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# The gene flow and mode of reproduction of *Dothistroma septosporum* in the Czech Republic

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Since 1911, dothistroma needle blight, caused by *Dothistroma septosporum*, has been recorded in most European countries. In the Czech Republic, the fungus has become an important disease of pines since 2000, especially Austrian pines in plantations of Christmas and ornamental trees. The aim of this study was to analyse the population structure, gene flow and mode of reproduction of this pathogen. Microsatellite and mating-type markers were analysed in a *Dothistroma* population in the southeastern part of the country using reference isolates from other European countries. The haplotypic diversity was high, with 87 unique and 13 shared haplotypes (probable clones) identified in 121 samples. Based on STRUCTURE analysis, the isolates were divided into two populations, with an uneven distribution over the sampling sites. The grouping of the sites to the populations did not follow a geographical pattern because certain isolates that were sympatrically co-occurring at the same site were placed in different populations. Tests for random mating (the index of association and a parsimony tree-length permutation test) showed a significant clonal mode of reproduction in most cases, but the intrapopulation haplotypic diversity is unexpectedly high. Although a teleomorphic stage of *D. septosporum* has not been previously observed in the Czech Republic, the high intrapopulation haplotypic diversity can be explained by infrequent sexual reproduction consistent with the occurrence of both mating types.

*Keywords*: *Dothistroma*, dothistroma needle blight, gene flow, mating type, *Mycosphaerella*, SSR genotype

## Introduction

Dothistroma needle blight (DNB) is caused by two closely related species, *Dothistroma septosporum* and *D. pini* (the anamorphic stage of *Mycosphaerella pini*) and results in severe needlecast in pines and, to a lesser extent, other conifers. According to European and Mediterranean Plant Protection Organization (EPPO) and European law, DNB is a quarantine fungal disease in Europe. DNB is distributed worldwide and is reported in at least 63 countries (Watt *et al.*, 2009), and more than 80 host species. DNB has emerged in Europe in the past 15–20 years and is currently widespread in most European countries (Watt *et al.*, 2009). Although its European isolates belong primarily to *Dothistroma septosporum*, *D. pini* has been detected in samples from France (Ioos *et al.*, 2010), Hungary, Russia and Ukraine (Barnes *et al.*, 2011). Woods *et al.* (2005) consider DNB to be a disease supported by global climate change and emphasize the influence of locally enhanced precipitation on the spread of the disease throughout the temperate zone.

In the Czech Republic (CR), *D. septosporum* was first noted in 1999 on imported nursery stock of *Pinus nigra* from Hungary, and the first detection of DNB in a plantation was recorded the following year in 6-year-old Austrian pines in Christmas tree plantations (Jankovský *et al.*, 2004). The increasing number of new findings is explained by the substantial number of trees imported in the 1990s and by the use of infested stock from nurseries until 2000, when strict phytosanitary precautions were instituted throughout the CR. Even though DNB monitoring was conducted prior to the 1990s, it is possible that the disease occurred during that period. In the CR, DNB has been identified in 21 pine species, four spruce species and Douglas-fir (Bednářová *et al.*, 2006); however, *D. pini* has not been recorded in the country to date (March 2012). Observations of the current DNB outbreak have been conducted throughout the CR, and the disease has been found mainly in Austrian pine plantations or on amenity trees in urban areas, whilst the infestation of native Scots pine stands is relatively rare (Jankovský *et al.*, 2004; Bednářová *et al.*, 2006). Since 2008, new observations in Scots pine plantations and Scots and bog pine stands in protected areas have been

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reported; the occurrence of native pine populations is anticipated at such natural sites (Mikeska *et al.*, 2008). Therefore DNB may be caused by an older population of the pathogen there, whereas Austrian pine plantations have been more recently infected by infested nursery stock.

Few studies have investigated the genetic structure of *D. septosporum* populations. In New Zealand, no genetic diversity was detected in the pathogen using RAPD and RAMS DNA markers (Hirst *et al.*, 1999). Microsatellite markers for *D. septosporum* were developed by Barnes *et al.* (2008) but have not been used in any population analysis. Dale *et al.* (2011) recently used AFLP and mating type markers to reveal a high haplotypic diversity, a mixed mode of reproduction, and an equal mating-type segregation ratio in *D. septosporum* populations in British Columbia, Canada. A population with a high genetic variation has a strong evolutionary potential, and it is probable that such a population can rapidly adapt to new conditions (McDonald & Linde, 2002). Gene flow among populations and recombination are two crucial sources of new allelic and genotypic variation that can increase the genotypic or haplotypic diversity of a population (McDonald & Linde, 2002). In pleomorphic ascomycetes, the role of propagation by asexual (conidia) or sexual (ascospores) diaspores is crucial for genotypic and haplotypic diversity. However, the role of ascospores for infections *in situ* is unclear. In Europe, DNB is usually considered a human-mediated disease, and *D. septosporum* is hypothesized to have short-lived conidia with limited dispersal abilities (Gadgil, 1970; Peterson, 1973). However, the precise data for the CR region are incomplete.

The aim of this study was to analyse the population structure, gene flow and mode of reproduction of *D. septosporum* in the CR using microsatellite (SSR) and mating-type markers.

