PRELIMINARY STUDIES ON GENETIC DIVERSITY OF ECTOMYCORRHIZAL FUNGUS *SUILLUS BOVINUS* IN LITHUANIA

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Abstract

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Genetic diversity of some Lithuanian populations of *Suillus bovinus* was assessed basing on nucleotide sequence of ITS1 and 2 regions, and the 5.8S RNA gene obtained from 42 samples. Four haplotypes were defined, each representing isolate groups of varying abundance and distribution. The most abundant and widely distributed was haplotype 2. All Lithuanian haplotypes nested in one, the largest clade of European isolates of *S. bovinus*.

Keywords: genetic diversity, biogeography, *Suillus bovinus*, Lithuania.

INTRODUCTION

The genus *Suillus* Gray (Boletales, Basidiomycota) comprises widespread species, which form mycorrhizas with coniferous trees (Pinaceae) in temperate and boreal regions (Singer, 1986). Eight species of the genus are known in Lithuania, four of these (*S. bovinus, S. granulatus, S. luteus* and *S. variegatus*) are common (Urbonis, 1997). *Suillus bovinus* shows the least intraspecific variation and forms well-defined taxonomic entity (Kretzer et al., 1996; Manian et al., 2001). Besides, *S. bovinus* is one of the most common and abundant representatives of the genus, usually found associated with the only naturally occurring in Lithuania pine – *Pinus sylvestris*. The fungus is also widely distributed over Europe and Asia forming associations with two-needle pines: *Pinus sylvestris, P. densiflora, P. massoniana, P. nigra, P. thunbergii, P. taiwanensis* (Hung & Chien, 1979; Duddridge & Read, 1984; Yamada et al., 2001; El Karkouri et al., 2005). As an introduced species, *S. bovinus* has spread to several continents following planted hosts (Vellinga et al., 2009).

Mycorrhizal associations are widespread and play a fundamental role in the life of forest trees, supporting them with minerals, water and rendering other services, thus playing important roles in ecosystems (Read & Perez-Moreno, 2003; Selosse & Duplessis, 2006). Tree roots associate with many different species of ectomycorrhizal fungi (Dahlgren et al., 1997) and genetically variable populations (Debaud et al., 1999; Fiore-Donno & Martin, 2001). The fact that trees are exposed to genetically diverse mycobiont is an important consideration in forest ecology (Gherbi et al., 1999). This is true both at stand and regional level. Assessment of genetic diversity and geographical variation of populations also help in better understanding of natural formation of post-glacial European biodiversity as well as modern invasions of the species. The geographical distribution
of DNA-types has been used to determine diversification areas and historical migration of fungal germplasm (KERRIGAN, 1995; WU et al., 2000; RUBINI et al., 2005; GEML et al., 2008; HALLING et al., 2008).

The objective of this study was to perform a preliminary evaluation of variation of *S. bovinus* populations in Lithuania and to compare it with known European and extra-European population data.

**MATERIALS AND METHODS**

**Material studied**

Sequences for the ITS region (ITS1 + 5.8S + ITS2) were obtained from *Suillus bovinus* gathered by field collecting in yrs 2007–2008 or from herbarium (BI-LAS) specimens.

