STRUCUTRE AND GENETIC VARIATION OF \textit{DIPLODIA MUTILA} ON DECLINING ASHES (\textit{FRAXINUS EXCELSIOR}) IN POLAND

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SUMMARY

This study encompassed 137 cultures of \textit{Diplodia mutila} isolated from necrotic tissue of 3- to 20-year-old trees of \textit{Fraxinus excelsior} with decline symptoms, originating from three geographic regions of Poland differing by climatic conditions. Fungus identification was conducted on the basis of morphological traits of cultures and confirmed by analysis of ribosomal DNA sequences. The structure and genetic variation of \textit{D. mutila} populations was determined with the use of RAMS markers. Neighbor Joining analysis of ITS sequences of the examined isolates showed the greatest similarity to sequences of \textit{D. mutila} isolated from \textit{F. excelsior} and deposited in GenBank. Intra-population variation expressed by Dice coefficient and Shannon index was high and increased along with the shifting of a population’s location from mild climate (northern Poland) to more severe climate, characteristic for upland and mountainous areas (southern Poland). The smallest genetic distance was shown by pairs of populations close to one another, inside individual regions. AMOVA showed great share (91%) of genetic variation inside a population, whereas variation was very small between populations (6.4%) and regions (2.6%). Principal component analysis showed grouping of fungus populations in accordance with their geographic location.

Key words: Diplodia mutila, Fraxinus excelsior, ash decline, genetic variation, RAMS markers.

INTRODUCTION

\textit{Diplodia mutila} Fr. (teleomorph: \textit{Botryosphaeria stevensii} Shoemaker) is an ascomycetous fungus of the family Botryosphaeriaceae with worldwide distribution (Sutton, 1980; Alves et al., 2004; Amponsoah et al., 2011). It infects a wide range of angiosperms (de Wet et al., 2008) and, sporadically, gymnosperms (Mohali and Encinas, 2001). On many plant species, like oak, pome and stone fruits, \textit{D. mutila} causes diseases characterized by fruit rots, stem and branch cankers, dieback of the apical twigs and, in some cases, tree death (Vajna, 1986; Ragazzi et al., 1997; Slippers et al., 2007). Ash species, including \textit{Fraxinus ornus} L. in Italy (Sidoti and Granata, 2004) and \textit{F. angustifolia} ‘Raywood’ in the USA (Aegerter et al., 2004) are host plants (Sutton, 1980).

Identification of the botryosphaeriaceous species by conventional methods based on morphological characters is allegedly difficult (Denman et al., 2000; Crous et al., 2006; de Wet et al., 2008) unless they are supported by molecular data (Denman et al., 2000; Alves et al., 2004; de Wet et al., 2008). Data from analysis of ITS sequences revealed that isolates previously identified as \textit{D. mutila} are not part of a homogeneous species. Rather, they represent a complex of several species including \textit{D. corticola} A.J.L. Phillips et A. Alves (from oak) (Alves et al., 2004) and \textit{D. cupressi} A.J.L. Phillips and A. Alves (from juniper) (Alves et al., 2006). Correct identification of fungal species and subsequent precise determination of affected host plants is the basis for further studies, including genetic variation of fungal populations, which is an important feature that can provide information on the dispersal ability and reproductive strategy of the organism in question (Baskarathewan et al., 2012).

At the beginning of 1990s, mass decline of stands of European ash (\textit{Fraxinus excelsior} L.) started in Poland, and is currently recorded in over 20 European countries (Przybył, 2002; Kowalski and Łukomska, 2005; Kowalski, 2006; Timmerman et al., 2011). The main cause of decline is \textit{Hymenoscyphus pseudoalbidus} Quezoloz et al. (anamorph: \textit{Chalara fraxinea} T. Kowalski) (Kowalski, 2006; Quezol et al., 2011). However, in certain European countries \textit{D. mutila} was isolated from necrotic tissues on trunks and branches of affected \textit{F. excelsior} plants (Przybył, 2002; Kowalski and Łukomska, 2005; Cech, 2006; Kowalski and Holdenrieder, 2008; Bakys et al., 2009).