## Materials and methods

### Sampling

Localities of *D. septosporum* occurrence were monitored between 2005 and 2010. Fungal cultures were isolated from green needles with mature acervuli that were producing fertile conidia, and the sampling was usually performed from June to September. In localities with a relatively high number of isolates, the isolates were obtained from different trees or from the same host tree. The needles were incubated in moist chambers for 2 days to produce fresh conidia in the acervuli, and the presence of the conidia was evaluated under a microscope prior to isolation. Small slivers of needles with acervuli (5 mm long) were soaked in ready-to-use sodium hypochlorite solution (Sigma-Aldrich) for 3–5 min. The samples were then washed twice with 96% ethanol for 1 min and soaked for 5–10 min in sterile distilled water. The samples were placed on Petri dishes with malt extract agar (MEA; 30 g malt extract, 15 g agar and 5 g peptone per litre; Himedia) and incubated at 21°C in the dark; after 2–3 days of inoculation, the culture was transferred to a fresh Petri dish. A culture originating from a single acervulus from one needle was maintained as a single isolate; the additional single spore purification was not carried out. For long-term storage, the isolates were maintained as agar slant cultures with malt extract medium at 7°C in the dark.

In all, 103 isolates from 46 sampling sites were included in the analysis (Table 1).

### DNA extraction and PCR

DNA was extracted from freshly grown cultures using the DNeasy plant mini kit (QIAGEN). The identity of *D. septosporum* (as distinguished from *D. pini*) was determined by the species-specific PCR targeting of the beta-tubulin gene (Ioos *et al.*, 2010). All of the sampled isolates were confirmed as *D. septosporum*. Labelled primers derived by Barnes *et al.* (2008) were used for the SSR analysis. The following primer pairs (loci) for the analysis were selected by M. Mueller (Finnish Forest Research Institute METLA, Vantaa, Finland, personal communication): Doth_F, Doth_G, Doth_J, Doth_K, Doth_L, Doth_S, Doth_DS1 and Doth_DS2. The forward primers were labelled with one of four fluorophores on the 5′ end (6-FAM, VIC, NED or PET). The combinations of primer pairs sharing the same fluorophores were chosen when the PCR products of the two primer pairs were clearly distinguishable according to their size. Each microsatellite locus was amplified by an independent PCR reaction (Barnes *et al.*, 2008) using *Taq* DNA polymerase (Promega). The SSR locus sizes were analysed using the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) and determined with GENEMARKER v. 1.3 software.

In 18 isolates, two alleles were detected at a single locus – such isolates probably do not have a single spore origin. These isolates were analysed as two samples (A, B) differing in these allele sizes. In total, 121 samples were analysed. The core data set (111 samples) originated in the Czech Republic. The 10 remaining samples were from other European countries (Austria, Finland, Norway and Slovenia).

The mating-type genes were amplified with species-specific, mating-type-specific primers using procedures and primers developed by Groenewald *et al.* (2007). The PCR products were viewed under UV light on 1% agarose gels stained with SERVA DNA Stain G. The fragments were compared with a 100 bp DNA ladder (New England Biolabs) and scored as either mating type 1 (820 bp) or mating type 2 (480 bp; Groenewald *et al.*, 2007).

### Data analysis

The haplotypes were determined using ARLEQUIN v. 3.5.1.2 (Excoffier & Lischer, 2010). Two sets of haplotype data were created. The first data set included all of