Collections were made in the areas with prevail-

### Table 1. List of *Suillus bovinus* samples, GenBank accession numbers and collection localities

| Isolate No | GenBank accession No | Collection locality and year                           |
|------------|----------------------|--------------------------------------------------------|
| BI-201-2   | GU016579             | Švenčionys distr., Pabradė environs, 2007              |
| BI-201-3   | GU016580             | Švenčionys distr., Pabradė environs, 2007              |
| BI-201-4   | GU016611             | Švenčionys distr., Pabradė environs, 2007              |
| BI-201-7   | GU016581             | Švenčionys distr., Pabradė environs, 2007              |
| BI-201-8   | GU016582             | Švenčionys distr., Pabradė environs, 2007              |
| BI-202-1   | GU016572             | Curonian Spit, Nagliai Nature Reserve, 2007            |
| BI-202-2   | GU016573             | Curonian Spit, Nagliai Nature Reserve, 2007            |
| BI-202-3   | GU016574             | Curonian Spit, Nagliai Nature Reserve, 2007            |
| BI-202-4   | GU016575             | Curonian Spit, Nagliai Nature Reserve, 2007            |
| BI-202-5   | GU016576             | Curonian Spit, Nagliai Nature Reserve, 2007            |
| BI-202-6   | GU016577             | Curonian Spit, Nagliai Nature Reserve, 2007            |
| BI-202-7   | GU016578             | Curonian Spit, Nagliai Nature Reserve, 2007            |
| BI-203-11  | GU016593             | Varėna distr., Varėna environs, 2008                   |
| BI-203-12  | GU016594             | Varėna distr., Varėna environs, 2008                   |
| BI-203-13  | GU016595             | Varėna distr., Varėna environs, 2008                   |
| BI-203-14  | GU016596             | Varėna distr., Varėna environs, 2008                   |
| BI-203-15  | GU016597             | Varėna distr., Varėna environs, 2008                   |
| BI-203-16  | GU016598             | Varėna distr., Varėna environs, 2008                   |
| BI-203-17  | GU016599             | Varėna distr., Varėna environs, 2008                   |
| BI-203-22  | GU016614             | Varėna distr., Marcinkonys environs, 2008              |
| BI-203-23  | GU016615             | Varėna distr., Marcinkonys environs, 2008              |
| BI-203-24  | GU016616             | Varėna distr., Marcinkonys environs, 2008              |
| BI-203-25  | GU016617             | Varėna distr., Marcinkonys environs, 2008              |
| BI-203-26  | GU016618             | Varėna distr., Marcinkonys environs, 2008              |
| BI-203-27  | GU016619             | Varėna distr., Marcinkonys environs, 2008              |
| BI-203-28  | GU016620             | Varėna distr., Marcinkonys environs, 2008              |
| BI-203-29  | GU016600             | Varėna distr., New Varėna environs, 2008               |
| BI-204-31  | GU016601             | Druskininkai, 2008                                    |
| BI-204-32  | GU016602             | Druskininkai, 2008                                    |
| BI-204-33  | GU016603             | Druskininkai, 2008                                    |
| BI-204-34  | GU016604             | Druskininkai, 2008                                    |
| BI-204-35  | GU016605             | Druskininkai, 2008                                    |
| BI-204-36  | GU016621             | Druskininkai, 2008                                    |
| BI-204-37  | GU016606             | Varėna distr., Mašnyčios environs, 2008                |
| BI-204-38  | GU016607             | Varėna distr., Mašnyčios environs, 2008                |
| BI-204-39  | GU016608             | Varėna distr., Mašnyčios environs, 2008                |
| BI-204-40  | GU016609             | Varėna distr., Mašnyčios environs, 2008                |
| BI-204-41  | GU016610             | Varėna distr., Mašnyčios environs, 2008                |
| BI-200-H4  | GQ994940             | Akmenė distr., Kamanos Nature Reserve, 1996            |
| BI-200-H5  | GQ994941             | Varėna distr., Zervynos environs, 2003                 |
| BI-205-19  | GU016612             | Šalčininkai distr., Dieveniškės environs, 2008         |
| BI-205-20  | GU016613             | Šalčininkai distr., Dieveniškės environs, 2008         |
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ing pine forests (Navasaitis et al., 2003): Curonian Spit, Akmenė, Švenčionys, Šalčininkai, Varėna districts and Druskininkai municipality – a total of 42 samples. Isolate numbers, GenBank accession numbers and collection localities are listed in Table 1.

Sequences for comparison with other European and extra-European populations of *S. bovinus* as well as sequences of *Suillus* species used for outgroup were directly downloaded from GenBank. Data on these sequences are provided in Table 2.

Ten sequences of extra-Lithuanian *S. bovinus* specimens were obtained from collections made by the authors in 2008 in Estonia (Saaremaa island, Odalätsi Nature Reserve) (Table 2).

DNA extraction, amplification, sequencing and analysis

Genomic DNA was extracted from two dried herbarium specimens (collected in 1996 and 2003) and frozen fresh fruitbodies (collected in 2007–2008) with NucleoSpin® Plant II Kit (Macherey–Nagel GmbH & Co. KG, Germany) according to manufac-

