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Diversification and species delimitation of lichenized fungi in selected groups of the family Parmeliaceae (Ascomycota)

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Department of Botany, Institute of Ecology and Earth Sciences, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred in the text by the Roman numerals:

I Mark, K., Saag, L., Saag, A., Thell, A., & Randlane, T. (2012) Testing morphology-based delimitation of *Vulpicida juniperinus* and *V. tubulosus* (Parmeliaceae) using three molecular markers. *The Lichenologist* **44** (6): 757−772. doi: 10.1017/S0024282912000448.

II Saag, L., Mark, K., Saag, A., & Randlane, T. (2014) Species delimitation in the lichenized fungal genus *Vulpicida* (Parmeliaceae, Ascomycota) using gene concatenation and coalescent-based species tree approaches. *American Journal of Botany* **101** (12): 2169−2182. doi: 10.3732/ajb.1400439.

III Mark, K., Saag, L., Leavitt, S. D., Will-Wolf, S., Nelsen, M. P., Tõrra, T., Saag, A., Randlane, T., & Lumbsch, H. T. (2016) Evaluation of traditionally circumscribed species in the lichen-forming genus *Usnea* (Parmeliaceae, Ascomycota) using six-locus dataset. *Organisms Diversity & Evolution* (in press). doi: 10.1007/s13127-016-0273-7.

IV Mark, K., Randlane, T., Hur, J.-S., Thor, G., Obermayer, W. & Saag, A. Lichen chemistry is concordant with multilocus gene genealogy and reflects the species diversification in the genus *Cetrelia* (Parmeliaceae, Ascomycota). Manuscript submitted to The Lichenologist.

V Mark, K., Cornejo, C., Keller, C., Flück, D., & Scheidegger, C. (2016) Barcoding lichen-forming fungi using 454 pyrosequencing is challenged by artifactual and biological sequence variation. *Genome* (in press). doi: 10.1139/gen-2015-0189.

The author of the dissertation was primarily responsible for sample selection, molecular data generation of all the papers (**I−V**), and data analyses and writing of the publications **I**, **III−V**. The author substantially contributed to developing the idea and study design of all the papers, and data analyses and interpretation of the results in the paper **II**.

1. INTRODUCTION

1.1. General introduction

Two of the main goals of systematics are dividing the diversity of life into species and discovering the phylogenetic relationships of these species (O'Meara 2010). The species category is a fundamental unit in biology. Developing robust and highly replicable measures for identifying distinct evolutionary lineages is a central goal of species delimitation (Coyne & Orr 2004; de Queiroz 2007; Fujita *et al.* 2012). Establishing a stable taxonomy is particularly important for any field that relies on accurate measures of biodiversity, including ecology and conservation, as well as for research of the evolution of organismal traits, including comparative biology and genomics (Blackburn & Gaston 1998; Cracraft 2002; Agapow *et al.* 2004; Mace 2004). An unstable taxonomy has also important financial ramifications, for example, when protecting rare or endangered species or monitoring and fighting invasive species or pathogens (Boykin *et al.* 2012).

Lichens are intimate and long-term symbiotic associations, consisting of a heterotrophic fungal partner – also called the mycobiont – and photosynthetic algae or cyanobacteria – also called the photobiont (Nash III 2008). Systematically, lichens are a non-monophyletic group of fungi with about 98% of the species belonging to the Ascomycota and 2% to the Basidiomycota. More than 20% of all fungal species are considered lichen-forming (Honegger 1996). While lichens include many bio-indicators for monitoring environmental quality, including air pollution and ecological integrity of forest landscapes (Nimis *et al.* 2002), accurate identification of lichenized fungal species remains challenging (Lumbsch & Leavitt 2011).

1.2. Species concepts and estimating phylogeny in lichenized fungi

The prevailing species concept assumes that species represent independently evolving metapopulation lineages, but the specific operational criteria used for delimiting species may differ considerably depending on the perceived importance of various attributes of evolving populations (de Queiroz 2007; Carstens *et al.* 2013). The recently reformulated species concept, the general lineage concept, allows researchers to delimit species using any of several criteria associated with lineage formation, such as morphological distinctions, geographic range, monophyly, or reproductive isolation, rather than a single indicator of species-level differentiation (de Queiroz 2007; Lumbsch & Leavitt 2011; Carstens *et al.* 2013).

Although the majority of currently accepted lichen-forming fungal species were originally described on the basis of classical phenotypic characters of the symbiotic organisms, DNA sequence-based phylogenetic studies for delimiting

taxa are increasingly employed. Incongruence between the morphological circumscriptions of species and phylogenetic delimitation based on DNA sequence data is known from several groups of lichen-forming fungi. In some cases, overestimation of the species diversity based on morphology and chemistry has been demonstrated (Velmala *et al.* 2009; Leavitt *et al.* 2011a; Velmala *et al.* 2014), while in others, new lineages, often representing undescribed species, have been discriminated (Lumbsch *et al.* 2011; Molina *et al.* 2011; Kraichak *et al.* 2015b; Singh *et al.* 2015). Finding and applying the appropriate character sets is one of the most challenging aspects of species delimitation. Furthermore, different strategies and analytical methods often lead to divergent conclusions regarding the boundaries and numbers of species (Fujita *et al.* 2012; Miralles & Vences 2013). Effectively integrating ecological, biogeographical, and other independent sources of data has provided more robust species delimitations than use of any single kind of data (Edwards & Knowles 2014).