Table 1 List of strains included in the study

| Sample no.[a] | Isolate no. | Country | Locality | Host | Host age[b] | Habitat[c] | Population |
|---|---|---|---|---|---|---|---|
| 24 | 1126 | Finland | Ähtäri | *Pinus sylvestris* | OXT | D | 2 |
| 76 | 1365 | CR | Borkovická Blata | *Pinus sylvestris* | OXT | D | 2 |
| 95 | 1385 | CR | Bynina – Christmas tree plantation | *Pinus nigra* | XT/OXT | B | 1 |
| 109 AB | 1400 | CR | Bynina – Christmas tree plantation | *Pinus nigra* | XT/OXT | B | 1 |
| 82 | 1359 | CR | Bynina – private garden | *Pinus nigra* | OXT | B | 1 |
| 86 | 1357 | CR | Bynina – private garden | *Pinus nigra* | OXT | B | 1 |
| 8 | 1123 | CR | Havlíčkův Brod | *Pinus nigra* | OXT | A | 2 |
| 23 | 1124 | CR | Havlíčkův Brod | *Pinus nigra* | OXT | A | 2 |
| 9 | 1125 | CR | Havlíčkův Brod – Mírovka | *Pinus mugo* | × | A | 1 |
| 70 | 1338 | Austria | Hollenstein an der Ybbs | *Pinus* sp. | OXT | D | 1 |
| 94 | 1374 | CR | Jakule | *Pinus nigra* | M | D | 1 |
| 103 | 1377 | CR | Jakule | *Pinus nigra* | OXT | D | 1 |
| 89 | 1348 | CR | Jandovka | *Pinus sylvestris* | M | D | 2 |
| 97 | 1371 | CR | Jandovka | *Pinus sylvestris* | OXT | D | 2 |
| 34 | 1165 | CR | Jarcová | *Pinus nigra* | OXT | A | 2 |
| 35 | 1166 | CR | Jarcová | *Pinus nigra* | OXT | A | 2 |
| 36 | 1167 | CR | Jarcová | *Pinus nigra* | OXT | A | 2 |
| 87 | 1354 | CR | Jasenice | *Pinus nigra* | OXT/M | C | 1 |
| 96 | 1413 | CR | Karolinka – glass factory | *Pinus nigra* | OXT/M | A | 2 |
| 102 | 1405 | CR | Karolinka – medical centre | *Pinus mugo* | × | A | 1 |
| 108 | 1421 | CR | Karolinka – medical centre | *Pinus mugo* | × | A | 1 |
| 1 | 1110 | CR | Koryčany | *Pinus nigra* | M | C | 2 |
| 40 AB | 1176 | CR | Křtiny – arboretum | *Pinus ponderosa* | OXT | A | 2 |
| 41 | 1177 | CR | Křtiny – arboretum | *Pinus ponderosa* | OXT | A | 1 |
| 42 | 1178 | CR | Křtiny – arboretum | *Pinus ponderosa* | OXT | A | 2 |
| 101 | 1407 | CR | Křtiny – arboretum | *Pinus ponderosa* | OXT | A | 2 |
| 49 AB | 1218 | CR | Karviná – Lázně Darkov | *Pinus mugo* | × | A | 2 |
| 50 AB | 1219 | CR | Karviná – Lázně Darkov | *Pinus mugo* | × | A | 2 |
| 51 | 1220 | CR | Karviná – Lázně Darkov | *Pinus mugo* | × | A | 1 |
| 28 | 1153 | CR | Lhotka nad Bečvou | *Pinus nigra* | XT | B | 1 |
| 13 | 1275 | CR | Lidmilův mlýn, Sádek | *Pinus mugo* | × | A | 1 |
| 22 | 1245 | CR | Lidmilův mlýn, Sádek | *Pinus mugo* | × | A | 1 |
| 26 | 1246 | CR | Lidmilův mlýn, Sádek | *Pinus mugo* | × | A | 1 |
| 47 | 1216 | CR | Lidmilův mlýn, Sádek | *Pinus nigra* | OXT | C | 1 |
| 64 | 1331 | CR | Luhačovice | *Pinus ponderosa* | OXT/M | A | 2 |
| 71 | 1332 | CR | Luhačovice | *Pinus ponderosa* | OXT/M | A | 1 |
| 72 | 1333 | CR | Luhačovice | *Pinus ponderosa* | OXT/M | A | 1 |
| 17 | 1250 | CR | Mezina | *Pinus nigra* | XT | C | 2 |
| 54 AB | 1224 | CR | Mezina | *Pinus nigra* | XT | C | 1 |
| 55 | 1225 | CR | Mezina | *Pinus nigra* | XT | C | 2 |
| 56 | 1226 | CR | Mezina at Bruntál | *Pinus nigra* | XT | C | 1 |
| 99 AB | 1384 | CR | Mštenovice | *Pinus nigra* | M | C | 1 |
| 16 | 1258 | CR | Nová Pec | *Pinus ponderosa* | M | A | 1 |
| 58 | 1231 | CR | Nová Pec | *Pinus ponderosa* | M | A | 1 |
| 59 AB | 1232 | CR | Nová Pec | *Pinus ponderosa* | M | A | 1 |
| 84 AB | 1367 | CR | Nová Pec | *Pinus mugo* | × | A | 2 |
| 91 | 1366 | CR | Nová Pec | *Pinus mugo* | × | A | 2 |
| 5 AB | 1118 | CR | Nové Hrady – Sušidla | *Pinus mugo* | × | A | 1 |
| 6 | 1120 | CR | Nové Hrady – Sušidla | *Pinus nigra* | OXT | A | 1 |
| 11 | 1300 | CR | Odry | *Pinus nigra* | XT | B | 1 |
| 62 | 1295 | CR | Odry | *Pinus nigra* | XT | B | 1 |
| 63 | 1296 | CR | Odry | *Pinus nigra* | XT | B | 1 |
| 33 | 1163 | CR | Oznice at Valašské Meziříčí | *Pinus mugo* | × | A | 1 |
| 45 AB | 1162 | CR | Oznice at Valašské Meziříčí | *Pinus mugo* | × | A | 2 |
| 18 | 1244 | CR | Pernek | *Pinus uncinata* subsp. *uliginosa* | × | A | 2 |
| 57 | 1230 | CR | Pernek | *Pinus uncinata* subsp. *uliginosa* | × | A | 2 |
| 73 | 1362 | CR | Pernek | *Pinus ponderosa* | M | A | 1 |
| 77 | 1361 | CR | Pernek | *Pinus ponderosa* | M | A | 1 |
| 83 | 1351 | CR | Pernek | *Pinus ponderosa* | M | A | 1 |

Table 1 (Continued)

