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

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Abstract

Motiejūnaitė J., Kačergius A., Kasparavičius J., 2013: Preliminary studies on genetic diversity of ectomycorrhizal fungus *Suillus bovinus* in Lithuania [Pradiniai ektomikorizinio grybo *Suillus bovinus* genetinio kintamumo Lietuvoje tyrimai]. – Bot. Lith., 19(2): 91–98.

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 (Urbonas, 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 (Dahlberg 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 postglacial 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 (BILAS) 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

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-

Table 2. Details of the *Suillus* spp. sequences from the GenBank

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, Menkis et al., 2005
S. bovinus	AJ419934	Finland, Groenberg et al., 2003
S. bovinus	AJ493679	Finland, Groenberg et al., 2003
S. bovinus	AJ419935	Finland, Groenberg 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, Hilszczańska et al., 2008
S. bovinus	DQ179128	Sweden, Fransson et al., 2007
S. bovinus	AY898623	Central Spain, Ruiz-Díez et al., 2006
S. bovinus	AB036902	Japan, Murata, 2000 (unpublished)
S. bovinus	FJ481028	China, Jiang et al., 2008 (unpublished)
S. bovinus	AB284446	Japan, Hirose & 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 & Berbere, 2008 (unpublished)
S. collinitus	DQ440567	Central Spain, Ruiz-Díez et al., 2006
S. collinitus	DQ440569	Central Spain, Ruiz-Díez 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-Díez et al., 2006
S. luteus	AY898620	Central Spain, Ruiz-Díez et al., 2006
S. mediterraneensis	AY935512	Central Spain, Ruiz-Díez 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	AY898622	Central Spain, Ruiz-Díez 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 $MgCl_2$, 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

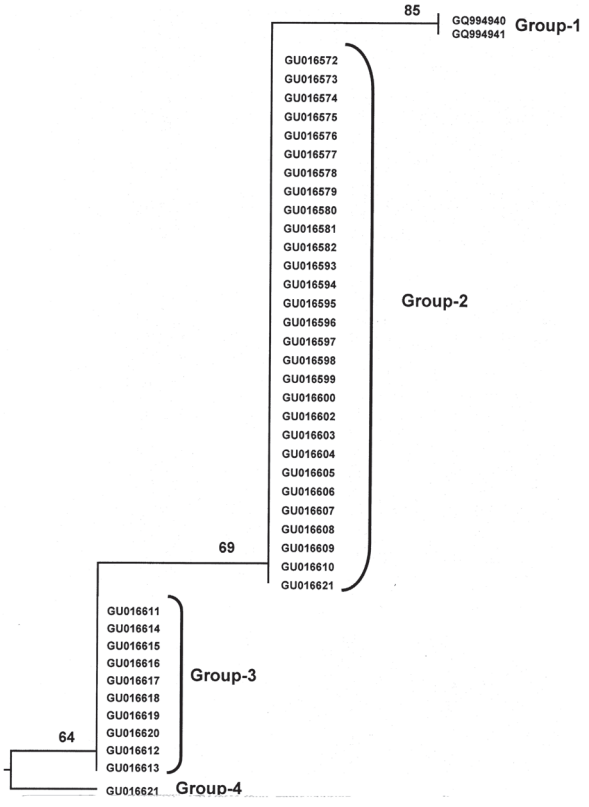

Fig. 1. Phylogram of four groups (haplotypes) of Lithuanian *Suillus bovinus* isolates. The numbers above branches are bootstrap support values based on parsimony (percentage)

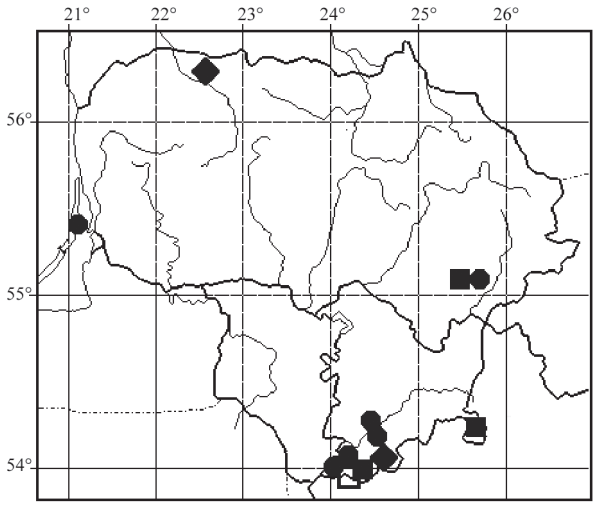

Fig. 2. Geographical distribution of the studied *Suillus bovinus* haplotypes in Lithuania: ◆ – haplotype 1; ● – haplotype 2; ■ – haplotype 3; □ – haplotype 4

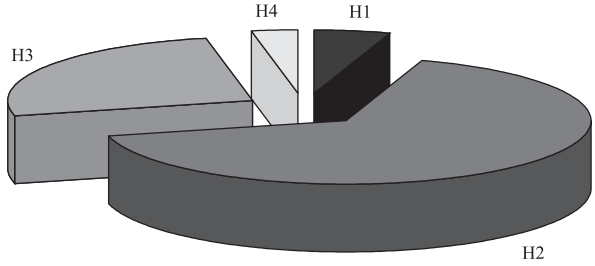

Fig. 3. Proportion of haplotypes 1–4 (H1–H4) in Lithuanian population of *Suillus bovinus*

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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Fig. 4. Parsimony tree derived from the neighbour-joining analysis of Lithuanian *Suillus bovinus* isolates and other species of the *Suillus* genus inferred from ITS sequence data. *S americanus* (AF166501) was used as an outgroup. The values above branches are bootstrap support values based on parsimony (percentage). Bootstrap values < 50% are not indicated

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EKTOMIKORIZINIO GRYBO *SUILLUS BOVINUS* GENETINIO KINTAMUMO LIETUVOJE TYRIMAI

Jurga Motiejūnaitė, Audrius Kačergius, Jonas Kasparavičius

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 jungėsi vienoje, pačioje didžiausioje europinių *S. bovinus* izoliatų filogenetinėje grupėje.