This widespread decline prompted the research object of this paper, whose main aims were: (i) confirmation of the identification by molecular methods of \textit{D. mutila} isolated from declining \textit{F. excelsior} in Poland; (ii) determination of the variation and genetic structure of isolates obtained from dying ashes in various Polish regions; (iii) determination of the relationships among Polish isolates of \textit{D. mutila} from \textit{F. excelsior} and fungus isolates obtained from other plant species for which ITS sequences are available in GenBank.
MATERIALS AND METHODS

Isolates. The research utilized isolates of *D. mutila* obtained between 2007 and 2011 from bark necrosis on live or dead shoots of 3- to 20-year-old *F. excelsior* (Table 1) in three different regions of Poland (Fig. 1). Region I comprises the seaside part of north-western Poland, region II, the upland part of central Poland, whereas region III, the upland and partially mountainous part of southern Poland (Fig. 1). These regions differ by elevation above sea level, as well as climatic conditions, such as average annual temperatures, annual sum of precipitation and number of days with snow cover (Table 1, 2).

Fungal isolations were performed within 24-72 h from collection of shoots in the field. In the majority of cases, there were no fungal fruiting bodies on shoots. On ca. 10% of the shoots, there were pycnidia with conidia, which enabled initial identification of fungal species as *D. mutila* (Sutton, 1980; Alves *et al*., 2004). From each shoot, a section with necrotic tissue was cut out whose length ranged between 8 and 10 cm.

Shoots without fruiting bodies were superficially disinfected with ethanol 96% for 1 min, NaOCl 4% for 5 min, and ethanol 96% for 30 sec. Shoots fragments (5x2x2 mm) were then excised and partly buried in 2% of malt extract agar [MEA; 20 g l⁻¹ malt extract (Difco, USA), 15 g l⁻¹ agar Difco supplemented with 100 mg l⁻¹ streptomycin sulphate] in Petri dishes. From each shoot, 6 to 12 fragments were collected and placed on the medium. Isolations were also made from fruiting bodies when they were present on the host, after surface disinfection of the shoots with cotton wool soaked in 96% ethyl alcohol. In such cases, fragments of bark with fruiting bodies were collected with the use of a sterile scalpel after cutting off the outer layer of bark. Incubation was in the dark, at room temperature. One *Diplodia mutila*-like colony was randomly selected among the colonies of various fungi growing out from any given shoot.

The following traits, all based on observation of well characterized isolates maintained at the Department of Forest Pathology in Cracow (Poland), were used to define a colony as *Diplodia mutila*-like: fluffy, quick-growing aerial mycelium initially white and darkening in time. The growing mycelium was transferred to new MEA plates and incubated at 20°C in the dark. Some 150 cultures were initially classified for subsequent molecular analyses. Studies on structure and genetic variation were carried out with these colonies, whose affiliation to *D. mutila* was confirmed by analysis of ribosomal DNA sequence fragments.

Molecular analyses. Extraction of genomic DNA was performed following the modified procedure by Khanuja *et al*. (1999). Besides morphology-based identification, strains were also defined based on sequence of the entire ITS operon including ITS1, 5.8S and ITS2. Fragments of

Table 1. Origin of *Diplodia mutila* isolates studied.