The original goal of DNA barcoding was to use standardized DNA sequences of a single locus to identify a species and also to accelerate species discovery (Marshall 2005; Schindel & Miller 2005). The nuclear ribosomal internal transcribed spacer region (ITS) is the most widely used DNA marker for species delimitation in different groups of organisms and has been proposed as the standard barcode marker for fungi (Schoch *et al.* 2012). DNA-based specimen identification to a species level is useful in a system of well-circumscribed taxa and a high-quality reference database (Seifert 2009; Begerow *et al.* 2010). It has been proven successful in some groups of lichens (Kelly *et al.* 2011; Divakar *et al.* 2016), while in other groups, with unresolved taxonomy and/or limited reference-database, purely DNA-based identification of lichens – without considering morphological and chemical characters – can result in erroneous results. Sampling difficulties occur where other very similar lichen species live mixed or close by, and/or saprophytic, endophytic, and parasitic fungi live intimately admixed with the lichen mycobiont, making the application of Sanger sequencing insufficient (Flück 2012; Orock *et al.* 2012). Recent advancements in pyrosequencing methods now allow the amplification of fragments up to 1,000 base pairs (bp) in the GS FLX+ system of Roche/454 pyrosequencing, therefore allowing the amplification of the full fragment of the ITS marker. However, only a limited number of studies are known to have successfully applied pyrosequencing to recover the identity of a lichen (Hodkinson & Lendemer 2013; Lücking *et al.* 2014a). 454 pyrosequencing is also notorious for its high indel (short insertions and deletions) error rate in homopolymeric regions (three or more identical nucleotides) and carry-forward-incomplete-extension (CAFIE) errors (Huse *et al.* 2007; Gilles *et al.* 2011; Lücking *et al.* 2014b). In addition to artifactual sequence variation, biological sequence variation – such as intragenomic and/or intra-mycelial (i.e. allelic heterozygosity) variation of this multicopy gene – may be possible (Wörheide *et al.* 2004; Simon & Weiß 2008; Lindner *et al.* 2013).

At the same time, relying only on ITS or any single marker for species identification, and especially delimitation, has also been criticized (Dupuis *et al.* 2012). The use of additional independent loci reduces stochastic errors (Chen *et al.* 2008) and increases the resolution of a phylogeny and delimitation success (Pino-Bodas *et al.* 2013). Even though multilocus phylogenies are clearly preferred over single locus gene trees when testing species boundaries (Lumbsch & Leavitt 2011), the information derived from different genetic markers should be considered with caution (Edwards 2009). Gene trees may not be congruent with each other or with underlying species tree (Maddison 1997; Cummings *et al.* 2008), most often due to incomplete lineage sorting (ILS), but also from introgression, gene paralogy, and other reasons (Maddison 1997; Edwards 2009; Knowles & Kubatko 2010). ILS can occur in any taxonomic group or molecular marker, The taxa in such groups do not appear monophyletic in gene trees even if they represent reproductively isolated evolutionary lineages (Funk & Omland 2003). ILS is especially common in closely related taxa since allelic coalescence is not simultaneous in different loci and gene monophyly is only the final stage in the divergence process (de Queiroz 2007; Hobolth *et al.* 2011). Novel methods based on the multispecies coalescent model have explicitly incorporated gene tree heterogeneity due to ILS into a species tree estimation (Funk & Omland 2003; Rannala & Yang 2003). In this fastly developing field, new model-based frameworks of maximum likelihood (ML) and Bayesian species tree inferences, and species delimitation approaches are being developed and are increasingly replacing the cladistics-based methods in species phylogeny estimations (Liu *et al.* 2009; Yang & Rannala 2014; Jones 2015).

1.3. The study groups

Phylogeny-based case studies in three groups of lichenized fungi in the family Parmeliaceae (Ascomycota) were conducted for this thesis. This family is the best known and probably the largest family of lichen-forming ascomycetes, including about 2760 species classified in 81 genera (Jaklitsch *et al.* 2016), approximately one tenth of all lichen-forming fungal species. Many of the groups included here belong to the earliest described lichen genera, e.g. *Parmelia, Cetraria* and *Usnea*, and many of the species are important components of diverse lichen communities in different vegetation types all around the world (Arup *et al.* 2007). The family is currently regarded as a uniform monophyletic group characterized morphologically by a certain type of ascoma ontogeny and the presence of an ascomatal structure called a cupulate exciple (Henssen *et al.* 1981; Crespo *et al.* 2007). Most genera in this family form lichens with large, complex thalli, developing either foliose or fruticose growth forms. Within the family, seven major groups can be distinguished (Arup *et al.* 2007; Crespo *et al.* 2007; Thell *et al.* 2012; Divakar *et al.* 2015), which are: alectorioid, anzioid, cetrarioid, hypogymnioid, parmelioid, psiloparmelioid, and usneoid. The phylogeny and species delimitation in three lichenized fungal genera in the family

Parmeliaceae (Ascomycota), belonging to the three major groups – cetrarioid, parmelioid, and usneoid – were investigated for this thesis. The genera *Cetrelia* W.L. Culb. & C.F. Culb., *Usnea* Dill. ex Adans., and *Vulpicida* J.-E. Mattsson & M. J. Lai were selected as the case study groups based on previous knowledge on their fuzzy species boundaries and unsettled species concept (e.g. (Thell *et al.* 2009; Saag *et al.* 2011; Thell *et al.* 2012).