| Species name | GenBank accession No | Country of origin, reference |
|--------------|----------------------|-----------------------------|
| *S. americanus* | AF166501 | USA, Wu et al., 2000 |
| *S. bovinus* | AJ419215 | Portugal, Martin & Raidl., 2002 |
| *S. bovinus* | EF493250 | Sweden, Nygren et al., 2008 |
| *S. bovinus* | EF493249 | Sweden, Nygren et al., 2008 |
| *S. bovinus* | L54077 | Sweden, Kretzer et al., 1996 |
| *S. bovinus* | DQ068967 | Lithuania, Minks et al., 2005 |
| *S. bovinus* | AJ419934 | Finland, Groenburg et al., 2003 |
| *S. bovinus* | AJ493679 | Finland, Groenburg et al., 2003 |
| *S. bovinus* | AJ419935 | Finland, Groenburg et al., 2003 |
| *S. bovinus* | AJ272404 | UK, Manian et al., 2001 |
| *S. bovinus* | AJ272402 | UK, Manian et al., 2001 |
| *S. bovinus* | AJ272401 | UK, Manian et al., 2001 |
| *S. bovinus* | AJ272403 | UK, Manian et al., 2001 |
| *S. bovinus* | EU379677 | Poland, Hilszczanka et al., 2008 |
| *S. bovinus* | DQ179128 | Sweden, Fransson et al., 2007 |
| *S. bovinus* | APY98623 | Central Spain, Ruiz-Diez et al., 2006 |
| *S. bovinus* | AB036902 | Japan, Murata, 2000 (unpublished) |
| *S. bovinus* | FJ481028 | China, Jiang et al., 2008 (unpublished) |
| *S. bovinus* | AB284446 | Japan, H prescription and Tokumasu, 2006 (unpublished) |
| *S. bovinus* | AF438604 | Germany, Harms et al., 2001 (unpublished) |
| *S. bovinus* | BI-300-1 – BI-300-10 (10 sequences) | Estonia, present publication |
| *S. caerulescens* | EU486453 | Canada, Denis & Berihe, 2008 (unpublished) |
| *S. collinitus* | DQ440567 | Central Spain, Ruiz-Diez et al., 2006 |
| *S. collinitus* | DQ440569 | Central Spain, Ruiz-Diez et al., 2006 |
| *S. granulatus* | AJ272410 | UK, Manian et al., 2001 |
| *S. grevillei* | EU488714 | Ireland, Mitchell et al., 2008 (unpublished) |
| *S. luteus* | AJ272411 | UK, Manian et al., 2001 |
| *S. luteus* | AJ272415 | UK, Manian et al., 2001 |
| *S. luteus* | DQ440568 | Central Spain, Ruiz-Diez et al., 2006 |
| *S. luteus* | AY988620 | Central Spain, Ruiz-Diez et al., 2006 |
| *S. mediterraneensis* | AY935512 | Central Spain, Ruiz-Diez et al., 2006 |
| *S. tomentosus* | DQ988251 | Canada, Paul et al., 2006 (unpublished) |
| *S. umbonatus* | L54115 | USA, Kretzer et al., 1996 |
| *S. variegatus* | AJ272420 | UK, Manian et al., 2001 |
| *S. variegatus* | AJ272421 | UK, Manian et al., 2001 |
| *S. variegatus* | AY988622 | Central Spain, Ruiz-Diez et al., 2006 |
| *S. variegatus* | AM086446 | UK, Izumi et al., 2007 |
turer’s instruction using approximately 100 mg wet or 20 dried weight of fruiting bodies. The internal transcribed spacers 1 and 2 of rDNA, including the 5.8S rDNA, were amplified in 25 µl reactions on TProfessional 96 Gradient Thermocycler (Biometra GmbH, Germany) in the following mixture: ~25 ng of template, 0.25 units of Taq polymerase (UAB „Thermo Fisher Scientific Baltics”), 2.5 µl 10× PCR buffer with KCl and MgCl₂, 0.2 mM of each dNTP, 10 µM of primers ITS5 and ITS4 (White et al., 1990). The PCR conditions were the following: 10 min at 95°C as initial denaturation, followed 35 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C, with final extension of 10 min at 72°C. Amplicons were separated on 1.5% agarose for gel electrophoresis in TAE buffer at 80 V, 120 mA. The PCR products were purified according to the Protocol for PCR Product Clean-up with Exonuclease I and FastAP™ Thermosensitive Alkaline Phosphatase (UAB „Thermo Fisher Scientific Baltics”). Purified PCR products were sequenced by Macrogen (Macrogen Inc., Seoul, Korea) on an ABI 3730XL DNA sequencer. Two different PCR products from each specimen from both ends (5’ and 3’) were sequenced to confirm the sequence. The rDNA homology searches were performed through the internet at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, USA). Sequences were aligned by using the Clustal W method, and phylogenetic tree analysis was performed with the Lasergene software package (DNASTAR, Inc., Madison, USA). All sequences have been deposited with the NCBI GenBank database. The accession numbers are provided in Table 1.