| Sample no.[a] | Isolate no. | Country | Locality | Host | Host age[b] | Habitat[c] | Population |
|---|---|---|---|---|---|---|---|
| 67 | 1327 | Finland | Pyhtää | *Pinus sylvestris* | OXT/M | D | 2 |
| 107 | 1420 | CR | Rožnov pod Radhoštěm – library | *Pinus mugo* | × | A | 1 |
| 37 | 1171 | CR | Rožnov pod Radhoštěm – Písečná | *Pinus nigra* | OXT | A | 2 |
| 38 AB | 1173 | CR | Rožnov pod Radhoštěm – Písečná | *Pinus nigra* | OXT | A | 1 |
| 39 | 1174 | CR | Rožnov pod Radhoštěm – Písečná | *Pinus nigra* | OXT | A | 2 |
| 85 | 1353 | CR | Rožnov pod Radhoštěm – Písečná | *Pinus mugo* | × | A | 1 |
| 90 | 1364 | CR | Rožnov pod Radhoštěm – Písečná | *Pinus mugo* | × | A | 1 |
| 92 | 1346 | CR | Rožnov pod Radhoštěm – Písečná | *Pinus mugo* | × | A | 1 |
| 79 | 1368 | CR | Rožnov pod Radhoštěm – Rybníčky | *Pinus nigra* | OXT | A | 2 |
| 81 | 1370 | CR | Rožnov pod Radhoštěm – Rybníčky | *Pinus nigra* | OXT | A | 2 |
| 88 AB | 1356 | CR | Rožnov pod Radhoštěm – Rybníčky | *Pinus nigra* | OXT | A | 2 |
| 69 | 1329 | Norway | Rundhaug | *Pinus sylvestris* | OXT | D | 2 |
| 3 | 1112 | CR | Řícmanice – arboretum | *Pinus cembra* | OXT/M | A | 1 |
| 4 | 1113 | CR | Řícmanice – arboretum | *Pseudotsuga menziesii* | M | A | 1 |
| 10 | 1127 | CR | Řícmanice – arboretum | *Pinus nigra* | OXT/M | A | 2 |
| 25 | 1128 | CR | Řícmanice – arboretum | *Pinus mugo* | × | A | 1 |
| 14 | 1270 | CR | Sádek | *Pinus nigra* | OXT | A | 1 |
| 15 | 1268 | CR | Sádek | *Pinus ponderosa* | M | A | 1 |
| 27 | 1247 | CR | Sádek | *Pinus ponderosa* | M | A | 1 |
| 30 | 1248 | CR | Sádek | *Pinus ponderosa* | M | A | 1 |
| 48 | 1217 | CR | Sádek | *Pinus ponderosa* | M | A | 1 |
| 61 | 1269 | CR | Sádek | *Pinus nigra* | OXT | A | 1 |
| 7 | 1121 | CR | Soběslavská Blata | *Pinus sylvestris* | OXT | D | 2 |
| 2 | 1111 | CR | Strhaře | *Pinus nigra* | OXT | B | 1 |
| 104 AB | 1403 | CR | Střitež nad Bečvou | *Pinus mugo* | × | A | 1 |
| 68 AB | 1328 | Finland | Suonenjoki | *Pinus sylvestris* | OXT/M | D | 2 |
| 80 | 1350 | CR | Sušidla | *Pinus sylvestris* | OXT | D | 2 |
| 43 | 1183 | Slovenia | the river Soča valley | *Pinus nigra* | M | D | 2 |
| 44 AB | 1184 | Slovenia | the river Soča valley | *Pinus nigra* | M | D | 2 |
| 46 | 1186 | Slovenia | the river Soča valley | *Picea abies* | M | D | 2 |
| 100 AB | 1411 | CR | Valašské Klobouky | *Pinus nigra* | M | A | 1 |
| 110 | 1380 | CR | Valašské Klobouky | *Pinus nigra* | M | A | 1 |
| 12 | 1294 | CR | Vídeň at Velké Meziříčí | *Pinus nigra* | OXT | A | 2 |
| 21 | 1242 | CR | Vídeň at Velké Meziříčí | *Pinus nigra* | OXT | A | 2 |
| 60 AB | 1241 | CR | Vídeň at Velké Meziříčí | *Pinus nigra* | OXT | A | 1 |
| 20 | 1223 | CR | Šance Dam | *Pinus mugo* | × | A | 2 |
| 52 | 1221 | CR | Šance Dam | *Pinus mugo* | × | A | 2 |
| 53 | 1222 | CR | Šance Dam | *Pinus mugo* | × | A | 2 |
| 19 | 1234 | CR | Zálší at Veselí nad Lužnicí | *Pinus sylvestris* | OXT | C | 2 |
| 74 | 1358 | CR | Zašová – Lužanka restaurant | *Pinus ponderosa* | OXT | A | 1 |
| 29 | 1154 | CR | Zubří – sawmill | *Pinus ponderosa* | OXT | A | 1 |
| 31 | 1156 | CR | Zubří – sawmill | *Pinus ponderosa* | OXT | A | 2 |
| 32 | 1157 | CR | Zubří – poultry farm | *Pinus nigra* | OXT | B | 2 |
| 105 AB | 1409 | CR | Zubří – poultry farm | *Pinus nigra* | OXT | B | 1 |

[a]AB, sample kept as two samples due to an allele polymorphism in a single locus.
[b]XT, Christmas tree size (≤2 m high); OXT, overgrown christmas tree; M, mature tree; ×, *Pinus mugo* and *Pinus uncinata* subsp. *uliginosa* not evaluated due to their scrub growth.
[c]A, urban greenery; B, Christmas tree plantation in use or abandoned; C, artificial reforestation; D, natural reforestation.

the haplotypes, whereas the second data set was purged of clones, with each individual haplotype present as only one copy (clone-corrected). Prior to the analysis of the mode of reproduction, population assignments were carried out to avoid the Wahlund effect (reduction of heterozygosity in a population caused by subpopulation structure, e.g. caused by different allele frequencies within subpopulations). The samples were grouped into populations according to STRUCTURE – a Bayesian, model-based algorithm (Hubisz *et al.*, 2009), and the number of populations resulting from the grouping was estimated according to Evanno *et al.* (2005). The haplotypic diversity was calculated on the full data set, whereas the genetic diversity and population differentiation statistics were computed on the clone-corrected data set. The methods used for assessing the mode of reproduction included both data sets to avoid a reduced probability of rejection of the null hypothesis of random mating in the clone-corrected data set (Milgroom, 1996). Each population was diagnosed for the number