The taxa in the genus *Cetrelia* were previously considered 'cetrarioid' with some of its species included in the genus *Cetraria* by reason of submarginal apothecia, but are, however, phylogenetically distinctly related to *Xanthoparmelia* and other 'parmelioid' genera within the family Parmeliaceae (Crespo *et al.* 2007; Divakar *et al.* 2015). The species in the genus *Cetrelia* are characterized by foliose, tan to greenish-gray thallus, with broad rounded lobes covered with pseudocyphellae. According to current knowledge, the genus is distributed over northern hemisphere, with a clear distribution center in the eastern and southeastern Asia, where majority of the species are found (Culberson & Culberson 1968; Randlane & Saag 1991). The genus *Cetrelia* presents a taxonomically interesting group of foliose macrolichens where morphologically uniform, but chemically different populations are considered as distinct species (Culberson & Culberson 1968). Such chemical species concept is not uncommon among lichenized fungi (e.g. in *Pseudevernia furfuracea* (L.) Zopf, *Thamnolia vermicularis* (Sw.) Schaer., *Dimelaena oreina* (Ach.) Norman, *Sulcaria sulcata* (Lév.) Bystrek ex. Brodo & D. Hawksw., etc.). However, it is disputable whether, and in what conditions, such chemotaxa should be considered as entities at the species level (Rogers 1989; Lumbsch 1998). The generally accepted 18 *Cetrelia* species (Randlane *et al.* 2013) are delimited in combining five morphotypes with six chemotypes (Randlane & Saag 1991). Each individual produces a set of biochemically related substances – a chemosyndrome – specific to its taxonomic affiliation, while morphologically the species are separated mainly by their reproduction mode. Although Luo *et al.* (2007) showed in analyzing the ITS marker of four *Cetrelia* species from South Korea that the reproductive mode as well as thallus chemistry seem to be important diagnostic characters, no comprehensive multilocus taxonomical research had been conducted in the genus previously.

Usnea represents an iconic example in which a lack of recognizable, diagnostic characters and the use of homoplasic characters have led to circumscription of many non-monophyletic species (Clerc 1998). It is one of the largest genera in the family Parmeliaceae, comprising ca. 350 species (Thell *et al.* 2012). Its members are characterized by beard-like, finely branched, pendent or erect thalli with a stiff central axis that is exposed when a branch is stretched and the cortex breaks apart. All *Usnea* species produce usnic acid in the cortex, giving the thallus a slightly yellow appearance. The monophyletic genus is well circumscribed, conspicuous, and easily recognized even by non-experts, but the delimitation of many species in this genus is very difficult due to transitional forms and the complexity of diagnostic characters. This genus is also famous for its complicated taxonomy – more than 770 names have been published

worldwide, and about half of these could be considered synonyms (Clerc 1998), illustrating the necessity of taxonomic revisions in this group. We focused on the section (sect.) *Usnea*, a group of closely related species with a wide distribution across the northern hemisphere and includes the type species of the genus – *U. florida* (L.) Weber ex F.H. Wigg. This group consists of taxa with a high variation in lichen secondary metabolites and transitional forms between morphotypes; both that have led to uncertainties in species boundaries and taxonomic confusion.

The genus *Vulpicida* belongs to the so-called 'cetrarioid core group' among the cetrarioid lichens (Thell *et al.* 2009) and consists of six species distributed in the temperate and arctic regions of the northern hemisphere (Mattsson & Lai 1993). The study on species boundaries in the genus *Vulpicida* was initiated by morphological observations on the islands of western Estonia, where two species – *Vulpicida juniperinus* (L.) J.-E. Mattsson & M.J. Lai and *V. tubulosus* (L.) J.-E. Mattsson & M.J. Lai – were found to form morphological intermediates that could not be assigned to a species unanimously. The problem was first postulated and investigated in the authors' master thesis (2011), titled "Separation of two species of lichenized fungi – *Vulpicida juniperinus* and *V. tubulosus* – using DNA characters", that, however, raised even further questions regarding the species boundaries and phylogeny in the genus *Vulpicida*. The morphological recognition of the genus is easy due to a unique set of secondary metabolites, pinastric and vulpinic acids, that cause the intense yellow colour of medulla in *Vulpicida*, but its monophyly has not been shown with confidence and the evolutionary relationships between the species were unclear.

1.4. The objectives of the thesis

The aim of the present thesis was to contribute to the knowledge of systematics and evolution of lichenized fungi, whereby further and more detailed studies on phylogeny and character evolution within this group may be aided. I attempted this by conducting phylogeny-based case studies in some groups of lichenized fungi and by investigating the applicability of DNA barcoding approach in identifying lichenized fungal species. More specifically, the main objectives of this thesis were the following:

(1) To estimate the phylogenetic relationships and delimit species boundaries using modern multi-locus coalescent-based species tree and species delimitation methods in addition to traditional gene trees and concatenation approach, and to compare the molecule-based results with the currently applied morphological taxon circumscriptions in the selected genera of the family Parmeliaceae – *Cetrelia*, *Usnea* (sect. *Usnea*), and *Vulpicida* (papers **I–IV**).
(2) By combining genetic, morphological, and chemical data, circumscription of natural, monophyletic taxa was aimed through evaluating the utility of

some traditionally used characters and providing morphology-based perspectives of species in the light of molecular data (papers **I, III, IV**).

(3) To assess the possibilities and accuracy of DNA based species identification in lichenized fungi, and test for the applicability of DNA barcoding using the Roche 454 pyrosequencing platform (paper **V**).

(4) Finally, to attempt to elucidate some aspects of divergence processes in lichenized fungi (incl. species, character, and molecule evolution (i.e. intragenomic sequence variation of ITS)) using genetic information (papers **I–V**).

2. MATERIALS AND METHODS

2.1. Taxon sampling and morphological identification

The phylogeny and species delimitation in three lichenized fungal genera in the family Parmeliaceae (Ascomycota) was investigated for this thesis (**I–IV**) – the genera *Cetrelia*, *Usnea*, and *Vulpicida*. The number of specimens and species of each study, together with outgroup taxa and source of the collections is summarized in Table 1.

