Comprehending phylogenetic diversity – case studies in three groups of ascomycetes

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Comprehending phylogenetic diversity –
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# CONTENTS

LIST OF ORIGINAL PUBLICATIONS ................................................................. 6

INTRODUCTION .......................................................................................... 7
  Species recognition criteria .................................................................... 7
  Selected taxa ......................................................................................... 8
    Geopora ............................................................................................. 9
    Hypomyces ........................................................................................ 10
    Encoelia .............................................................................................. 10
  Gene regions used for delimitation of taxa ........................................... 11
  Importance of taxonomy in biodiversity studies ............................... 12
  Aims of the study .................................................................................. 12

MATERIALS AND METHODS ................................................................. 13
  Sampling of specimens and species identification ............................ 13
  DNA extraction, PCR and sequencing ............................................... 13
  Sequence alignment and phylogenetic analysis .................................. 14

RESULTS AND DISCUSSION ................................................................. 16
  Delimitation of genera ........................................................................ 16
  Species delimitation based on morphological characters ............... 17
  Recognition of monophyletic taxa based on molecular characters .... 18
  Host/symbiont associations .................................................................. 20
  Geographical distribution ...................................................................... 21
  Applications of advances in taxonomy .............................................. 23

CONCLUSIONS .......................................................................................... 24

SUMMARY IN ESTONIAN ......................................................................... 26

ACKNOWLEDGEMENTS ........................................................................... 30

REFERENCES ............................................................................................. 31

PUBLICATIONS ........................................................................................... 41

CURRICULUM VITAE ................................................................................ 121
LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications that are referred to in the text by Roman numerals:


Author’s contribution to each paper

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idea and design</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sampling</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Morphological analyses</td>
<td>+</td>
<td>+</td>
<td>NA</td>
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<tr>
<td>Molecular analyses</td>
<td>+</td>
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<td>−</td>
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<td>Phylogenetic analyses</td>
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INTRODUCTION

Species recognition criteria

Traditionally, mycologists have used morphological and biological species recognition criteria to infer fungal taxonomy. Whereas both criteria are rationally used in many cases (Anderson et al. 1980, Hibbett & Donoghue 1996, Vilgalys & Sun 1994, Taylor et al. 2006), they still lack universal applicability. Although morphological species recognition method can be viewed as universal in the sense that most of the described fungal species have a morphological description, its usability is often restricted because of variability, which can be extremely high or low, and species limitation based on morphological characters is often artificial. Biological species recognition method seems more natural but is impossible to apply to the many fungi that are asexual, homothallic or cannot be cultivated. In addition to this, many fungi are able to outbreed, thus the fact of mating does not provide evidence of delimitation of species. Also, sexually compatible populations occurring in distinct regions might not be mating in nature because of geographic isolation (Vilgalys & Sun 1994, Petersen & Hughes 1999). Phylogenetic species concept is widely applicable, however, defining species limits is subjective, requiring decisions about whether observed polymorphisms are shared within a species or fixed in isolated species (Taylor et al. 2000). To overcome this subjectivity, genealogical concordance phylogenetic species recognition relying on multiple recombining genes have been proposed (Taylor et al. 2000).

The method of species recognition affects the inferred geographic range of a fungal species (Taylor et al. 2006). Whereas several morphologically defined fungal species have been found to be distributed worldwide, the phylogenetically delimited species of same organisms might have different distribution patterns (Taylor et al. 2006). The reason is that changes in DNA sequences can be detected more easily than changes in morphology which can be vague, especially in organisms with relatively simple structure. In the course of evolution the first changes always appear in the genome, and by the time at which reproductive isolation or distinguishable changes in morphology are starting to emerge, genetic isolation has already occurred. Therefore, species recognition based on morphology or reproductive isolation can be more inclusive than that based on genetic isolation. On the other hand, reproductive isolation can precede changes in morphology. Aspergillus flavus and A. fumigatus are morphological species with world-wide distribution, however, analyses have shown the occurrence of several genetically and reproductively isolated groups (Geiser et al. 1998, Pringle et al. 2005, Rydholm et al. 2006). Similarly, Fusarium oxysporum and F. graminearum both represent morphologically homogenous species complex showing diverse patterns of genetic variability, host specificity and geographic distribution, whereas genetic segregation does not always correspond to the host and distribution patterns (O’Donnell et al. 1998, Starkey et al. 1998).
2007, Lievens et al. 2009, Kvas et al. 2009, Sarver et al. 2011). In Neurospora, several groups of morphologically indistinguishable species have been discovered based on genetic segregation (Dettman et al. 2003, 2006). Another example is Beauveria, a genus of insect pathogens which displays high variation in morphological characters so that these cannot be used for identification of well-supported phylogenetic lineages (Rehner et al. 2011). Trichoderma is a large genus of soil-inhabiting filamentous fungi, the species of which are widely used as biological control agent of plant pathogenic fungi as they have ability to parasitize on other fungi (Kredics et al. 2010). Whereas several strains of T. harzianum have been found to colonize industrial mushroom beds causing green mold disease, aggressive colonization has been attributed only to a couple of biotypes which have been later redescribed as T. aggressivum f. europaeum and T. aggressivum f. aggressivum (Samuels et al. 2002). The two taxa differ in morphology and are genetically as well as geographically segregated. In addition to these ascomycetous taxa, many basidiomycetes display analogous pattern. Armillaria mellea was considered as a single species, although heterogeneous in terms of morphology and geographic distribution, yet it was later reassigned to several species based on reproductive isolation or genetic variance (Anderson et al. 1980, Coetzee et al. 2005). Also in Serpula himantioides, reproductively isolated groups have revealed several cryptic species (Kauserud et al. 2006, Carlsen et al. 2011). In rust and smut fungi, morphological characters together with narrow host ranges generally provide sufficient distinction between species as confirmed by phylogenetic analyses (Stoll et al. 2005, Seier et al. 2009).