<table>
<thead>
<tr>
<th>No.</th>
<th>Forest District</th>
<th>Region</th>
<th>Sample size</th>
<th>Coordinates</th>
<th>Elevation [m]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Latitude</td>
<td>Longitude</td>
</tr>
<tr>
<td>1</td>
<td>Gryfice</td>
<td>I</td>
<td>24</td>
<td>53°54'N</td>
<td>15°11'E</td>
</tr>
<tr>
<td>2</td>
<td>Resko</td>
<td>I</td>
<td>10</td>
<td>53°46'N</td>
<td>15°24'E</td>
</tr>
<tr>
<td>3</td>
<td>Rokita</td>
<td>I</td>
<td>20</td>
<td>53°45'N</td>
<td>14°50'E</td>
</tr>
<tr>
<td>4</td>
<td>Staporkow</td>
<td>II</td>
<td>7</td>
<td>51°08'N</td>
<td>20°34'E</td>
</tr>
<tr>
<td>5</td>
<td>Staszow</td>
<td>II</td>
<td>28</td>
<td>50°34'N</td>
<td>21°11'E</td>
</tr>
<tr>
<td>6</td>
<td>Wloszczowa</td>
<td>II</td>
<td>10</td>
<td>50°51'N</td>
<td>19°58'E</td>
</tr>
<tr>
<td>7</td>
<td>Limanowa</td>
<td>III</td>
<td>13</td>
<td>49°42'N</td>
<td>20°25'E</td>
</tr>
<tr>
<td>8</td>
<td>Miechow</td>
<td>III</td>
<td>12</td>
<td>50°21'N</td>
<td>20°02'E</td>
</tr>
<tr>
<td>9</td>
<td>Ojcow</td>
<td>III</td>
<td>13</td>
<td>50°13'N</td>
<td>19°49'E</td>
</tr>
</tbody>
</table>
ribosomal DNA were amplified with the use of ITS1 and ITS4 primers (Sigma-Aldrich, USA) (White et al., 1990). The PCR reaction was conducted in 50 µl of reactive mixture containing: PCR buffer, MgCl₂ 2 mM, dNTP 0.2 mM (Fermentas, Canada), primer 1 µM, Dream Taq Green DNA Polymerase 1 U (Fermentas, Canada), template DNA 50 ng. Cycling conditions were: initial denaturation in 95°C for 5 min followed by 36 cycles of denaturation at 95°C for 1 min, annealing for 45 sec at 54°C, elongation at 72°C for 2.5 min and final extension for 8 min. Purification of PCR products and their sequencing with the use of ITS1 primer was conducted by Macrogen Europe (The Netherlands). BLAST searches were conducted using Geneious 6.1.5. software (Biomatters, New Zealand) (Drummond et al., 2012).

Analysis of genetic variation was performed with the use of RAMS (Random Amplified Microsatellites) method (Zietkiewicz et al., 1994; Hantula et al., 1996). Initially, nine primers were tested. On the basis of the number of the obtained amplification products and recurrence of electrophoretic images, the four most appropriate primers were selected. DNA amplification was carried out in 10 µl of reactive mixture with the following composition: PCR buffer, MgCl₂ 2 mM, dNTP 0.2 mM, primer 1 µM, Taq DNA polymerase 0.2 U, template DNA 10 ng. The thermocycler was programmed as described above, except that annealing temperature was dependent on the primer (Table 3). All PCR reactions were performed in the Eppendorf Mastercycler EP Gradient S (Eppendorf AG, Germany) thermocycler. The products of PCR reactions were resolved electrophoretically in 1.5% agarose gel (Prona, Spain) containing fluorescent Midori Green DNA stain (Nippon Genetics, Japan) in TBE buffer (0.089 mM Tris, 0.089 mM boron acid, 0.022 mM EDTA, pH 8.3). GeneRuler 100 bp ladder plus (Fermentas, Canada) was used as a marker for the length of DNA fragments, which was determined with the use of BIO-PROFIL Bio-1D++ version 11.11 programme (Vilber Lурmat, France).

Data analysis. PCR reactions were performed twice for each primer and isolate. In the analyses, amplification products occurring in both reactions were taken into account and were assigned one of two scores: present (1) or absent (0). Statistical analyses were conducted only on the basis of clear and easy-to-read bands. The following indices were used to evaluate intra-population variation of the examined fungus populations: percentage of polymorphic loci, Dice coefficient of genetic similarity and Shannon's information index (Shannon and Weaver, 1949). In order to determine the percentage share of variation inside a population (isolates from each study site), between populations and between regions in relation to total variation, molecular variance analysis (AMOVA) was performed. Genetic distance between fungi populations was expressed with the use of Nei's unbiased genetic distance (Nei, 1972). To perform principal component analysis (PCA), Huff's genetic distance was calculated between pairs of populations, and subsequently Huff’s matrix was used (Huff et al., 1993). Calculations of indices of inter-population variation of D. mutila isolates and AMOVA and PCA were performed with the use of GenAlex 6.4 (Peakall and Smouse, 2006).

To evaluate evidence for recombination, the linkage disequilibrium test was performed for each population. The association index (Iₐ) was calculated using the Multilocus 1.3 programme. Iₐ has an expected value of zero if there is no association of alleles at unlinked loci as expected in a randomly mating population. The significance of Iₐ was tested by randomization (1000 times) procedures and comparing the observed value of Iₐ to that expected under the null hypothesis that there is random association of loci (Agapow and Burt, 2001).