Table 1. Number of specimens, outgroup taxa, and herbaria or institute abbreviations of the collections

Paper	**I**	**II**	**III**	**IV**	**V**
Study group	*Vulpicida*	*Vulpicida*	*Usnea sect. Usnea*	*Cetrelia*	*Lecanorales*
Study area	Europe	Northern hemisphere	Europe and Noth America	Eurasia	Switzerland
Total no of specimens	78	70	144	64	100
Total no of species	8	16	18	16	93
No. of ingroup specimens	75	58	142	58	n.a.
No. of ingroup species	5	6	17	11	n.a.
Outgroup taxa	Species from genera *Allocetraria* and *Cetraria*	Species from genera *Allocetraria*, *Cetraria*, *Cetrariella*, and *Usnocetraria*	*Usnea ceratina*	Species from genera *Arctoparmelia* and *Xantho-parmelia*	n.a.
No. of out-group specimens	3	12	2	6	n.a.
No. of out-group species	3	10	1	5	n.a.
Herbaria or institutes of the collections	GZU, LD, and UPS	ASU, GZU, LD, MIN, NY, OSC, TU, and UPS	TU and WIS	GZU, KoLABIC, LE, MAF, TU, and UPS	WSL

Papers **I** and **II** deal with species delimitation in the genus *Vulpicida*. Paper **I** focuses on a pair of species – *V. juniperinus* and *V. tubulosus* – that show morphological intermediates which seem to be especially problematic in Estonia; therefore, most of the included samples represented the two species and their morphological intermediates. In the paper **II**, the sampling of the *Vulpicida*

species was extended to study the phylogeny of the whole genus; specimens of the other *Vulpicida* species – *V. canadensis*, *V. pinastri*, *V. tilesii*, and *V. viridis* – were added to the study. The species boundaries in the genus *Usnea*, sect. *Usnea* were under investigation in the paper **III**. A dataset of 144 specimens of 18 phenotypically-circumscribed species, collected from different parts of Europe and North America, was analysed. The phylogeny of 11 *Cetrelia* species, collected mainly from different parts of Eurasia, was studied in the paper **IV**.

In addition to the phylogenetic approach applied in papers **I–IV**, we tested the applicability of barcoding lichenized fungi using Roche 454 pyrosequencing and species identification success via DNA barcoding of the fungal ITS marker, and subsequently, investigated the intragenomic variation in lichenized fungi of one hundred lichen specimens in the paper **V**. The sampling included 52 crustose and 48 macrolichens (with foliose and fruticose thalli) from mainly the order Lecanorales. Among other taxa, 34 specimens from the family Parmeliaceae, with representatives from the three groups under phylogenetic investigations in papers **I–IV** were studied (i.e. *Cetrelia monachorum, Usnea barbata*, *U. intermedia*, *U. lapponica*, and *Vulpicida pinastri*).

For each study the morphological and chemical characters relevant to the species group were used for taxon identifications. In papers **I**, **III**, and **IV**, morphological and/or chemical diagnostic characters were studied in detail to evaluate the utility of characters, and provide morphology- and/or chemistry-based perspective for the species and their phylogeny.

2.2. Molecular methods

The methods used for molecular data generation followed in principle the steps as shown in Fig. 1. In papers **I–IV**, Sanger sequences were generated for the studied samples, while in paper **V**, Roche 454 pyrosequencing was used. Since pyrosequencing principally differs from Sanger sequencing, additional required steps are explained separately below. Cloning of ITS sequences for paper **II** is further described in the section 2.7.

The sample preparation step included examining the specimens under a stereomicroscope for the presence of lichenicolous and other contaminating fungi, followed by sampling 3–5 mg of visually uncontaminated lichen thallus for subsequent molecular and chemical analyses. A thallus piece was soaked in acetone to extract the chemical substances for thin layer chromatography (TLC) in the solvent system A (Orange *et al.* 2001). The same piece of thallus was subsequently used for DNA extraction. The dried piece of lichen thallus was grounded using stainless steel beads in a bead mill and the total genomic DNA was extracted using the High Pure PCR Template Preparation Kit (**I–III**), the Prepease DNA Isolation Kit (**III**), or the Qiagen DNEasy Plant Mini Kit (**IV** and **V**). Altogether, sequence data of seven markers were generated for this thesis (Table 2) – two loci from the nuclear ribosomal cistron: the internal

transcribed spacer region (ITS; **I–V**) and the intergenic spacer (IGS; **II–IV**), fragments from four low-copy protein-coding genes: beta-tubulin (Bt; **III**), MCM7 (**I–IV**), RPB1 (**II–IV**), and RPB2 (**III**), and a fragment of the mitochondrial SSU rDNA (mtSSU; **I** and **II**). Primers used for PCR reactions, as well as for sequencing, are shown in Table 2. For more specific amplification, new primers were generated for RPB1 and RPB2 markers in the *Vulpicida* and *Usnea* studies (**II** and **III**). Amplification of low-copy genes MCM7, RPB1, and RPB2 usually required a nested-PCR approach to obtain high-quality sequences. The second PCR step with inner primers (for ITS) or the same primers (for IGS) were also used to amplify the ribosomal genes of old samples (highly fragmented DNA) in the paper **IV**.