Selected taxa

Pezizales, Hypocreales and Helotiales are three of the largest orders among ascomycetes with the systematics of many taxa at different taxonomic levels remaining obscure (Lumbsch & Huhndorf 2010). This can be ascribed to scarceness of morphological characters that are used for recognition of taxa. In distinguishing among closely related species in Pezizales and Helotiales, the micromorphology of ascospores along with fruit-body characteristics are usually given most emphasis (Hansen et al. 2001, Hansen & Pfister 2006, Baral 1985, Stone & Gernandt 2005). In Hypocreales, also the ascus micromorphology and presence of stroma in teleomorphic stage are used for species identification, with additional characters of anamorphic stage (Põldmaa 2000). Pezizales has been established as a monophyletic group (Spatafora et al. 2006, Hansen & Pfister 2006, Hansen et al. 2013), that was among the first ones to diverge among the euscomycetes, currently classified in the subphylum Pezizomycotina (Liu et al. 1999, Lumbsch et al. 2000, Platt & Spatafora, 2000). While higher level taxonomy in this order is relatively well resolved both on morphological and molecular basis, species recognition is often complicated.
Similarly, Hypocreales forms a monophyletic group (Zhang et al. 2006, Schoch et al. 2009a) displaying a broad range of lifestyles (Rossman 1996). However, the relationships of taxonomic groups within Hypocreales are unclear (Sung et al. 2007, Summerbell et al. 2011). Likewise, molecular analyses do not support the higher level system of the Helotiales based on morphological grounds. Moreover, multigene studies have shown that the order Helotiales is paraphyletic in regard to Rhytismatales, Erysiphales, Cyttariales and Thelebolales, which are nested within it (Wang et al. 2006a, b, Schoch et al. 2009a, b, Peterson & Pfister 2010).

In this study, taxon boundaries of different ranks were assessed in selected genera, one from each of the orders Pezizales, Hypocreales and Helotiales. In Hypomyces (Hypocreaceae, Hypocreales), closely related species were delimited in a small subgroup representing about 1/10 of the genus. In Geopora (Pyronemataceae, Pezizales), boundaries were assessed for majority of accepted species as well as for closely related genera. In Encoelia (Helotiales, incertae sedis), the phylogenetic relationships of available species were assessed and described at family level, covering about 1/5 of the genus.

Geopora

Geopora is a genus comprising important mycorrhizal symbionts associated with deciduous and coniferous trees as well as orchids. Although about 25 Geopora species have been described, the majority of hypogeous species have been synonymized by Burdsall (1968) and most authors recognize roughly ten species in this genus (Seaver 1928, Kers 1974, Schumacher 1979, Dennis 1968, Yao & Spooner 1996a, b, 2003, Benkert 2010). The section Sepultaria Cooke within Peziza Dill. ex Fr. was raised to the genus level in 1885 by Boudier, comprising 20 species. He selected Peziza sepulta Fr. as the type species (Kers 1974; Burdsall 1968). Two months earlier, Harkness (1885) had proposed a new genus Geopora Harkn. coincide to great extent, Burdsall (1968) designated Sepultaria as the synonym of Geopora. Following his amended concept of Geopora, the genus comprises hypogeous species with closed ascocarp, epigeous cup-shaped species of the former Sepultaria, and some species of Hydnocystis Tul. having hollow hypogeous ascocarps with randomly oriented opening (Burdsall 1968).

In Geopora, various ascocarp forms exist. These include strictly epigeous apothecia (G. tenuis (I), Hoffmannoscypa (= Geopora) pellita (Stielow et al. 2012)), ascocarps that are at first almost closed and deeply immersed in the substrate and later expanding more or less above the surface (G. arenicola, G. sepulta (I), G. cercocarpi (Southworth & Frank 2011)), and closed ptychothecia remaining hypogeous even at late developmental stages (G. cooperi complex, G. gilkeyae, G. tolucana (II)).
**Hypomyces**

*Hypomyces* (Hypocreaceae, Hypocreales) is the largest ascomycetous genus of exclusively fungicolous fungi, comprising about hundred species. The genus is paraphyletic, comprising several subgroups with different morphology and host range (Põldmaa 2000). One of such subgroups includes a monophyletic clade of approximately 25 species producing a chionic red pigment, aurofusarin, forming *Cladobotryum* anamorph and growing on agaricoid as well as poly-poroid fruit-bodies of various basidiomycete taxa. During sexual state the bright red perithecia develop in lighter subiculum that might extend to several centimetres. Asexual state is characterised by fast-growing whitish and cottony mycelium. The group comprises species occurring in either temperate or tropical regions (Põldmaa 2011). Study III focuses on the monophyletic core group of temperate aurofusarin-producing *Hypomyces/Cladobotryum* species, several of which are frequently reported to cause cobweb disease in mushroom growing farms resulting in substantial yield loss. The diversity of hosts and variation in some morphological characters, combined with a broad geographic distribution challenge the present concept of *H. rosellus*, the taxonomic anchor of the group, and suggest the existence of cryptic lineages.

For many years, *H. rosellus* was the most commonly reported cause of cobweb disease (McKay et al. 1998; Bhatt & Singh 2002; Potočnik et al. 2008), but during the last decade the anamorph of *H. odoratus* has been reported with increasing frequency (McKay et al. 1999; Grogan & Gaze 2000; Adie et al. 2006; Khan et al. 2008; Back et al. 2010, 2012; Gea et al. 2011, 2012). Since the late 1960s cobweb disease have been controlled mainly using methyl benzimidazole carbamate (MBC) fungicides. The emergence of strains strongly resistant to these fungicides was considered responsible for occasional epidemics in mushroom farms in the British Isles in 1990s. The cobweb disease seems to be a continuous problem in many countries, suggesting that MBC resistant strains of *H. odoratus* occur widely also outside this region.

**Encoelia**

Members of the genus *Encoelia* (Fr.) P. Karst. exhibit wide morphological variation. Although the species are mostly well delimited, phylogenetic placement of most of them is unclear and so far this large genus encompassing about 50 species has not been included in any modern revision.

*Encoelioidae* was proposed by Nannfeldt (1932) as a new subfamily in Helotiaceae to include most of the genera previously assigned to Cenangiaceae. Later, several other genera were included to the subfamily by Korf (1973), which according to his concept was distinguished in Leotiaeeae (=Helotiaceae) mainly by excipulum characters. The genus *Encoelia* is very heterogenous in terms of morphology and fruit-body development (Bellemere 1977, Korf & Kohn 1976, Spooner & Trigaux 1985, Verkley 1995, Baral & Richter 1997).
Apothecia of *E. fimbriata* and *E. siparia* grow gregariously on well developed stroma and *E. fascicularis* on sclerotium. By contrast, the fruit-bodies of the type species *E. furfuracea* are not as densely clustered and not forming stromata or sclerotia. In addition, development of fruit-bodies of *E. furfuracea* is cleistothecial, as opposed to gymnothecial development of *E. fimbriata* (Spooner & Trigaux 1985) and other species. Moreover, ascus apparatus structures are very different in *E. tiliacea* (Bellemere 1977) and *E. fimbriata* (Verkley 1995). The only molecular study involving more than one species of *Encoelia* also suggests that the genus is not monophyletic (Peterson & Pfister 2010).