of polymorphic loci and number of unique haplotypes (occurring only once in the entire data set). MULTILOCUS v. 1.3b (Agapow & Burt, 2001) was used for the calculations of haplotypic diversity. Nei's gene diversity (Nei, 1987) was computed as the average gene diversity over all of the loci in ARLEQUIN v. 3.5.1.2. The pairwise $F_{ST}$ – measure of population differentiation based on allele frequency differences (Wright, 1949) and number of migrants $Nm$ – derived from $F_{ST}$ computation (Slatkin, 1991) between the populations, were also calculated. The presence of random mating was tested using the index of association and the parsimony tree-length permutation test. The index of association, $I_A$ (Brown *et al.*, 1980; Smith *et al.*, 1993; Haubold *et al.*, 1998; Agapow & Burt, 2001), was calculated using MULTILOCUS v. 1.3b. A randomization (10 000 repeats) of alleles among individuals, independently for each locus (Burt *et al.*, 1996), was performed to assess any significant deviations from the gametic equilibrium expected under the null hypothesis of random mating ($\alpha = 0{\cdot}05$). To exclude the risk of a physical linkage between the loci, which would skew the results, an exact test between all of the pairs of loci in each population was performed using ARLEQUIN v. 3.5.1.2. Because no loci were found to be linked, no further precautions were taken before the analysis.

The parsimony tree-length permutation test (PTLPT) was used to compare the lengths of the most parsimonious trees obtained from the observed data to the lengths of comparable trees from randomized data sets in a manner similar to that used in the previous randomizations. Again, 10 000 randomizations were performed to assess the statistical significance associated with the test of the null hypothesis of random mating. Under random mating, the expected tree length is substantially longer, and more than one tree would be identified. In contrast, a clonal population should produce one well-resolved, significantly shorter tree (Burt *et al.*, 1996). The data sets for this analysis were generated using MULTILOCUS v. 1.3b and subsequently analysed using PAUP v. 4.0b10 (Swofford, 2003).

To examine the dispersal patterns, the relationships between the sites were tested to evaluate the distribution of rare alleles and shared haplotypes. A local spatial analysis was performed, and the correlation between the geographic and genetic distance matrices was assessed. The allele frequencies were determined on the clone-corrected data set for each population (ARLEQUIN v. 3.5.1.2). The genetic-distance-matrix computation and a Mantel test were performed in GENALEX v. 6.41 (Peakall & Smouse, 2006) to detect any possible geographic structure shown by the samples (i.e. isolation by distance). To study the within-site spatial patterns, the three sites with the highest number of samples from a single population were selected (Sádek, Rožnov p. Radhoštěm – Písečná and Nová Pec). A 2-D local spatial analysis (GENALEX v. 6.41) was used on the full data set to determine whether the individuals were genetically correlated with their two nearest neighbours. This analysis was then repeated to determine whether the individuals were genetically correlated with their three nearest neighbours. If clonal reproduction is prevalent, samples located closer to each other are expected to be more genetically similar. The three sites were then combined in a Multiple Dclass analysis (GENALEX v. 6.41) using the full data set. A total of 40 runs were performed, with the distance increasing by 5 km on each successive run.

## Results

All eight of the microsatellite markers were polymorphic, and 81 different alleles were detected at the loci analysed. The eight preselected markers were determined to be sufficient for the population genetic analysis because the plot of genetic diversity versus the number of loci showed that six of the markers explained 99% of the observed variation. In all, 100 haplotypes were found. Of these, 87 were unique in the entire collection (i.e. present in only one copy); of the remaining 13 shared haplotypes, nine were found only within sites, and four were shared within and between sites. The population allele frequencies are shown in Table 2.

STRUCTURE analysis was performed in order to assign individual isolates to populations. Accordingly, samples were divided into two populations based on K parameter (Fig. 1), each with 29 sampling sites. Therefore, the two populations sympatrically co-occurred at 12 of the sampling sites. The placement of the samples into populations is shown in Table 1. Both populations have a similar proportion of polymorphic loci, and the percentage of variation is almost three times higher within populations than between them. The population statistics and characteristics are shown in Table 3. The observed haplotypic diversity was high, and the values of Nei's gene diversity were 0·49 (Pop1) and 0·64 (Pop2). The $F_{ST}$ and predicted $Nm$ values between these populations are 0·2939 and 1·20124. These numbers, supported by results of a Mantel test, are indicative of prevalent range-limited spread by conidia and significant differentiation between two populations with very low numbers of migrants. Assessment of $Nm$ is susceptible to violation of the underlying model for $F_{ST}$ computation (Whitlock & McCauley, 1999) but nevertheless the computed number indicates very limited exchange of migrants between populations. The exact tests (ARLEQUIN v. 3.5.1.2) performed for each of the populations using the clone-corrected data set did not indicate that any of the loci are in linkage disequilibrium.

In Pop1, including Czech and Austrian samples, significant correlation between the genetic and geographic distances was evidenced by a Mantel test conforming to assumed spatially-limited conidial spread. In comparison, Pop2 contained Czech and the remaining foreign samples from distant localities which were genetically similar. In the 2-D local spatial analysis, the sites with the highest number of samples in Pop1 (Sádek, Rožnov p. Radhoštěm - Písečná and Nová Pec sites) showed significant correlations with their two nearest neighbours as