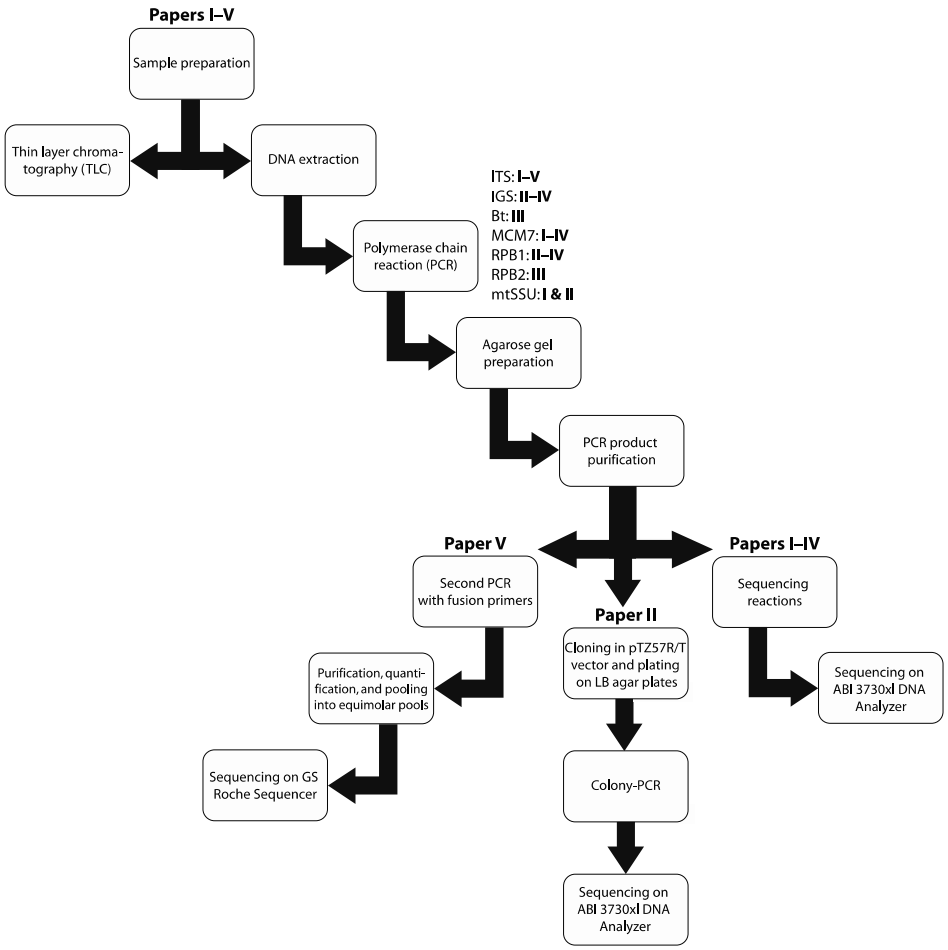

Figure 1. The outline of the molecular methods used in this thesis.

Successful PCR products were purified and complementary DNA strands were sequenced in the DNA Genotyping and Sequencing Core Facility of the Estonian Biocentre and Institute of Molecular and Cell Biology at the University of Tartu (Tartu, Estonia; **I–IV**), and partially in the Pritzker Laboratory for Molecular Systematics at the Field Museum (Chicago, IL, USA) for the paper **III**.

In paper **V**, where Roche 454 pyrosequencing was used, a two-step PCR approach was applied for unidirectional amplicon sequencing. The sample preparation and DNA extraction, as described above, were followed by a PCR with a high-fidelity polymerase and fungal-specific ITS primers, ITS1F and ITS4 (Table 2; additional info in paper **V**). In the second PCR step, the products were re-amplified with full-length fusion primers, followed by purification, quantification, pooling the aliquots in equimolar pools, and running on a GS Roche Sequencer (454 technology, Roche Diagnostics) using the Titanium FLX+ reagents. In this study, the pre-sequencing steps starting from the second PCR, sequencing, and raw data processing were carried out by Mircosynth AG (Balgach, Switzerland). For papers **I–IV**, the PCR purification and sequencing steps were conducted by the Estonian Biocentre and Institute of Molecular and Cell Biology at the University of Tartu, or by the author in the Pritzker Laboratory for Molecular Systematics at the Field Museum for the paper **III**.

2.3. Sanger sequence data analyses (I–IV)

2.3.1. Sequence alignment and recombination detection

The complementary sequence strands from Sanger sequencing were viewed, assembled, and edited in various programs, such as 4Peaks, Sequencher v4.2, Mesquite v2.75 (Maddison & Maddison 2011), and Geneious v7.1 (Kearse *et al.* 2012). The software MAFFT v7 (Katoh & Standley 2013) was generally used for aligning the sequences, followed by trimming the edges of the alignments in editing programs to level sequence lengths. The programs RDP (v3 and v4; Martin *et al.* 2010) and GARD (Kosakovsky Pond *et al.* 2006) were used to scan for possible recombination events in all the matrices in **I–III**. In paper **I**, the gaps in the matrices were coded as standard characters using the software SeqState (Müller 2005), while in other papers the gaps were treated as missing information.