**Gene regions used for delimitation of taxa**

Ribosomal DNA (rDNA) is most frequently used genomic region for fungal species recognition. Internal transcribed spacer (ITS) flanking ribosomal RNA genes has recently been selected as the official barcoding gene for fungi (Schoch et al. 2012). ITS region has frequently been used as a phylogenetic marker at species level (e.g. Hansen et al. 2002, Acero et al. 2004, Francis et al. 2007, Niskanen et al. 2009, Yuan & Wan 2012). However, it has limitations in terms of insufficient resolving power in some fungal groups or unsatisfactory performance of universal ITS primers in other groups (Nilsson et al. 2008, Schoch et al. 2012). For example, ITS variation within ascomycete genera *Aspergillus, Penicillium, Fusarium* and *Cladosporium* has been shown to be very low in many cases (O’Donnell & Cigelnik 1997, Skouboe et al. 1999, Geiser et al. 2007, Schubert et al. 2007, Gazis et al. 2011). In contrast, some fungal groups such as Glomeromycota and basidiomyceteous *Tulasnella* display extreme variability of ITS region (Stockinger et al. 2009, 2010, Taylor & McCormick 2008, J. Oja unpublished). This, along with amplification challenges, limits the use of ITS for species identification and inferring phylogenies in these fungal groups.

Alternatively, the more conservative small and large subunits of ribosomal RNA genes (SSU and LSU, respectively) have been widely used to construct phylogenies at species and higher level (e.g. O’Donnell et al. 1997, Campbell et al. 2005, Hansen & Pfister 2006, Ferrer et al. 2012). Limitations of these in resolving higher level relationships e.g. in Leotiomycetes (Wang et al. 2006a, b) and Sordariomycetes (Summerbell et al. 2011) have channeled the search for other gene regions. Implementation of several single-copy protein-coding genes has considerably improved the resolution of higher level phylogenies. The genes encoding translation elongation factor 1-α (*tef1*), the largest and second largest subunits of RNA polymerase II (*rpb1* and *rpb2*, respectively), and β-tubulin are most frequently used for constructing phylogenies in fungi (e.g. Schoch et al. 2009a, Hansen et al. 2013, O’Donnell et al. 2012, Taşkin et al. 2012).
Importance of taxonomy in biodiversity studies

Advances in molecular taxonomy provide tools to detect monophyletic groups of organisms whose accurate identification using traditional methods has been challenging. Such applications of molecular taxonomy include identification of environmental samples, which in many cases do not lend themselves to easy identification because of low abundance, lack of cultivation methods and paucity of morphological characters. Ecological studies regularly report high genetic diversity, however, the species identities and species boundaries often remain undefined because of lack of available taxonomically authenticated voucher specimens. This applies to members of *Geopora* and Helotiales, which are often detected in soil and root samples (e.g. Gehring et al. 1998, Fujimura et al. 2005, Bidartondo et al. 2004, Tedersoo et al. 2006, 2009, Vrålstad et al. 2002, Gazis et al. 2011). Similarly, sequences with reliable identity provide means for identification of pathogens, in case of which the promptness of accurate determination is economically crucial. In mushroom cultivation industry, cobweb disease caused by anamorphs of *Hypomyces* spp. has been problematic for years, rapidly ruining the crop and causing economical losses (McKay et al. 1998, 1999, Grogan & Gaze 2000, Potočnik et al. 2008).

Aims of the study

1) To elucidate phylogenetic relationships and delimitation of species in the genus *Geopora* (I, II) and in the core group of temperate aurofusarin-producing *Hypomyces* (III).
2) Comprehending observed phylogenetic diversity to delineate the genera *Geopora* and *Encoelia* and their segregates (II, IV).
3) To assess host preferences and patterns of geographical distribution in *Geopora* (I, II) and temperate aurofusarin-producing group of *Hypomyces* (III).
4) To compare the roles of host specialisation vs geographic isolation in genetic segregation in the core group of temperate aurofusarin-producing *Hypomyces* (III).
5) To identify cobweb-causing *Hypomyces* strains isolated from mushroom farms and to clarify their origin as well as sources of recently emerged fungicide resistance (III).
6) To test and compare the resolving power of ITS for species recognition in studied groups of ascomycetes.
7) To investigate the performance of selected nuclear single-copy protein-coding gene regions in distinguishing monophyletic groups in *Hypomyces* (III) and the Helotiales (IV).
MATERIALS AND METHODS

Sampling of specimens and species identification

The specimens of Geopora and encoelioid fungi were collected mostly from Europe. About 100 specimens of Geopora (I, II) and 40 encoelioid ascomycetes (IV) were sequenced and examined microscopically when dry. All collections are deposited in fungaria (mainly in TAAM and H; acronyms following Index Herbariorum) or private collections. For each collection, the length and width of ten or more ascospores were measured. The data on ascospore measurements of Geopora were compared in a multistate analysis of variance (MANOVA) between the ten phylogenetic species. To test whether two particular lineages differ in ascospore dimensions, additional pairwise MANOVA procedures were conducted. The analyses were performed using SAS 9.1 (SAS Institute Inc, Cary, NC, U.S.A.).

The 119 strains of Hypomyces (III) were isolated from material originating from Europe, North America, Asia, Australia, New Zealand and Africa. Isolates derived from mass of conidia or single/mass ascospores were grown on 1.5% MEA (Oxoid, Cambridge, UK). The strains are deposited in fungal culture collections, mainly in TFC and CBS (acronyms following World Federation for Culture Collections).

DNA extraction, PCR and sequencing

Genomic DNA from Geopora and encoelioid fungi was extracted from fresh material stored in CTAB buffer (Gardes and Bruns 1993) and from dried herbarium specimens. DNA of Hypomyces was extracted from 4–7 d old mycelium scraped from agar surface. The DNA from samples stored in CTAB was extracted according to Gardes and Bruns (1993), or using High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland) or Qiagen DNeasy 96 Plant Kit (Qiagen, Crawley, West Sussex, UK) according to manufacturer’s instructions. PCR was performed using PuRe Taq Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech., Piscataway, NJ, USA.) or 5 x HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia) in 25-μl reaction volume.