Table 2 *Dothistroma septosporum* population allele frequency based on eight microsatellite markers

| Locus | Population allele frequency | | |
|---|---|---|---|
| | Allele (bp) | Pop1 | Pop2 |
| J | 182 | 0·038462 | 0·104167 |
| | 186 | 0 | 0·083333 |
| | 188 | 0·250000 | 0·312500 |
| | 190 | 0·480769 | 0·479167 |
| | 192 | 0·230769 | 0 |
| | 204 | 0 | 0·020833 |
| DS2 | 361 | 0·153846 | 0·041667 |
| | 371 | 0·115385 | 0·020833 |
| | 375 | 0·230769 | 0·020833 |
| | 377 | 0·153846 | 0·104167 |
| | 379 | 0 | 0·020833 |
| | 381 | 0·019231 | 0·020833 |
| | 385 | 0 | 0·020833 |
| | 387 | 0·019231 | 0·125000 |
| | 397 | 0 | 0·104167 |
| | 399 | 0 | 0·125000 |
| | 400 | 0 | 0·020833 |
| | 401 | 0·076923 | 0·020833 |
| | 403 | 0·038462 | 0 |
| | 407 | 0·192308 | 0·125000 |
| | 421 | 0 | 0·020833 |
| | 427 | 0 | 0·020833 |
| | 429 | 0 | 0·041667 |
| | 446 | 0 | 0·020833 |
| | 450 | 0 | 0·020833 |
| | 489 | 0 | 0·020833 |
| | 493 | 0 | 0·041667 |
| | 495 | 0 | 0·041667 |
| K | 336 | 0·557692 | 0·125000 |
| | 350 | 0 | 0·020833 |
| | 354 | 0·057692 | 0·041667 |
| | 356 | 0·192308 | 0·145833 |
| | 358 | 0·173077 | 0·104167 |
| | 360 | 0 | 0·062500 |
| | 362 | 0 | 0·041667 |
| | 364 | 0 | 0·041667 |
| | 366 | 0·019231 | 0·187500 |
| | 368 | 0 | 0·020833 |
| | 370 | 0 | 0·020833 |
| | 374 | 0 | 0·062500 |
| | 397 | 0 | 0·020833 |
| | 408 | 0 | 0·020833 |
| | 412 | 0 | 0·020833 |
| | 415 | 0 | 0·062500 |
| G | 172 | 0·019231 | 0 |
| | 179 | 0·019231 | 0·020833 |
| | 182 | 0·211538 | 0·333333 |
| | 184 | 0·750000 | 0·625000 |
| | 186 | 0 | 0·020833 |
| F | 172 | 0 | 0·020833 |
| | 174 | 0·288462 | 0·854167 |
| | 176 | 0·711538 | 0·125000 |
| S | 330 | 1·000000 | 0·770833 |
| | 346 | 0 | 0·020833 |
| | 364 | 0 | 0·208333 |
| DS1 | 143 | 0 | 0·020833 |
| | 147 | 0 | 0·062500 |
| | 151 | 0·480769 | 0·166667 |
| | 153 | 0·019231 | 0·270833 |
| | 155 | 0·038462 | 0·104167 |
| | 157 | 0·115385 | 0·291667 |
| | 159 | 0·230769 | 0·020833 |
| | 161 | 0·115385 | 0·020833 |
| | 174 | 0 | 0·041667 |
| L | 304 | 0·038462 | 0 |
| | 308 | 0 | 0·020833 |
| | 321 | 0·846154 | 0·520833 |
| | 337 | 0 | 0·062500 |
| | 341 | 0·057692 | 0 |
| | 342 | 0 | 0·020833 |
| | 345 | 0 | 0·020833 |
| | 347 | 0 | 0·020833 |
| | 349 | 0 | 0·041667 |
| | 351 | 0·019231 | 0·125000 |
| | 357 | 0·019231 | 0·041667 |
| | 359 | 0 | 0·020833 |
| | 362 | 0 | 0·020833 |
| | 363 | 0·019231 | 0 |
| | 367 | 0 | 0·041667 |
| | 369 | 0 | 0·020833 |
| | 379 | 0 | 0·020833 |

Table 2 (Continued)

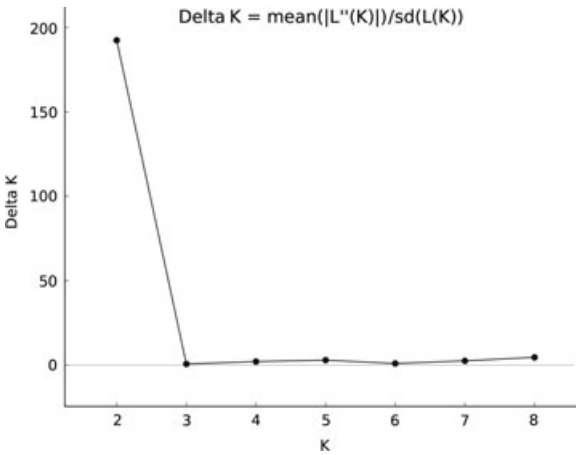


Figure 1 Delta K plot of the STRUCTURE analysis, showing K = 2 as the most probable number of populations in the data set.

follows: four of five samples from Rožnov, all of the samples from Nová Pec and one of six from Sádek showed correlations. In the analysis of the three nearest neighbours, three of seven of the samples showed correlations at Rožnov, and three of four samples at Nová Pec. No correlations were found for Sádek. In the Multiple Dclass analysis, the correlation was the highest at the shortest distances and decreased as the distance increased, but it remained significant, even at the maximum distance tested.

Tests for random mating were performed using the index of association and a PTLPT analysis (Table 4) and

Table 3 Population genetic statistics for *Dothistroma septosporum* populations

| | Sample size | | | | Diversity | |
|---|---|---|---|---|---|---|
| Population | Full data | CC | Loci[a] | No. of haplotypes[b] | Haplotypic[c] | Nei's gene[d] |
| Pop1 | 66 | 52 | 0·875 | 44 | 0·989744 | 0·494344 |
| Pop2 | 55 | 48 | 1·00 | 43 | 0·993939 | 0·648715 |

CC: clone corrected.
[a]Proportion of polymorphic loci.
[b]Number of unique haplotypes.
[c]Haplotypic diversity (MULTILOCUS v.1.3b) calculated using complete data set.
[d]Gene diversity calculated on CC data using ARLEQUIN v. 3.5.1.2; average gene diversity over loci.