2.3.2. Gene trees and concatenation-based phylogeny

Gene trees (single-locus and concatenated) were reconstructed for papers **I–IV** using primarily two approaches – Bayesian (B/MCMC) and maximum likelihood (ML). Maximum parsimony (MP) with nonparametric bootstrapping in PAUP* 4.0 (Swofford 2002) was additionally used in paper **I**. ML analyses were conducted and bootsrap probabilities (BP) were calculated using the program RAxML v7.3.1 (Stamatakis 2006); Bayesian analyses were performed in

MrBayes v3.2.1 (Ronquist *et al.* 2012) and in BEAST v1.7.2 (Drummond *et al.* 2012). While in RAxML, the evolutionary model for datasets was set to GTRGAMMA (Stamatakis *et al.* 2008), in Bayesian analyses, the DNA sequence evolution models for each marker were chosen in jModeltest v2.1.4 (Darriba *et al.* 2012; **I–III**) or in PartitionFinder v1.1.1 (Lanfear *et al.* 2012; **IV**). Convergence of the runs was assessed using Tracer v1.5 (Rambaut & Drummond 2007). LogCombiner v1.7.4 (Rambaut & Drummond 2012b) was used to combine trees and log files from multiple runs when neseccary, and a maximum clade credibility (MCC) tree with posterior probabilities (PP) for branch support was constructed in TreeAnnotator v1.7.4 (Rambaut & Drummond 2012a) and visualized in FigTree v1.3.1 (Rambaut 2009). Additionally, set of trees from BEAST analyses were visualized in DensiTree v2.1.11 (Bouckaert 2010; **IV**).

Table 2. The studied loci and primers used in publications **I–V**. F – forward; R– reverse

Marker	Primer name	Direction	Primer sequence (5′–3′)	Primer reference	Publication
Bt	Bt2a	F	GGT AAC CAA ATC GGT GCT GCT TTC	Glass and Donaldson 1995	**III**
	Bt2b	R	ACC CTC AGT GTA GTG ACC CTT GGC	Glass and Donaldson 1995	**III**
IGS	IGS12a	F	AGT CTG TGG ATT AGT GGC CG	Carbone and Kohn 1999	**III**
	XIGS_R	R	TAC TGG CAG AAT CAR CCA GG	Leavitt et al. 2011c	**III**
	IGSf	F	TAG TGG CCG WTR GCT ATC ATT	Wirtz et al. 2008	**II, III, IV**
	IGSr	R	TGC ATG GCT TAA TCT TTG AG	Wirtz et al. 2008	**II, III, IV**
ITS	ITS1F	F	CTT GGT CAT TTA GAG GAA GTA A	Gardes and Bruns 1993	**I, II, III, IV, V**
	ITS4	R	TCC CCG CTT ATT GAT ATG C	White et al. 1990	**I, II, III, IV, V**
	ITS1LM	F	GAA CCT GCG GAA GGA TCA TT	Myllys et al. 1999a	**IV**
	ITS2KL	R	ATG CTT AAG TTC AGC GGG TA	Lohtander et al. 1998	**IV**
MCM7	Mcm7-709for	F	ACI MGI GTI TCV GAY GTH AAR CC	Schmitt et al. 2009	**I, II, III, IV**
	Mcm7-1348rev	R	GAY TTD GCI ACI CCI GGR TCW CCC AT	Schmitt et al. 2009	**I, II, III, IV**
	X_Mcm7_F	F	CGT ACA CYT GTG ATC GAT GTG	Leavitt et al. 2011c	**III**
	X_Mcm7_R	R	GTC TCC ACG TAT TCG CAT TCC	Leavitt et al. 2011c	**III**
	LecMCM7f	F	TAC CAN TGT GAT CGA TGY GG	Leavitt et al. 2011a	**I, II, III, IV**
	LecMCM7r	R	GTC TCC RCG TAT TCG CAT NCC	Leavitt et al. 2011a	**I, II, III, IV**
mtSSU	mrSSU1	F	AGCAGTGAGGAATATTGGTC	Zoller et al. 1999	**I, II**
	mrSSU3R	R	ATGTGGCACGTCTATAGCCC	Zoller et al. 1999	**I, II**
RPB1	gRPB1-A for	F	GAK TGT CCK GGW CAT TTT GG	Matheny et al. 2002	**II, III, IV**
	fRPB1-Cr-Par1	R	CRG CRA TRT CRT TRT CCA TRT A	**II**	**II**
	fRPB1-C rev	R	CCN GCD ATN TCR TTR TCC ATR TA	Matheny et al. 2002	**III, IV**
	RP1C-uc1	R	CRG CRA TRT CRT TRT CCA TRT A	**III**	**III**
	RPf-Usn3	F	CTC GCA GTA CCY GTT TAC C	**III**	**III, IV**
	RPr-Usn2	R	TGG CTC GAA CTC ATT SAC	**III**	**III**
	RPB1f-Cet2	F	GTT TAY CAY GTY GGT ATG TG	**II**	**II**
	RPB1r-Cet2	R	GCT GCT CAA ACT CRT TGA C	**II**	**II, IV**
RPB2	RPB2-6F	F	TGG GGK WTG GTY TGY CCT GC	Liu et al. 1999	**III**
	fRPB2-7cr	R	CCC ATR GCT TGY TTR CCC AT	Liu et al. 1999	**III**
	RPB2-UsnF	F	CTG CGG AAA CTC CTG AAG GC	**III**	**III**
	RPB2-UsnR	R	GGT AAG TRT TTC TAG GAG ACT G	**III**	**III**

2.3.3. Species delimitation and phylogeny under multispecies coalescent model (II, III)

To assign individuals to candidate species or independent populations, as is necessary for species tree inference (Leaché & Fujita 2010), coalescent-based species delimitation methods were used for the *Vulpicida* and *Usnea* datasets. In paper **II**, the nonparametric heuristic method in Brownie 2.1.2 (O'Meara *et al.* 2006), and in **III**, the newly developed package STACEY v1.0.1 (Jones 2015) in BEAST v2.2 (Bouckaert *et al.* 2014), were implemented to estimate the putative species in the study groups. The Species or Minimal Clusters trees (SMC-trees) resulting from STACEY were subsequently used in the SpeciesDelimitationAnalyser (Jones *et al.* 2014) to assign individuals into minimal clusters, and the output of posterior probabilities of individuals belonging to the same cluster were visualised in a similarity matrix constructed in R v2.15.1 (R Core Team 2014).