The ITS regions of Geopora and Hypomyces were amplified using one of the forward primers ITS1F, ITS1 (Gardes and Bruns 1993) or ITS0F (Tedersoo et al. 2008), and the reverse primer ITS4 (White et al. 1990). In Hypomyces, selected regions of the five protein-coding genes were amplified using the following primers: RNA polymerase II subunit 1 (rpb1): RPB1-AFasc and RPB1-6R1asc (Hofstetter et al. 2007); RNA polymerase II subunit 2 (rpb2): RPB2-5F and RPB2-7cR (Liu et al. 1999); translation elongation factor 1α (tef1) introns 5 and 6 and exon 5: EF1-728F (Carbone and Kohn 1999) and EF2 (O’Donnell et al. 1998) as well as exon 6: EF1-983F and EF1-2218 (Rehner

In 60 strains of encoelioid fungi, selected regions of nuclear 18S and 28S ribosomal subunits, exon 6 of translation elongation factor 1α (tef1) gene and RNA polymerase II subunit 1 (rpb1) were amplified using the following primers: 18S rDNA: PNS1 (Hibbett 1996), nssu131 (Kauff & Lutzoni 2002), NS1, NS3, NS4, NS8 (White et al. 1990), NS24 (Gargas & Taylor 1992), NS41, NS19b (Hibbett 1996), NRC3R and NRC4R (Peterson & Pfister 2010); 28S rDNA: LR0R (Vilgalys unpbl.), CTB6, LR5 and LR7 (Vilgalys & Hester 1990); tef1: EF1-983F and EF1-2218R (Rehner 2001), rpb1: RPB1-AFasc and RPB1-6R1asc (Hofstetter et al. 2007). In addition, ITS region was amplified using ITS0F and ITS4 but omitted from phylogenetic analyses because of too high variability.

PCR included an initial step of 3 or 15 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C for ITS and tef1 intron, or at 57°C for tef1 exon, rpb1 and rpb2, or at 59°C for FG1093, and 1 min at 72 °C, and a final step of 10 min at 72 °C. PCR products were purified using either Exo-Sap enzymes (Sigma, St. Louis, MO, USA) or MoBio UltraClean™ PCR CleanUp™ Kit (MoBio Laboratories, West Carlsbad, CA, USA) according to manufacturers’ instructions. Sequencing was performed by MWG Biotech (Ebersberg, Germany) or by Macrogen Inc. (Seoul, Korea or Amsterdam, The Netherlands).

**Sequence alignment and phylogenetic analysis**

Sequences were edited and assembled with Sequencher 4.10.1 (Gene Codes, Ann Arbor, MI, USA). Datasets for each marker were aligned with Mafft online version (Katoh and Toh 2008) and edited manually using Se-Al 2.0a11 (Rambaut 1996) or Genedoc 2.7 (Nicolas et al. 1997).

For *Geopora*, maximum parsimony (MP) analysis was performed with PAUP* 4.0b10 (Swofford 2003) applying heuristic search with random stepwise sequence addition, all characters equally weighted, tree bisection-reconnection (TBR) branch swapping, and gaps treated as missing data. 1000 random sequence addition replicates were performed saving no more than 100 trees per replicate. Support of individual clades was assessed by parsimony bootstrap analysis that was performed using heuristic search with 1000 bootstrap replicates, each consisting of a single random sequence addition replicate. Bayesian analyses were performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Metropolis-coupled MCMC method was applied using SYM+I+G model that was previously selected by MrModeltest 2.2 (Nylander 2004). Two parallel MCMC analyses each consisting of one cold and three heated chains were performed for 1,000,000 generations. Both analyses initiated with random starting trees. Every 100th generation was sampled. From the trees sampled, 5000 first trees were dis-
carded as the burn-in. Remaining trees were used to calculate Bayesian posterior probabilities of the clades.

For *Hypomyces*, the combined five-gene data were divided into seven partitions, distinguishing coding and non-coding regions of each gene. The evolutionary models for constructing Bayesian and maximum likelihood phylogenies were selected using AIC in MrModeltest (Nylander 2004) as follows: *rpb1* exon and *rpb2*, SYM+I+G; *rpb1* intron, SYM; *tef1* and FG1093 introns, HKY+G; *tef1* exon, HKY+I+G; FG1093 exon, K80. For ITS region GTR+I+G was selected. For Bayesian inference, MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) was used to analyse the partitioned five-gene dataset as well as ITS dataset. Two parallel MCMC analyses were performed, each consisting of four chains initiated from random starting trees. For five-gene data, the analyses were run for 100 hours at CIPRES Science Gateway v3.3 (http://www.phylo.org/index.php/portal/). Every 1000th generation was sampled from the total of 53,753,000. The first 14,000 trees were discarded as burn-in based on at which generation the log likelihood scores reached stationary level; the average standard deviation of split frequencies had reached 0.04. Posterior probabilities (PP) were calculated from remaining 39,753 trees.

Maximum likelihood (ML) analysis of the five-gene partitioned data was performed with Garli 2.0 (Zwickl 2006), with maximum number of generations set for 5,000,000. Automatic termination condition was used, setting the number of generations without topology improvement, required for termination, to 20,000. Support for individual clades was assessed by running 100 bootstrap replicates in Garli. Subsequently, PAUP* 4b10 (Swofford 2003) was used to compute the consensus tree. ML analysis of ITS rDNA data was run in RAxML (Stamatakis et al. 2008), applying the gamma model of rate heterogeneity.

In *Encoelia*, MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) was used to analyse the partitioned four-gene dataset as well as SSU+LSU dataset implementing GTR+I+G model. Two parallel MCMC analyses were performed, each consisting of four chains initiated from random starting trees. The analyses were run for 10,000,000 generations at CIPRES Science Gateway v3.3. Every 1000th generation was sampled. The first 1000 trees in four-gene analysis and 5000 in SSU+LSU analysis were discarded as burn-in. Posterior probabilities (PP) were calculated from remaining trees. The confidence of branching was also assessed by 1000 MP bootstrap replicates conducted in PAUP* 4.0b10 (Swofford 2003).

Similarity of ITS sequences in all studied groups was assessed using Blastclust at PlutoF workbench (Abarenkov et al. 2010) by clustering the sequences at different identity thresholds.
RESULTS AND DISCUSSION

Delimitation of genera

Delimitation of genera was studied in Geopora and Encoelia. Geopora was shown to be paraphyletic (II) but Encoelia polyphyletic (IV). Our studies revealed phylogenetic relationships of distinct lineages observed in both genera.