To determine dependencies between Polish isolates of D. mutila from European ash and isolates deriving from other host plant species, on the basis of ITS sequences obtained in current studies and ITS sequences available in the GenBank database, the NJ (neighbor-joining) analysis was performed and a dendrogram was plotted. ITS sequences were analysed for three isolates from each population (in total, 27 sequences) and 22 sequences retrieved from database. The Tamura and Nei (1993) nucleotide substitution model was used for distance analysis, which was performed using Geneious 6.1.5.

### Table 2. Climatic characteristics of regions where from Diploodia mutila isolates originated.

<table>
<thead>
<tr>
<th>Region</th>
<th>Average annual temperature (°C)</th>
<th>Annual sum of precipitation (mm × m²)</th>
<th>Number of days with snow cover</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region I</td>
<td>7.2</td>
<td>570</td>
<td>50</td>
</tr>
<tr>
<td>Region II</td>
<td>7.6</td>
<td>594</td>
<td>80</td>
</tr>
<tr>
<td>Region III</td>
<td>6.5</td>
<td>792</td>
<td>86</td>
</tr>
</tbody>
</table>

### Table 3. Primers used, number of amplified products and their size.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5' → 3')</th>
<th>Temperature of annealing (°C)</th>
<th>Number of amplified products</th>
<th>Length of amplified products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA</td>
<td>BDBACAACAACAACAACA</td>
<td>49</td>
<td>20</td>
<td>270-2518</td>
</tr>
<tr>
<td>AGC</td>
<td>DHBAGCAGCAGCAGCAGC</td>
<td>60</td>
<td>31</td>
<td>138-1267</td>
</tr>
<tr>
<td>AGT</td>
<td>DHBAGTAGTAGTAGTAG</td>
<td>30</td>
<td>20</td>
<td>312-3077</td>
</tr>
<tr>
<td>CCCCT</td>
<td>CCCTCCCTCCCCTCCCT</td>
<td>64</td>
<td>21</td>
<td>224-1022</td>
</tr>
</tbody>
</table>
RESULTS

On the basis of morphological traits of cultures and analysis of sequences of ribosomal DNA fragments, 137 isolates were determined as representing a single species, *D. mutila*. The remaining 13 isolates belonged to other representatives of the Botryosphaeriaceae. The results of amplification of ribosomal DNA were products with a length of ca. 515 bp. The analysed sequences were most similar to the following sequences of *D. mutila* deposited in GenBank: FJ228165 (percent of identical sites: 99.4-99.8%), EU856765 (98.9-99.2%), EU030326 (98.9%) and DQ458886 (99.0-99.8%).

NJ analysis clearly separated isolates into three groups (Fig. 2). All isolates deriving from *F. excelsior* (examined in this study and analysed on the basis of sequences from GenBank) formed one group. Isolates deriving from *Malus pumila*, *Malus sylvestris*, *Vitis vinifera*, *Fraxinus angustifolia* and *Quercus agrifolia* created a separate group. An isolate from *Quercus suber* was completely separate from all the others.

On the basis of initial analyses, four primers were selected which gave recurring and clear bands in both repetitions. Sample electrophoretic images for ACA, AGC, AGT and CCCT primers are presented in Fig. 3. Depending on the applied primer, from 20 to 31 amplification products with various lengths were obtained, in total 92
RAMS markers (Table 3). Examined populations of *D. mutila* showed high genetic intra-population and between population variations. It was not found any monomorphic marker characteristic for all 137 isolates. Each of the examined isolates was characterized by a different haplotype (pattern of bands). The degree of polymorphism of markers showed a growing tendency along with changes in the place of origin of fungal specimens from the north (region I) to the south (region III) (Table 4). Nevertheless, the regions differed with respect to the Dice coefficient of genetic similarity and Shannon’s index. Intra-population genetic variation expressed by these indices was smallest in northernmost region I and greatest in southernmost region III (Table 4). However, no significant differences between the above-mentioned indices for the populations on the small area within individual regions were determined.

Smallest genetic distance characterized pairs of populations located close to one another inside individual regions (Table 5). Dependence between genetic distance and geographic distance between populations was, however, not confirmed by Mantel test. Principal components analysis (PCA) showed that fungus populations create three groups related to their geographic location (Fig. 4). The first comprised populations located on a lowland region of northwestern Poland (Region I); the second, populations from upland area of central Poland (Region II), whereas the third, one upland populations from Miechów and Ojcow and a mountainous population from Limanowa.