RESULTS AND DISCUSSION

The nucleotide sequence of ITS1 and 2 regions, and the 5.8S RNA gene were obtained for a total of 52 samples (of these, 42 Lithuanian samples, Table 1). Changes of bases in three positions of DNA: 141 and 230 positions of spacer 1 sequences and 499 positions in spacer 2 sequences were observed. Thus, intraspecific sequences’ variation was found to be very low among tested isolates. Basing on these differences, four haplotypes of Suillus bovinus were defined, their similarity being 99.7–99.9%. First haplotype comprised two specimens from Akmenė and Varėna districts, second – the largest one – comprised specimens from eight different, geographically widely spaced localities (notably, Estonian samples were identical with this haplotype). Third haplotype included 10 specimens from the southern and eastern parts of Lithuania, and a single sample from Druskininkai environs belonged to fourth haplotype (Figs 1 and 2). Haplotype 2 showed distribution virtually in the whole area of Lithuania, limited by the presence of suitable forests (for the distribution of forest stands in Lithuania see Navasaitis et al., 2003), and comprised the largest part of studied S. bovinus population in the country (Fig. 3).

Different haplotypes of S. bovinus were neither restricted to specific habitats nor showed strong spatial delimitation: e.g. specimen BI-204-36 (haplotype 4) was found in very close proximity (ca. 50 m) to the specimens BI-204-(29-35) (haplotype 2) (Table 1). This is explainable by high genetic diversity in populations of ectomycorrhizal fungi (Gherbi et al., 1999). Notably, none of haplotypes defined by us...
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Fig. 2. Geographical distribution of the studied *Suillus bovinus* haplotypes in Lithuania: ♦ – haplotype 1; ● – haplotype 2; ■ – haplotype 3; □ – haplotype 4

Fig. 3. Proportion of haplotypes 1–4 (H1–H4) in Lithuanian population of *Suillus bovinus*

were identical to the Lithuanian haplotype obtained by MENKIS et al. (2005) (Table 2, Fig. 4), which is explainable both by limited area covered by the present investigation and by temporal variability of fruiting genets, as was noted in the study of *Laccaria amethystina* (GHERBI et al., 1999). In most of our collection localities, however, only one haplotype was found.

To compare the obtained Lithuanian genetic material of *S. bovinus* to that from other parts of the Eurasian continent as well as to define the place of Lithuanian population in general genetic pattern of the species, we employed the data downloaded from GenBank and the data from specimens collected in Estonia (Table 2).

Though it is reported that *S. bovinus* shows little intraspecific variation (MANIAN et al., 2001), we compared the sequences downloaded from GenBank against sequences of other *Suillus* species previous to phylogenetic analysis. JAROSCH (2001) stated that *S. bovinus* forms a monophyletic group together with *S. hirtellus*, *S. tomentosus* and *S. variegatus*, however, study by MANIAN et al. (2001) has shown that some of isolates of this group, namely *S. variegatus*, nested in the clade of *S. luteus*, *S. granulatus* and *S. subluteus*. Therefore, for our analysis we employed species of *Suillus* also outside the group that was defined by JAROSCH (2001). All *S. bovinus* sequences obtained by us and accessible in GenBank nested into a well-defined clade except for one (Acc. No AF438604, for details see Table 2), which was excluded from the further analyses as apparently not belonging to *S. bovinus*.

Four clades were detected within all analysed isolates of *S. bovinus*. Clade I represented bulk of the European isolates (Fig. 4). Clade II consisted of three Asian isolates from China and Japan. Two isolates from Sweden and from Portugal formed separate lineages. However, support of all clades was not so high (bootstrap value 62 or less). Groupings within the clade I were even less significantly supported, with low nucleotide diversity values. Thus, it can be stated that all Lithuanian isolates belong to the same major European clade, which apparently comprises the largest part of *S. bovinus* population in the subcontinent. This part of population is widespread from northwestern Atlantic pine forests to subcontinental stands in the east, southwards extending to Iberian Peninsula, apparently being widely adaptive to various climates and habitats. This, along with very high spore production (DAHLBERG & STENLID, 1994) might be the reason of successful introduction and establishment of *S. bovinus* in several continents.

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**EKTOMIKORIZINIO GRYBO *SUILLUS BOVINUS* GENETINIO KINTAMUMO LIETUVOJE TYRIMAI**

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**Santrauka**

Tampriojo kazlėko (*Suillus bovinus*) genetinė įvairovė Lietuvos populiacijose buvo ištirta lyginant RNR geno tarpiklių ITS1 ir ITS2 sekas, gautas iš 42 grybų pavyzdžių. Nustatyti keturi haplotipai, kurie reprezentavo skirtingo paplitimo ir gausumo izoliatų grupes. Gausiausias ir plačiausiai paplitęs buvo haplotipas 2, kuris taip pat buvo genetiškai identiškas pavyzdžiams, surinktiems Estijoje. Visi Lietuvoje nustatyti haplotipai jungesi vienoje, pačioje didžiausioje europinių *S. bovinus* izoliatų filogenetinėje grupėje.