In case of species assigned to Geopora, ascocarp type was shown to provide morphological distinction of monophyletic groups. All apothecial Geopora species form a well-supported monophyletic group. The ptychothecial Geopor spp. form the sister clade to ptychothecial Picoa, these together constituting a basal branch to the group of apothecial Geopora. Unlike apothecial Geopora, this basal lineage is only moderately supported. This does not support uniting Picoa with ptychothecial members of Geopora, furthermore, there are remarkable differences in their ascocarp anatomy and habitats (Alsheikh & Trappe 1983, Montecchi & Sarasini 2000, Sbissi et al. 2010). Therefore, we suggest the apothecial species within the paraphyletic Geopora to be reassigned to Sepultaria, yielding two monophyletic genera (II).

Encoelia appeared highly polyphyletic in all analyses with eight included species being distributed among six distinct clades in the phylogenies (IV). This is in accordance with the very high morphological and developmental heterogeneity reported in literature (e.g. Korf & Kohn 1976, Bellemere 1977, Spooner & Trigaux 1985, Verkley 1995, Baral & Richter 1997). As none of the seven analysed Encoelia species appeared congeneric with E. furfuracea, these have to be excluded from the genus and assigned to appropriate genera. The type species, E. furfuracea, is closely related to Velutarina rufo-olivacea, Cenangiopsis quercicola and Crumenulopsis sp., which form the sister group of Hemiphacidiaceae. Whereas these four species share some morphological similarities, the differences in fruitbody development, ascus pore amyloid reaction and the nature of ascospores do not advocate merging these taxa.

Majority of the analysed encoelioid genera were shown to belong to the clade ‘Encoelioideae’ sensu Peterson & Pfister. Most of the included members share several morphological characters, e.g. branched stipe, ionomidotic reaction and ectal excipulum cells embedded in gel. However, none of the three genera (Cordierites, Encoelia, Ionomidotis), each represented by three species in the analysis of rDNA data, appeared monophyletic within this clade. The remaining species of Encoelia (E. fascicularis, E. fuckelii, E. glauca, E. tiliae) were shown to be even more distantly related to the type species. This is consistent with morphological differences such as the structure of excipular cells and fruit-body development including the presence or absence of sclerotial or stromatal tissues. In order to delimit genera currently accepted in Encoelia, more inclusive morphological and molecular studies are needed.
Species delimitation based on morphological characters

Features of ascospores provide the main morphological characters for the distinction of species in studied collections of Geopora and Hypomyces represented only by the teleomorph. Ascospore size is applicable as the only character distinguishing between some species of apothecial Geopora (G. arenicola, G. tenuis, G. sepulta). In some cases, a combination of ascospore size and other morphological characters are needed to identify the species (G. gilkeyae and G. tolucana). In contrast, the monophyletic groups within species complexes, such as G. cervina and G. cooperi, cannot be explicitly distinguished on the basis of morphology. The species of Encoelia are easily distinguishable using morphological characters. However, this is expected as they show affinities to members of different families, thus being only distantly related.

Ascospore size is also the only distinguishing character among the species in the studied group of temperate aurofusarin-producing Hypomyces in case only the teleomorph is available for study. While teleomorphs are frequently found only in H. rosellus s. l., the anamorphic stage provides a valuable set of characters for the identification of all species in the group. If teleomorphs were the only stage in Hypomyces, then morphology-based distinction of current species would not really be possible, thus giving way to different concepts of species and their limits. Furthermore, with genetically well-segregated groups that cannot be distinguished on morphological basis, the situation would be analogous to that observed in Geopora.

Morphologically, the species are relatively well delimited in Hypomyces and Encoelia, but not in Geopora. In Geopora and also in the studied subgroup of Hypomyces the selected genetic markers strongly support lineages that cannot be distinguished based on morphology. Several such lineages were observed in H. rosellus s.l., comprising several cryptic species distinguished by differences in their hosts and geographical distribution ranges and to lesser extent in morphology (III).

High infraspecific morphological variation in Geopora complicates species delimitation as it masks the variation among the species. In apothecial Geopora, most of the clades comprised specimens that were assigned to different species based on identification relying mostly on ascospore dimensions. Although the spore measures overlap to great extent among the clades of both epigeous and hypogeous taxa, in most cases the pairwise comparisons of lineages of epigeous species revealed that the spore size was significantly different. Other characters, such as colour of the hymenium, hair length and shape and position of the fruit-body, were mostly homogenous within each clade; however, these characters coincide to a great extent among the clades.
Recognition of monophyletic taxa based on molecular characters

The resolving power of ITS rDNA varied among studied groups of ascomycetes (Table 1). In *Geopora*, ITS region provides sufficient characters to reconstruct well-resolved phylogenies in which most of the lineages are well supported. In contrast, the ITS variation in *Hypomyces* is extremely low, reaching 1.4% of genetic distance in the studied group comprising six described and at least six cryptic species, and merely 0.4% in the clade comprising all members of the ingroup except for *H. odoratus*. The variability of the ITS regions is extremely high among species of *Encoelia*. This is explained by the polyphyly of the genus, as the species were shown to belong to six distantly related groups across the Helotiales. In this case, the ITS region proved to be too variable to permit the inclusion of all of them in a joint alignment matrix.

Differences in variability of ITS can be striking at species level in different groups of ascomycetes, therefore, different threshold levels are needed for molecular species recognition (Nilsson et al. 2008, Schoch et al. 2012). In *Geopora*, variation in ITS is high, enabling to use the ITS as a barcoding gene around the 97% threshold level. The very low ITS variation in the studied group of closely related species of *Hypomyces* indicates the need for applying a more restricted species recognition threshold than the 97% sequence similarity, routinely used for species identification in most current ecological studies involving fungal diversity. Moreover, lack of hiatus between the infra- and interspecific variation limits the use of ITS as a barcoding gene region in this group advocating application of the more variable regions in *tefl* introns and *rpb2* (III). The situation is similar in many groups of ascomycetes, e.g. *Fusarium, Penicillium, Aspergillus* and *Cladosporium*, where genealogical concordance phylogenetic species recognition criteria should be implemented (Geiser et a. 2007, Schubert et al. 2007, O’Donnell & Cigelnik 1997, Skouboe et al 1999, Gazis et al. 2011).