AMOVA showed that almost 91% of total genetic variation was located inside a population (Table 6). Variation between populations had a much smaller share (6.43%), whereas only 2.6% of genetic variation resulted from...
Table 5. Pairwise population matrix of Nei’s unbiased genetic distance.

<table>
<thead>
<tr>
<th></th>
<th>Gryfice</th>
<th>Resko</th>
<th>Rokita</th>
<th>Staszow</th>
<th>Staporkow</th>
<th>Włoszczowa</th>
<th>Limanowa</th>
<th>Miechow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resko</td>
<td>0.010</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rokita</td>
<td>0.032</td>
<td>0.024</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staszow</td>
<td>0.056</td>
<td>0.101</td>
<td>0.068</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staporkow</td>
<td>0.069</td>
<td>0.055</td>
<td>0.060</td>
<td>0.034</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Włoszczowa</td>
<td>0.057</td>
<td>0.031</td>
<td>0.050</td>
<td>0.056</td>
<td>0.008</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limanowa</td>
<td>0.087</td>
<td>0.084</td>
<td>0.101</td>
<td>0.066</td>
<td>0.077</td>
<td>0.059</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miechow</td>
<td>0.057</td>
<td>0.059</td>
<td>0.063</td>
<td>0.028</td>
<td>0.034</td>
<td>0.046</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Ojcow</td>
<td>0.053</td>
<td>0.046</td>
<td>0.037</td>
<td>0.073</td>
<td>0.014</td>
<td>0.026</td>
<td>0.041</td>
<td>0.010</td>
</tr>
</tbody>
</table>

affiliation of populations to specific regions. Differences on all levels of AMOVA were statistically significant for P=0.001.

The index of association ($I_A$) values for populations and regions were significantly greater than that for randomized data sets giving the appearance of clonality for all populations and regions (Table 4). Therefore, the null hypothesis that loci are randomly associated in the populations, regions and in all investigated isolates was rejected.

DISCUSSION

This study represents the first molecular characterization of *D. mutila* isolated from *Fraxinus excelsior* with dying symptoms in Poland. It confirms the results of earlier studies, in which *D. mutila* was identified via morphological traits indicating frequent occurrence of this species on ashes in Poland (Przybył, 2002; Kowalski and Łukomska, 2005; Kowalski and Czekaj, 2010). ITS sequences from GenBank, which were most similar to sequences from the examined isolates, differed by only 1-2 base pairs from the ITS sequence of typical *D. mutila* culture (CBS 112553) (Alves et al., 2004). These results greatly improved the reliability of molecular identification of the fungal isolates and decreased the possible mistakes caused by insufficient annotations in public DNA repositories (Nilsson et al. 2006).

Disease expression for the Botryosphaeriaceae species is almost exclusively associated with some form of stress or non-optimal growth conditions of the trees (Stanisz et al., 2001; Slippers and Wingfield, 2007; Ragazzi et al., 1999). In the case of *F. excelsior*, among biotic factors, which may facilitate the infection of *D. mutila*, *Hymenoscyphus pseudoalbidus* should be included. The significant role of *H. pseudoalbidus* as a factor making ashes susceptible to infections by other fungi seems supported by the fact that before the appearance of *H. pseudoalbidus* and the resulting phenomenon of ash dieback in Europe, *D. mutila* did not belong to the fungal species frequently recorded on *F. excelsior* (Sutton, 1980; Butin and Kowalski, 1986).

Polish isolates of *D. mutila* originated from *F. excelsior* are genetically most similar to *F. excelsior* isolates from Netherlands, Lithuania and Sweden, which may indicate a certain degree of host specialization of this fungal species. Such cases are well documented in mycology. They lead to formation of “forme speciales”, which are characterized by the fact that they do not differ morphologically, but genetically and by the targeted plants (Correll, 1991; Namiki et al., 1994). It is also interesting to note that isolates from *F. excelsior* are phylogenetically quite distant from those from *F. angustifolia*. These results significantly comply with the observations of Ragazzi et al. (1997) in Italy, who identified nine vegetative compatibility groups on six *Quercus* species. There was some indication that certain vegetative compatibility groups are associated with particular oak species. Different results were obtained by de Wet et al. (2009), who showed that *D. cupressi* is more closely related to species from hardwoods, such as *D. mutila* from *Fraxinus*, than to *D. pinea*, which, similarly to *D. cupressi*, occurs on gymnosperms.