In paper **III**, the species tree, implemented in BEAST v1.8.0 (*BEAST; Heled & Drummond 2010), was estimated using the results from STACEY and gene concatenation analyses. In paper **II**, three species tree methods were used: two Bayesian methods – *BEAST implemented in BEAST v1.7.2 (Heled & Drummond 2010; Drummond *et al.* 2012) and BEST 2.3 (Liu 2008) – and one ML-based method – STEM-hy 1.0 (Kubatko *et al.* 2009).

Species boundaries were tested for alternative models of species delimitation in *Vulpicida* and *Usnea* using the program BP&P v2.1b (Yang & Rannala 2010; **II**) and BP&P v3 (Yang & Rannala 2014; **III**).

2.4. 454 pyrosequencing data processing and analyses (V)

The sequence generation in pyrosequencing platforms is principally different form Sanger sequencing and data processing of pyrosequencing reads is needed prior to conclusive analyses. In pyrosequencing data processing we first followed rather traditional steps: (1) dividing the sequencing reads into samples based on their MID tags, (2) sorting the reads based on specified quality and length parameters using the programs Cutadapt v1.7 (Martin 2011) and PRINSEQ-lite v0.20.4 (Schmieder & Edwards 2011; parameters in paper **V**), (3) screening for chimeras using the 'uchime_ref' command in the sequence analysis tool USEARCH v8.0.1623 (Edgar *et al.* 2011) to search against the UNITE/INSDC reference database of fungal ITS sequences (Nilsson *et al.* 2015), (4) sorting the remaining reads by length and cluster with USEARCH at a 95% similarity threshold using the 'centroids' function in the UCLUST algorithm (Edgar 2010), (5) comparing the resulting centroid sequences against the NCBI nucleotide database (Coordinators 2013) using the 'blastn' algorithm to obtain their initial taxonomic affiliations. We found that the centroid sequences of the clusters included a high rate of differences compared to the available Sanger sequences (see Table 2 in paper **V**), likely resulting from the sequencing

errors the 454 pyrosequencing is notorious for (Tedersoo *et al.* 2010; Gilles *et al.* 2011; Lücking *et al.* 2014b), and decided for consensus sequence approach to obtain reliable reference sequences for the species. For this, the target species sequences were gathered and aligned together with references from GenBank using the MAFFT v7 automatic algorithm in the Geneious v7.1.6 platform. Clear outliers in the alignment were removed, the primer binding sites were trimmed, and the remaining region – including the end of ribosomal RNA gene 18S, ITS1, 5.8S rRNA gene, ITS2, the beginning of the 28S rRNA gene, and in some species, also the group I intron at the end of the 18S gene – was designated as the barcode region. The consensus sequence of this region was assigned as the barcode for a species. We tested the identification of the generated barcodes using the 'megaBLAST' function to search against the NCBI nucleotide database (Madden 2002). Additionally, we estimated nucleotide diversity for alignments with removed reference sequences using DnaSP v5.10.1 (Librado & Rozas 2009) and quantified the sequence similarity (incl. number of indels and nucleotide differences) between generated consensus barcodes and representative centroid sequences.

2.5. Screening for paralogous copies of ITS (II, V)

Paralogous ITS copies distorting phylogenetic trees have been reported in fungi (Ko & Jung 2002; Lindner & Banik 2011; Lindner *et al.* 2013). In paper **I**, a species cluster including *Vulpicida juniperinus*, *V. tubulosus*, and *V. tilesii* was polyphyletic, divided into two clearly distinct groups of mixed morpospecies by their ITS sequences. The two ITS sequence groups were not supported by other loci or morphology; thus, screening for possible ITS paralogs in these groups via cloning was undertaken in the paper **II**. For this, the amplified ITS PCR products of 12 specimens (six from each of the mixed clades) were purified and cloned in pTZ57R/T vector. The cloning procedures were carried out in Icosagen Cell Factory OÜ (Tartumaa, Estonia). Sixteen positive clones, grown on LB agar plates, were used for colony-PCRs. ITS sequences of 14 successfully amplified clones were obtained. In paper **V**, ITS sequence variation in 99 lichen fungi from pyrosequencing was studied. In cases where (a) less-dominant version(s) of ITS was/were higher in frequency than 10% of the target reads, possibly representing intragenomic variation of ITS sequences or a mixture of individuals of the same or closely related species, the target species alignments were studied carefully, and consensus barcode(s) for the less-dominant versions were also generated (details and argumentation in paper **V**). The different barcode versions were aligned using the MAFFT v7.017 automatic algorithm, and sequence variation, nucleotide diversity, and p-distances were estimated using DnaSP v5.10.1 and MEGA5 (Tamura *et al.* 2011).

3. RESULTS

In total, 1,464 sequences were newly generated in Sanger technique and used for phylogenetic analyses in papers **I–IV (**Fig. 2A**)**. Pyrosequencing of the fungal ITS region of 100 lichens for paper **V** resulted in 128,449 reads, with an average of 1,285 reads per sample (Fig. 2B). The results of each study are summarised in the sub-paragraphs below.