By contrast, ITS variation in *Encoelia* is extremely high as expected due to its polyphyly. Whereas extremely high infrageneric ITS variation in *Encoelia* prevents to set a definite species recognition threshold, it is suitable for barcoding gene as infraspecific similarity in studied species is 98.5%. Very high variability of ITS sequences has also been found in resupinate cantharelloid basidiomycete *Tulasnella* (Taylor & McCormick 2008, J. Oja, unpublished), in Glomeromycota (Stockinger et al. 2010) and in the pezizalean genus *Neottiella* (Kullman & Tamm, unpublished).
Table 1. Comparison of resolving power of rDNA ITS region at different similarity threshold levels in three groups of ascomycetes.

<table>
<thead>
<tr>
<th>Species/lineages</th>
<th>Number of specimens</th>
<th>Number of clusters at different ITS similarity threshold levels&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
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<td>99.5%</td>
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<tr>
<td></td>
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<tr>
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<tr>
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</table>

<sup>a</sup> The colours mark the range of clusters for each given ITS similarity value. Transparent cells denote lineages from papers I-IV in which more than one cluster exists based on ITS similarity. The first figure in a cell designates the number of clusters of two or more sequences within each lineage. The second figure, if present, indicates the number of singletons. NA, not applicable as only one sample available from the clade.
Combined multigene analyses result in better resolved trees and larger number of highly supported lineages compared to separate analyses of the same genes. In Hypomyces, phylogeny of combined four protein-coding DNA regions gave strong support to species recognised on morphological grounds as well as several lineages apparently representing undescribed taxa. When analysed separately, rpb2 and tef1 gene regions yielded phylogenetic trees with mainly congruent topology compared to combined 4-gene analyses. Mostly, the support values in separate analyses of the two genes were comparable to those of combined multigene trees except for some of the terminal clades, which received slightly weaker support. In contrast, separate analyses of rpb1 and FG1093 genes resulted in poorly resolved trees with low support values to most of the clades.

In Encoelia, the basal branches of the phylogenetic tree based on rDNA sequences were poorly supported compared to the phylogeny based on a reduced set of taxa and the inclusion of tef1 and rpb1 genes. The eight Encoelia species were distributed among six distinct clades. Type of the genus, E. furfuracea, appeared closely related to Velutarina rufo-olivacea, Velutarina sp., Cenangiopsis quercicola and Crumenulopsis sp. that all form the sister group of Hemiphacidiaeae. ‘Encoelioideae’ sensu Peterson and Pfister includes E. fimbriata, E. helvola and E. heteromera that do not form a monophyletic group. Considering the consistency in the results of various analyses and support values for individual clades, only two Encoelia species can unequivocally be placed in well-established monophyletic groups in the Helotiales recognised at family level. Namely, E. fascicularis belongs to the Sclerotiniaceae and E. tiliacea to Rutstroemiaceae. As the order Helotiales is very diverse but seriously undersampled in terms of molecular sequences, it is difficult to assess phylogenetic relationships at family and genus level (LoBuglio & Pfister 2010).

**Host/symbiont associations**

Most of Geopora lineages have been shown to include ectomycorrhizal members. With one exception, Geopora species are generalists displaying some preference at the host genus level but less so at species level. Photobionts in EcM associations involving studied Geopora species include coniferous and deciduous trees such as Pinus, Quercus, Betula, Salix, Tilia and Populus. In addition, Geopora has been shown to form ectomycorrhizas with Pseudotsuga, Tsuga, Abies and Cedrus (Fogel 1994). Moreover, Geopora species from several apothecial and ptychothecial lineages have shown to associate with roots of orchids from the genus Epipactis. By contrast, G. cercocarpi is found to be host-specific, forming ectomycorrhiza exclusively with Cercocarpus ledifolius (McDonald et al. 2010, Southworth & Frank 2011), a wooden member of Rosaceae, which occurs in western part of North America. As in Geopora
there are clades with either numerous or occasional mycorrhizal sequences, as well as clades with no mycorrhizal associations detected, it is possible that some species are more apt to form mycorrhiza than others. However, no evidence has been found for EcM fungi to reverse to saprotrophic lifestyle (Tedersoo et al. 2010). Moreover, the most closely related lineages of Geopora, e.g. Picoa and a part of the polyphyletic Tricharina, have been proved to be mycorrhizal (Gutierrez et al. 2003, Trocha et al. 2006, Smith et al. 2009). Therefore it seems reasonable to consider EcM lifestyle as plesiomorphic for this larger group in Pyronemataceae, inherent to all species of Geopora and the related genera.

In Hypomyces, various host preference strategies exist. Hosts of temperate aurofusarin-producing Hypomyces belong primarily to five orders of Agaricomycetes (Basidiomycota): Agaricales, Russulales, Polyporales, Hymennochaetales and Thelephorales. Occasionally members of this group have been collected on fruitbodies of Boletales and Gomphales. Despite considerable overlap in host ranges, several of the species are distinguished by their host preference. While H. rosellus in the strict and broad sense has been found growing on species from all five basidiomycete orders, H. odoratus is almost exclusively confined to annual agaricalean basidiomata of the Agaricales. In addition to generalists (H. odoratus, H. rosellus s. str.), there are also species defined by hosts’ taxonomic identity (C. rubrobrunnescens on Inocybe spp.) or ecology (C. tenue, several subclades in H. rosellus s.l.).

Most of the species described in Encoelia are host specific, occurring on selected genera of deciduous trees such as Ulmus, Tilia, Prunus, Carpinus, Acer, Populus, Salix, Corylus or on bamboo culms. By contrast, E. furfuracea has been found on both Corylus and Alnus, and E. fascicularis on Populus, Fraxinus and Cornus.

**Geographical distribution**

Studied species from three groups of ascomycetes exhibit various patterns of geographic distribution. Whereas there are no cosmopolitan species, a number of species occur in several continents, and only a few are restricted to smaller regions (Fig.1).