The genetic variation of *D. mutila* isolates was investigated with the use of RAMS markers, which offer the possibility of obtaining indefinite number of polymorphic loci. Thanks to this, they are commonly applied to study population structure, epidemiology and infection biology of plant pathogenic ascomycetes (Hantula et al., 1996; Kraj and Kowalski, 2008; Guglielmo et al., 2012).

The present results provide data regarding variation and genetic structure of *D. mutila* populations from declining trees of *F. excelsior* in Poland. The high intra-population variation of the fungus is related to the variation of climatic conditions of the areas of its occurrence and the ecological features of *D. mutila* (Alves et al., 2004). Thanks to this, *D. mutila* is adapted to various climatic conditions and can act as an active or latent pathogen or an endophyte in a variety of woody hosts (Crous et al., 2006; Slippers and Wingfield, 2007). On the basis of oak
resistance to infections and differences in pathogenicity between fungal strains, Ragazzi et al. (1997) showed that *D. mutila* is a heterogeneous and genetically diverse species. It seems that one of the important consequence of pathogen variation is the ability to invade different species of host plants.

Genetic diversity is influenced by the relative contribution of the asexual and sexual reproduction of a species. Reproduction of *D. mutila* may take place in the field asexually, as well as sexually (Shoemaker, 1964; Stanosz et al., 1998; Alves et al., 2004; Crous et al., 2006; Amponsah et al., 2011). However, the teleomorphic stadium is rarely found in Poland (T. Kowalski, unpublished information) as in other countries (Shoemaker, 1964; Vajna, 1986; Slippers et al., 2007). The main role in dispersion of the species is played by conidia (Kuntzmann et al., 2009). The results of *I*<sub>2</sub> analysis showed that clonality decides on the genetic structure of *D. mutila* populations in Poland. Ascertainment of the presence of clonal reproduction was confirmed by the occurrence of conidia-bearing pycnidia in ca. 10% of the shoots. However, AMOVA and the absence of identical haplotypes suggest the presence of sexual stage. We rejected the hypothesis that loci are randomly associated, but caution is required in the interpretation of the association index (Agapow and Burt, 2001). Linkage disequilibrium may be influenced not only by non-random mating, including asexual reproduction, but also by chromosomal linkage, selection pressures, epigenetic effects and mixture of differentiated genetic isolates (Hedrick, 2000; Maynard Smith, 1993). The source of genetic variation of fungi that reproduce asexually can also be somatic hybridization and heterokariosis (Burdon and Silk, 1997; Bock et al., 2002). In a related species, *D. pinea*, occurring on gymnosperms, the process of cryptic recombination was ascertained, which is one of the significant sources of genetic variation (Bihon et al., 2012).

It was ascertained that smallest genetic distances occurred in pairs of populations located inside the examined regions. Even though Mantel test was not significant, yet PCA showed grouping of the examined populations in agreement with the geographic location of the regions of their origins. The results suggest that *D. mutila* is not dispersal-limited. This is confirmed by lack of limitations in the gene flow between populations and regions. The share of variation among populations, in particular between regions of origin of isolates, was significant, yet very small. Increased variability in regions with harsher climate may be linked to increased local extinction of individual genotypes by climatic conditions and increased sexual reproductive rates. Enhancement of intra-population variation along with shift in the location of the *D. mutila* population from the north to the south of Poland, i.e. from maritime (mild) climate to the harsher and more variable climate characteristic for upland and mountainous regions occurs also in many other fungi species. Studies by Kraj and Kowalski (2008) prove that main factors influencing genetic variation of *Gremmeniella abietina* populations were elevation above the sea level and climatic conditions in the place of their occurrence. Similar results were obtained by Kraj (2009) in the case of genetic variation of *Scleropithyma pythiophila* populations. In both fungal species, similarly to *D. mutila*, high inter-population genetic variation was ascertained, dependent upon climatic conditions with simultaneous lack of correlation between genetic and geographic distance.

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