Table 4 Random mating test of *Dothistroma septosporum* on full data set and clone-corrected (CC) data set

| | Index of association ($I_A$)[a] | | PTLPT[b] | | |
|---|---|---|---|---|---|
| Population | $I_A$ | *P* value | L | L* | *P* value |
| Pop1 | 0·07102 | 0·145 | 73 (1) | 86 | <0·0001 |
| Pop2 | 0·36831 | <0·0001 | 104 (2) | 129 | <0·0001 |
| Total | 0·38554 | <0·0001 | 181 (1) | 229 | <0·0001 |
| Pop1 CC | 0·0492321 | 0·5639 | 75 (2) | 71 | 0·002985 |
| Pop2 CC | 0·286235 | 0·0005 | 104 (1) | 116 | <0·0001 |
| Total CC | 0·33665 | <0·0001 | 176 (2) | 199 | <0·0001 |

[a]Values obtained after 10 000 randomizations from MULTILOCUS.
[b]Parsimony tree-length permutation test (PTLPT); L = observed length of tree produced through parsimony analysis (number in parentheses corresponds to number of most parsimonious trees found); L* = length of the shortest tree in the randomized data (10 000 replicates).

generally showed a significant clonal mode of reproduction, even in the clone-corrected data set. However, the $I_A$ index for Pop1 suggested random mating. With the exception of Pop1, the PTLPT tests showed results that were consistent with the $I_A$ values, even in the clone-corrected data set.

Both mating types were found in the Czech sampling sites; in contrast, all of the Fennoscandian samples included only mating type 2. Mating type 2 occurred more frequently than mating type 1 in both Pop1 and Pop2. However, the ratio of mating type 1 to mating type 2 including samples from both populations did not differ significantly from a 1:1 segregation ($\chi^2$ = 0·54, *P* = 0·462), and a chi-square analysis of the clone-corrected data yielded similar results ($\chi^2$ = 0·87, *P* = 0·349). Nevertheless, both of the mating types were detected within a sampling site in 10 cases (21·7% of the sampling sites; Bynina – Christmas tree plantation, Jakule, Karolínka – medical centre, Luhačovice, Mštenovice, Pernek, Valašské Klobouky, Vídeň at Velké Meziříčí, Zubří – sawmill and Zubří – poultry farm). Among these sites, five had samples that all belonged to Pop1 (Bynina – Christmas tree plantation, Jakule, Karolínka – medical centre, Mštenovice and Valašské Klobouky) and one site had samples that belonged to Pop2 (Zubří – sawmill). The four remaining sites included samples that fell into both populations.

## Discussion

The genetic separation of *Dothistroma* samples into two populations by STRUCTURE analysis does not correspond to their geographical location. The highly geographically separated samples from Austria, Finland, Norway and Slovenia were placed into Pop2, as were 23 of the Czech sites. This result could be an artefact of the analysis resulting from insufficient sampling of these sparsely sampled distant locations. Alternatively, the Pop2 sites could represent descendants of the same former colonization event. However, samples placed in both populations occurred sympatrically at 12 of the Czech sites. For example, three sites in the town of Rožnov pod Radhoštěm (library, Písečná and Rybníčky), separated by a distance of 2 km, included samples from both populations. Moreover, the site sampled in detail (Písečná) included samples from both populations. The occurrence of both populations is primarily limited to urban greenery and arboretums, where the occurrence of the pathogen is strongly influenced by human activities and where the disease was introduced by the planting of infected nursery stock. The effects of human-mediated haplotype transfer are misleading in terms of the population assignment. It is probable that the conidia are dispersed only over short distances (maximum 150 m) by rain or splashing water (Peterson, 1973) and that the unreleased conidia in acervuli lose viability during the 4–6 months on the ground (Gadgil, 1970). Therefore, the role of the conidia in long-distance dispersal is limited. These assumptions were partially corroborated by the spatial analyses that showed a significant genetic correlation between the samples in Pop1 located in close proximity. The $F_{ST}$ and *Nm* values indicate limited ability of the fungal diaspores to disperse over long distances as they show significant differentiation between populations, and marginal numbers of migrants between populations. The haplotypes shared among the samples (presumably clones) were distributed primarily within the sampling sites, and only four such haplotypes occurred at two different sites. Nová Pec and Pernek, sharing two common haplotypes, are approximately 4 km apart. The first possible clone was recorded only on *Pinus ponderosa* at both of the sites, whereas the second possible clone was detected on two pine species, *Pinus mugo* and *P. uncinata* subsp. *uliginosa*. The other example of identical haplotypes was detected at Lhotka nad Bečvou and Jasenice (5 km apart): both of the occurrences were found on *P. nigra*, and it is possible that these pines shared a common origin. These findings of shared haplotypes are explainable by human-mediated haplotype transfer. Nevertheless, one example of a shared haplotype occurred in Havlíčkův Brod and Křtiny, 90 km apart, a result that is surprising.