Ptychothecial Geopora species exhibit geographic segregation. The two complexes of G. cooperi are restricted to North America and Europe, respectively (II). Similarly, G. tolucana has been found only in North America. Among apothecial Geopora no geographical segregation could be found. With the exception of G. ledifolius, all of the lineages of apothecial Geopora occur in Europe and possibly also in North America and Asia, relying on reports mostly not including molecular evidence (Ohenoja & Ohenoja 2010, Bates 2006, Seaver 1928, Zhang & Yu 1992, Wei et al. 2010, Lee et al. 2012, Ishida et al. 2009, Kumar & Sharma 2009). The only records of apothecial Geopora from Southern Hemisphere originate from Argentina (Gamundi 1975, 2010).
Figure 1. Geographic distribution of species studied in papers I-IV. Each mark represents a species in respective geographic region. For clarity, all the species of apothecial Geopora are marked with the same symbol; all these species occur frequently in Europe. The clade numbers from paper I are given only for localities other than Europe. All subclades of Hypomyces rosellus s. l. from paper III are denoted with the same symbol, followed by name of the subclade, if applicable.
The most common aurofusarin-producing *Hypomyces* species in northern temperate regions, *H. rosellus* s. str. and *H. odoratus* are represented in Europe and Asia, the latter also in North America. In contrast, the genetically segregated lineages in the remaining part of *H. rosellus* s.l. are confined exclusively to east or the west coast of North America, Eurasia or Europe. According to the records to date, *C. tenue* and *C. rubrobrunnescens* occur only in Europe, *H. dactylarioides* in New Zealand, and *C. multisepatum* in Australia and New Zealand.

In *Geopora* and *Hypomyces*, there are genetically segregated groups exhibiting either distinct or broad and overlapping distribution. Similar situation has been described for other ascomycetes, e.g. *Fusarium oxysporum* and *F. graminearum* species complexes and in *Morchella* comprising several morphologically indistinguishable species with various patterns of distribution (Starkey et al. 2007, Kvas et al. 2009, Lievens et al. 2009, Sarver et al. 2011, O’Donnell et al. 2011, Taşkin et al. 2012). Similarly to *Aspergillus fumigatus* (Pringle et al. 2005), apothecial *Geopora* and *Hypomyces odoratus* exhibit no geographic segregation. The latter species includes the majority of cobweb strains isolated from industrial mushroom beds. The lack of geographic segregation within this pathogenic species contrasts with distribution pattern of another pathogen of cultivated mushrooms, *Trichoderma aggressivum*, in which clearly distinguishable forms have been described that occur either in Europe or North America (Samuels et al. 2002).

**Applications of advances in taxonomy**

Reference sequences obtained from *Geopora* fruit-bodies in the studies I and II facilitate species identification from growing number of mycorrhizal samples. Several ectomycorrhizal and orchidoid mycorrhizal samples, originating mostly from Europe and North America, were assigned to described species (I, Southworth & Frank 2011).

Many helotialean species live as root endophytes in diverse plant taxa (Tedersoo et al. 2009, Mayerhofer et al. 2013). To date, their identification has been difficult because of scarceness of sequences, especially those from fruit-bodies. Sequences obtained from reliably identified helotialean fruit-bodies during the study IV help to fulfil the demand for reference sequences.

A number of *Hypomyces* strains isolated from various mushroom farms with cobweb disease symptoms were involved in study III. By applying different molecular markers we determined that cobweb disease is mostly caused by strains belonging to *H. odoratus* or several subgroups of *H. rosellus* s. l., present in the local species pool of the particular region. In several cases the pathogen was reidentified as *H. odoratus* despite the original identification as *H. rosellus*. Moreover, we found that all the strains resistant to MBC fungicides belong to a single subclade in *H. odoratus*. This finding assists making informed choices to prevent economical losses in mushroom industry in the future.
CONCLUSIONS

- The genus *Geopora* is paraphyletic and therefore should be recircumscribed. Ascocarp type provides morphological distinction of two well-supported monophyletic groups within *Geopora* (II). We suggest to recognise apothecial and ptychothecial lineages as separate genera and to reassign the apothecial *Geopora* to *Sepultaria*. Apothecial *G. arenicola*, *G. tenuis* and *G. sepulta* (I) and ptychothecial *G. toluca* (II) are recognised as monophyletic species. Two genetically and geographically distinct lineages, recognised in *G. cooperi*, probably present distinct species (II). Unlike apothecial *Geopora*, ptychothecial species of *Geopora* represent geographically isolated lineages. With a few exceptions, the species of *Geopora* are host generalists forming mycorrhiza with different trees and orchids.

- *Hypomyces rosellus* is paraphyletic, comprising *H. dactylarioides* and *C. multiseptatum* as well as several cryptic species (III). *Hypomyces rosellus* s. str. is characterised by wide host range encompassing five basidiomycetous orders. The species occurs in Eurasia but not in North America. By contrast, the lineages within *H. rosellus* s. l. are distinguished by differences in their hosts and geographical distribution ranges, including East and West coast of North America. *Hypomyces odoratus* occurs across temperate Northern Hemisphere and is confined to annual agaricalean basidiomata. Both specialist and generalist host use strategies have evolved in the group. In *Hypomyces*, there are groups defined by hosts’ taxonomic identity (*C. rubrobrunescens on Inocybe* spp.) or ecology (*C. tenue*, several subclades in *H. rosellus* s.l.). Separate lineages appear to be maintained by geographic isolation in North America and temperate Australasia but by host specialisation in the species occurring sympatrically in Europe and Asia. The majority of cobweb isolates belong to *H. odoratus*, including a weakly supported group of fungicide-resistant strains from Europe and North America.

- The genus *Encoelia* is highly polyphyletic (IV). Of eight analysed *Encoelia* species, only *E. fascicularis* and *E. tiliacea* can be unequivocally placed to monophyletic families, Sclerotiniaceae and Rutstroemiaceae, respectively. The type species of the genus, *E. furfuracea*, forms a strongly supported sister group of Hemiphacidiaceae, which includes also *Velutarina rufolivacea*, *Velutarina* sp., *Cenangiopsis quercicola* and *Crumenulopsis* sp. According to morphology and ecology, these species are too different to merge them into one genus. *Encoelia helvola*, *E. heteromera* and *E. fimбриata* were found to belong to the monophyletic group ‘Encoelioideae’ sensu Peterson & Pfister, which also includes species of *Cordierites*, *Ionomidotis* and *Phaeangella*. These genera share similarities in morphology as well as in cytochemical reactions to different reagents. Exclusion of *E. furfuracea* from this clade necessitates reconsidering the name to be applied to the group that includes the majority of encoelioid taxa. Their
inclusion in the closely related Cyttariales provides an alternative to describing a new taxon at family or order level.

