tested populations in Slovenia, even though *L. acicola* has been reported from that area for more than 40 years. Furthermore, when compared to Croatia, *L. acicola* populations from Germany and Austria include both mating types, have considerably lower clonal fractions and higher haplotypic (genotypic) diversity and allelic richness (Janoušek *et al.*, 2016). The observed genetic bottleneck in the Croatian population could have resulted from an introduction of a population with a limited genetic diversity. This would be consistent with the fact that accidental introductions of alien species typically begin with a small number of individuals (Dlugosch & Parker, 2008). Thereafter, in the absence of sexual recombination or additional introductions, the genetic composition of the populations would remain largely unchanged. In addition, several attempts to eradicate the disease in Kožino were made by means of silvicultural phytosanitary measures (Glavaš, 1979; Glavaš & Margaletić, 2001). These eradication measures could have contributed to a reduction in the population size of *L. acicola,* creating a genetic bottleneck and leading to a population with only one mating type and reduced genetic diversity.

Pinus mugo seems to be the most frequently affected host of *L. acicola* in the studied areas (Trenta, Čatež, Ljubljana and Bled) of Slovenia. This pine species is generally considered to be highly susceptible to *L. acicola* in Europe, with infections occurring from Italy (La Porta & Capretti, 2000) to the Baltics (Adamson *et al.*, 2015) and with the most recent findings in Ireland and Russia (Mullett *et al.*, 2018). The Tolmin (Slovenia) population considered in this study represents one of the few known populations of *L. acicola* on *P. nigra* in Europe. Two other reports of this tree species being infected are from Austria and Spain (Hintsteiner *et al.*, 2012; Ortíz de Urbina *et al.*, 2016). In Austria, Hintsteiner *et al.* (2012) reported only two diseased *P. nigra* trees even though heavily infected *P. mugo* trees were growing in close proximity to them. In Spain, Ortíz de Urbina *et al.* (2016) detected BSNB in only 4% of the investigated *P. nigra* plots, in contrast to 65% infected *P. radiata* plots. Because infections of *L. acicola* are uncommon on *P. nigra* in Europe (Hintsteiner *et al.*, 2012), the abovementioned infected areas in Tolmin should be carefully monitored. This is especially important as they represent a source of *L. acicola* populations capable of infecting and spreading to *P. nigra* in the rest of Europe. Introductions of isolates having the opposite mating type into this population (e.g. from Trenta) could enable sexual reproduction to take place, which would lead to the production of wind-transmitted ascospores. Sexual recombination has already been detected in Germany and Austria, and the recent upsurge of disease reported from some European countries could be accounted for by the dispersal of long-range windblown ascospores (Janoušek *et al.*, 2016). Infected *P. nigra* stands in Slovenia border on plantations and natural stands of this

species in Italy and it will be important to verify the presence of BSNB in other Julian prealpine regions of Italy and Slovenia.

The subdivision of *L. acicola* populations in Slovenia and Croatia into four genetic clusters, with relatively low genetic diversity and uneven distribution of mating types, reflects introductions from multiple different sources. The four distinct clusters emerging from population structure analysis roughly corresponded to the locations and hosts from which the isolates were collected. The *EF1-α* sequences for the isolates sampled in Croatia were genetically different from all other populations studied and these isolates also formed their own population cluster. Similar levels of molecular variance were found to exist on all three tested levels (among populations, among hosts groups, and within these groups). Such hierarchical organization reflects the presence of a clearly defined population structure in the region and may be the result of populations being established from multiple different infection sources, with restricted gene flow between them.

The high clonal fraction values and unequal ratio of mating types, in combination with relatively low haplotypic diversity, suggest that asexual reproduction has had a strong influence on the *L. acicola* pathogen populations at the studied sites. Although both mating types were detected in Slovenia, they were not observed in isolates from the same sampling sites. Only MAT2 isolates were found in Croatia in this study, which is also consistent with the results of Janoušek *et al.* (2016). Sexual reproduction could lead to the production of novel genotypes, which could alter the behaviour of the pathogen (McDonald & Linde, 2002). Sexual recombination was not confirmed in the Slovenian and Croatian populations. However, the populations in Tolmin and Trenta (Slovenia), which have opposite mating types, are geographically closest to each other, and currently hold the greatest risk of incurring the introductions of opposite mating types that could lead to sexual reproduction.

Despite the fact that *L. acicola* has been present in Kožino (Croatia) for decades, infections have been detected only on *P. halepensis* (Milatović, 1976; Glavaš, 1979; Glavaš & Margaletić, 2001). Other *Pinus* species (i.e. *P. pinaster* and *P. nigra*) growing in close proximity to diseased pine stands in this area have not been infected (Glavaš & Margaletić, 2001; authors' personal observations). A similar situation has been observed for *L. acicola* populations in Trenta and Ljubljana (Slovenia), where *P. nigra* and *P. sylvestris* have not been infected even though they grow adjacent to the severely infected *P. mugo* stands. The observed patterns could be a result of microclimate and specific ecosystem conditions influencing certain host species to be more susceptible or resistant to *L. acicola*. Further screening with a larger collection of isolates from the same host and different locations would be required to test whether the hypothesis of specific preferences of the pathogen to certain hosts would hold true. Nevertheless, high levels of adaptability to climatic conditions and host species have already been observed in several studies on *L. acicola* isolates (Kais, 1972; Huang *et al.*, 1995; Janoušek *et al.*, 2016). In light of the recent new reports of host jumps to native species in the Baltics previously unaffected by the disease in that region (Adamson *et al.*, 2018), and with new cases of BSNB infection emerging throughout Europe (Mullett *et al.*, 2018), host preference and climatic adaptability will be important issues for future research.

From this, and other studies (Janoušek *et al.*, 2016; Mullett *et al.*, 2018), it is clear that different populations of *L. acicola* present in Europe are most likely still limited to particular geographical locations. Many of these locations still contain isolates having a single mating type. However, the increasing threat of spread of the pathogen in Europe, and the possibility of new adaptive strains emerging through sexual reproduction, is a cause for concern. With this in mind, the adequacy of current quarantine measures should be reconsidered. They clearly should not only be focused on the pathogen species, but should also consider pathogen mating types, lineages and haplotypes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Delineated results of Evanno (Evanno *et al.*, 2005) (left) and Puechmaille (Puechmaille, 2016) (right) methods for determining optimal number of *K*.

Figure S2. STRUCTURE bar plots representing $K = 2$ to $K = 5$, obtained from the Bayesian analysis of *Lecanosticta acicola* samples collected from Slovenia and Croatia. The percentage on the right represents the proportion of independent STRUCTURE runs (30 in total) that correspond to the significantly similar clustering pattern (SSC > 0.9). The population codes correspond to the order of populations listed in Table 1.

Figure S3. Value of Bayesian informative characters (BIC) versus the number of clusters.

Table S1. *Lecanosticta acicola* isolates (ID) from Slovenia and Croatia genotyped using 10 microsatellite markers (non-clone-corrected data).