The observed overall haplotypic diversity was high, which agrees with the results of Dale *et al.* (2011). If the hypothesis of a relatively recent introduction into Europe is considered, this unexpected outcome could be explained either by occasional genetic recombination, by the colonization of an array of genetically distinct individuals, or by the simultaneous colonization of different sites by genetically diverse diaspores. The most probable explanation would include a combination of all these hypotheses. The division of the data set into two almost-equal numbers of samples assigned to each population may be explained by two separate introductions of *D. septosporum* into Europe. This hypothesis could be tested by an analysis of the genetic similarity between isolates from potential native populations located in either Central America or Nepal (Evans, 1984; Ivory, 1994). The first European record of *D. septosporum* was published in 1911 by Doroguine in the boreal zone around St. Petersburg, Russia (Doroguine, 1911). During the 1950s and 1960s, occasional occurrences of the species were recorded in Austria (Petrak, 1961), the UK (Murray & Batko, 1962) and France (Morelet, 1967). Although the presence of *D. septosporum* was confirmed in the Czech Republic during 1999 on imported stock and during 2000 in plantations (Jankovský *et al.*, 2004), the fungus may have persisted in the country for a longer period of time, and the accumulation of the observed haplotypic diversity could have occurred. The biology of the fungus allows it to respond sensitively to weather conditions (Woods *et al.*, 2005; Welsh *et al.*, 2009), and it is probable that the recent fluctuations in climatic conditions provided DNB better opportunities to spread. In general, it is probable that forest trees respond slowly to disease, because the generation time of trees is much longer than the timescale on which such pathogens can spread (Shaw & Osborne, 2011).

Although the random mating tests performed on the data indicate a dominant clonal mode of reproduction, the high level of intrapopulation haplotypic diversity can be explained by infrequent sexual reproduction. This explanation is in agreement with Dale *et al.* (2011). A teleomorphic stage of *D. septosporum* has not been observed in the CR, and reports of a teleomorphic stage in other European countries are unusual (Butin, 1985; Karadžić, 1989; Kowalski & Jankowiak, 1998). The asci and ascospores have only rarely been observed, although an extensive observation was performed (Kowalski & Jankowiak, 1998). Nevertheless, the occurrence of a teleomorphic stage was occasionally recorded in two neighbouring countries, Germany and Poland, with similar environmental conditions. Additionally, Groenewald *et al.* (2007) confirmed the presence of both mating types in Austria and Poland. The results presented here confirm the occurrence of both mating types at 10 sampling sites (21·7% of all of the sampling sites) with the possibility for sexual reproduction of the pathogen to occasionally occur. Four of these sites (Luhačovice, Pernek, Vídeň at Velké Meziříčí and Zubří – poultry farm) included samples placed in both Pop1 and Pop2. Such localities may serve as local hotspots of genotype diversity. The current haplotype diversity within these sites can be explained by possible mating and subsequent recombination, resulting in mixed genotypes that tend to assign to either Pop1 or Pop2. In addition, the $I_A$ results for the clone-corrected data in Pop1 suggest random mating. Nevertheless, $I_A$ is known to be less sensitive to detecting clonal mode than PTLPT analysis (Taylor *et al.*, 1999). However, according to previous findings (Jankovský *et al.*, 2004; Bednářová *et al.*, 2006), the spread of the fungus results primarily from the movement of infected planting stock. In urban areas and arboretums, it is probable that the diversity of *D. septosporum* is increased by the introduc-

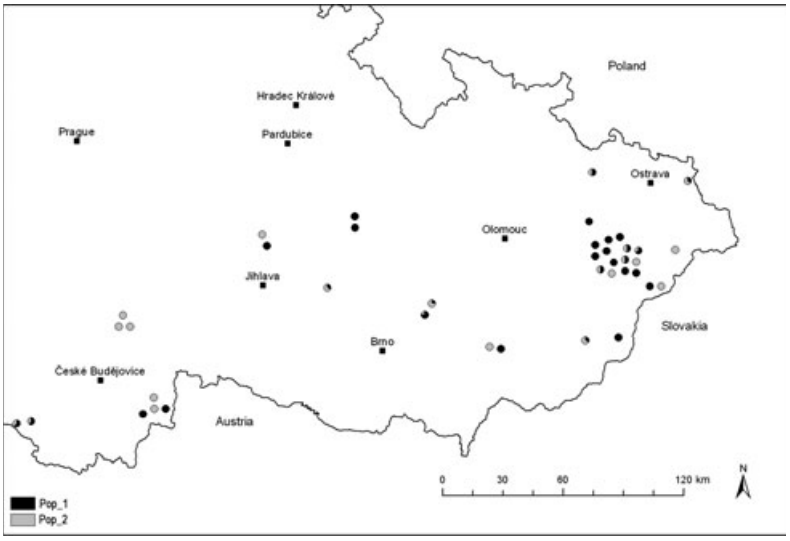


Figure 2 Map of the southeastern region of the Czech Republic, showing the sampling sites and the association of the isolates studied with the populations Pop1 and Pop2.

tion of diverse fungal diaspores of multiple origins. Apart from these instances of long-distance human-mediated transfer, the local genotypic structure is consistent with a hypothesis of predominantly conidial, range-limited spread. This hypothesis is supported by the results of the Mantel test and the spatial analyses.

The future spread of DNB can be predicted as a slow dispersal across short distances via conidia if human-mediated transfer is reduced. From the practical point of view, the control of nurseries and plantations is necessary to prevent epidemic dispersal of the pathogen. The respective phytosanitary precautions are currently applied in many EU countries. Phytosanitary authorities should pay extensive attention to imported coniferous planting stock to prevent increase of haplotypic diversity of the pathogen due to import of diverse genotypes of *D. septosporum* from foreign countries.

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