- The resolving power of ITS rDNA varies to great extent among studied taxa. In apothecial Geopora, variation in ITS among species is high, enabling to use the ITS as a barcoding gene at the 97% similarity threshold level (I). By contrast, variation among ITS regions of closely relates species of Hypomyces is extremely low, reaching about 1% of genetic distance. Moreover, lack of hiatus between the infra- and interspecific variation limits the use of ITS as a barcoding gene region in this group (III). Although the variability of the ITS region is extremely high among species of polyphyletic Encoelia, it is suitable for barcoding gene as infraspecific similarity is higher than 98% (IV).

- Incorporation of protein-coding sequence data in phylogenetic analyses greatly improves the resolution and support values for most branches in the calculated phylogenies. Phylogenetic analyses of combined four protein-coding genes in Hypomyces provide strong support to previously recognised species and several cryptic lineages (III). While tef1 and rpb2 can be recommended for species delimitation and identification of temperate aurofusarin-producing Hypomyces, the other two genes, rpb1 and FG1093 cannot. In Encoelia, addition of protein-coding gene data in the analysis increased support for many relationships that could not be resolved based only on rDNA regions (IV).

- Identification of fungal environmental and agricultural samples relies on DNA sequences deposited in public nucleotide sequence databases. Therefore, the quality of sequences and reliable metadata of voucher specimens, as well as sufficient representation of diverse taxon groups in databases, are crucial for accurate identification of samples and assessment of biodiversity.
SUMMARY IN ESTONIAN

fülogeneetilise mitmekesisuse korrastamine kolme kottseenerühma näitel

Seente süstemaatikas on tavaliselt kasutatud morfoloogilist ja bioloogilist liigimääratlust, kuid on juhtumeid, kus need ei saa rakendada. Morfoloogilise liigimääratlust kasutamist piirab sageli kas liiga suur või liiga väike varieeruvus, peale selle on morfoloogiliste tunnuste põhjal liigipiiride kehtestamine tihti kunstlik. Liikide määratlemine ristumisbarjääri alusel ehk bioloogilise liigi määratlust kohaselt on küll loomulikum, kuid kõigil seentel ei ole seda võimalik rakendada, kuna nad ei paljune suguliselt või ei osata need kultuuris kasvata. Peale selle võivad mitmed seeneliigid ületada ristumisbarjääri, moodustades liikidevahelisi hübriide. Viimasel ajal on liike hakatud tihti määratlema fülogeneetilistest alustest. Sarnaselt morfoloogiliselt liikide määratlemisele on siingi probleemiks kunstlikkus, kuna otsuse tegemine, kas tunnuse eri seisundid tähistavad liigisisest varieeruvust või eri liikidesse kuulumist, on subjektiivne.


Seltsi *Helotiales* kuuluv perekond *Encoelia* (põhik) sisaldab liike, mis kasvavad kõdulagundajatena surnud puidul. Viljakehad on arenguliselt ja morfoloogiliselt liigitite erinevad, mistõttu perekonda ei peeta monofüleetiliseks.


Perekondade *Geopora* ja *Encoelia* uurimiseks kasutati enamjaolt Euroopast pärit seenematerjali Eesti Maaülikooli mükoloogia osakonna (TAAM) ja Soome Loodusmuuseumi (H) fungaariumidest ning H. O. Barali erakogust. Eksemplaridel mõõdeti eoste pikkus ja laius. Perekonna *Hypomyces* eluskultuurid pälinevad eri maailmajagudest ning saadi Eesti Maaülikooli ja Tartu Ülikooli seekekkultuuride kogust (TFC), ja analoogsetest kollektsioonidest mittel pool maailmas (CBS, IMI, NBRC, MUCL). Niit herbaareksemplaridest kui eluskultuuridest eraldati DNA, seejärel amplifitseeriti ja sekkveteeriti erinevaid geenipiiirkondi: rDNA ITS, SSU ja LSU, ning valke kodeerivatest genidest *rpb1*, *rpb2*, *tef1* ja FG1093. DNA järjestused joondati ning viidi läbi fülogeneetilised analüüsid.


rDNA ITS regiooni lahutusvõime liikide eristamisel osutus uuritud seentel väga erinevaks. Perekonna *Geopora* avatud viljakehadega liikidel on ITS varieeruv ja seda saab kasutada liikide määramiseks 97% sarnasuse alusel. Perekonna *Hypomyces* uuritud rühmas varieerub ITS regioon äärmiselt vähe. Rühmasesene sarnasus ulatub ligi 99%-ni ning kuna ei ole selget piiri liigisesese ja liikidevahelise varieeruvuse vahel, ei saa ITS regiooni selles rühmas liikide tuvastamiseks kasutada. Perekonna *Encoelia* polüfüleetilisse tõttu on ITS regioon ootatult väga varieeruv, kuid sobib liikide määramiseks, kuna liigiisene sarnasus on üle 98%.

Perekonnas *Hypomyces* andis nelja valku kodeeriva geeni järjestuste kombineeritud fülogeneetiline analüüs tulemuseks kõrgelt toetatud liigid ja mitu morfoloogiliselt eristamatu rühma. Samu geene üksikult analüüsides andsid sarnase tulemuse tegutsues *rpb2* ja *tef1*, seevastu *rpb1* ja FG1093 analüüsides olid halva lahutusvõimega puud, mille harud olid madala toetusega.

Molekulaarsete meetodite abil saab liike määrama organismidel, mida traditsiooniliste meetoditega ei olnud võimalik määrama. Selliseks näiteks on keskkonnaproovid, millest ühendatud DNA-d. Selleks, et liike tuvastada, on vaja DNA järjestust võrrelda sellistest viljakehadest pärast DNA-ga, mille liigiline kuuluvus on täpselt teada. Käesoleva uurimuse käigus sekveneeritud *Geopora* liikide ITS järjestused võimaldavad määrata juureproovidest tuvastada seeneliigid, mis olid varem määramatud vaid perekonna või kõrgemal tasemel. Sekveneeritud rDNA ITS järjestused seltsist *Helotiales* teeid võimalikuks juure-endofüütidest ja puidu saprotroofide liikide tuvastamise. Samuti on perekonna *Hypomyces* sekveneeritud rDNA ITS järjestused abiks seente patogeneendid selleks ja täpseks määramiseks. Šampinjonidel parasilteerivad perekonna *Hypomyces* liigid on seenekasvandustes olnud aastaid probleemiks. Mõned tuved on fungitsiidiid vastu resistentsed ja nende liigiline
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34
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37


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