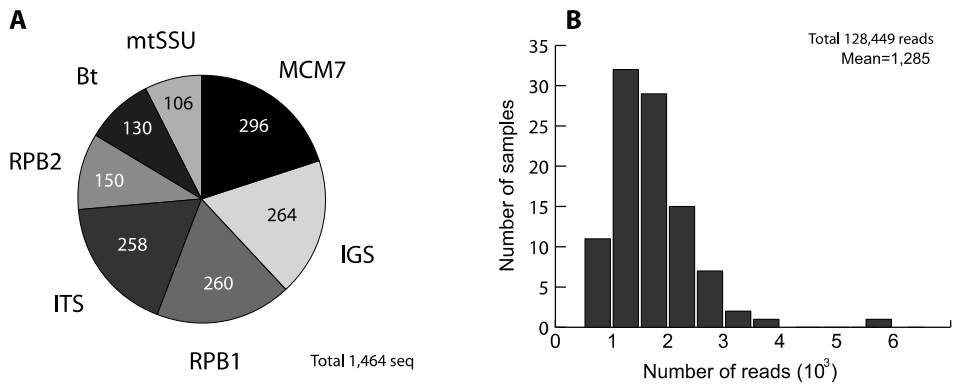

Figure 2. Number of newly generated Sanger sequences of each analysed locus (A; **I–IV**) and 454 pyrosequencing reads (B; **V**).

3.1. Phylogeny and species delimitation in the genus *Vulpicida* (I and II)

The three-locus data matrix in paper **I** consisted 1846 nucleotide positions. In paper **II**, with two more loci, the dataset consisted of 2820 nucleotide positions. The distinctiveness of the two North American *Vulpicida* species – *V. canadensis* and *V. viridis* – was clearly confirmed; samples of these two species formed monophyletic clades in all gene trees. Four other previously accepted species – *V. juniperinus*, *V. pinastri*, *V. tilesii*, and *V. tubulosus* – presented a greater challenge. They formed a strongly supported clade, a core group of the genus, in all analyses (here and further on, branch supports were considered strong when PP≥95 and BP≥70). However, single-gene tree topologies within this clade conflicted considerably in both of the studies. In the ITS gene tree, *V. juniperinus*, *V. tubulosus*, and *V. tilesii* specimens were divided between two clades, of which one was recovered as a sister to *V. pinastri* clade, while another was closely related to *V. canadensis* and *V. viridis*. The group of *V. juniperinus*, *V. tubulosus*, and *V. tilesii* appeared monophyletic in IGS and RPB1, and inter-mixed with *V. pinastri* in MCM7 and mtSSU. Conflicting were also the relationships between some closely related members from the cetrarioid core

group. *Vulpicida* appeared as monophyletic in the RPB1 tree, while the genus was not monophyletic in the IGS, MCM7, or mtSSU trees, where *V. canadensis* and *V. viridis* were found related more closely to different outgroup members (**II**).

As the division of *V. juniperinus* and *V. tubulosus* sequences into two distinct clades was only present in ITS, one could consider this to be the result of paralogy – two different copies of the same locus in one genome. Thus, the presence of ITS paralogs was tested in paper **II** by cloning the ITS fragements of 12 specimens, but no paralogous copies of ITS were found in the clone fragments. Intragenomic variation of ITS was also not detected in the analysed *V. pinastri* specimen in paper **V** via pyrosequencing. One of the major biological reasons for species polyphyly is considered to be recombination, which can be detected using numerous algorithms. However, no credible recombination events within or between loci were found.

The non-parametric heuristic method implemented in Brownie distinguished four "species" (or independent populations) within the core group of *Vulpicida*, while in the BP&P, the candidate species were more narrowly defined, showing six putative species in the *V. juniperinus-tubulosus-tilesii* complex (results in paper **II**). These groups were assigned as putative species in the coalescent-based species tree analyses, together with *V. canadensis* and *V. viridis*. Results of the multispecies coalescent-based species tree inferences from *BEAST, BEST, and STEM are summarized in Fig. 3 (illustration from paper **II**). In the species trees, the monophyly of *Vulpicida* was supported by *BEAST only. The *Vulpicida* core group was strongly supported in all species tree analyses. *Vulpicida pinastri* was clearly monophyletic while the rest of the taxa in the core group were on relatively short branches and formed a strongly supported complex.

The conclusive results on the species monophyly from the two *Vulpicida* papers differed significantly. Based on the results of paper **I**, one could suggest from the ITS and concatenated trees that *V. pinastri* splits two morphologically indistinguishable species that correspond to the two clades of *V. juniperinus/ tubulosus/tilesii*. However, this might not necessarily be the case, as also proved in paper **II**. Relatively short branches in the phylogenetic trees and little differences in other characters indicate that we might be dealing with a young species complex consisting of morphospecies *V. juniperinus*, *V. pinastri*, *V. tilesii*, and *V. tubulosus*, that are still evolving under the effect of ILS. It is possible that an early stage of speciation is reflected only in ITS and not yet in the other markers because of different molecular evolution rates between the loci.

In conclusion of the two *Vulpicida* papers, the current, phenotype-based species *V. juniperinus* and *V. tubulosus* could not be separated by any locus in any analysis. The third currently recognized taxon, *V. tilesii*, did not form a monophyletic clade in any of the gene trees. The two clearly separate cryptic lineages in the ITS topology were not recovered in other loci, suggesting that strong phylogenetic signal from ITS may have been dominating in the analysis of the concatenated genes in paper **I**. Undermining confidence in their validity

as distinct lineages, the "cryptic species" failed to appear as species or independent populations in O'Meara's method, reflecting the lack of congruent support for them from different loci, and were clustered in a strongly supported complex of putative species in the multispecies coalescent-based species tree analyses. Therefore, based on the results of the two papers, we proposed four instead of the currently accepted six species in the genus *Vulpicida*: *V. canadensis*, *V. juniperinus*, *V. pinastri*, and *V. viridis*, while *V. tilesii* and *V. tubulosus* were reduced to synonymy under *V. juniperinus*, which is the type species of the genus (nomenclatural changes in the Taxonomy section of the paper **II**).

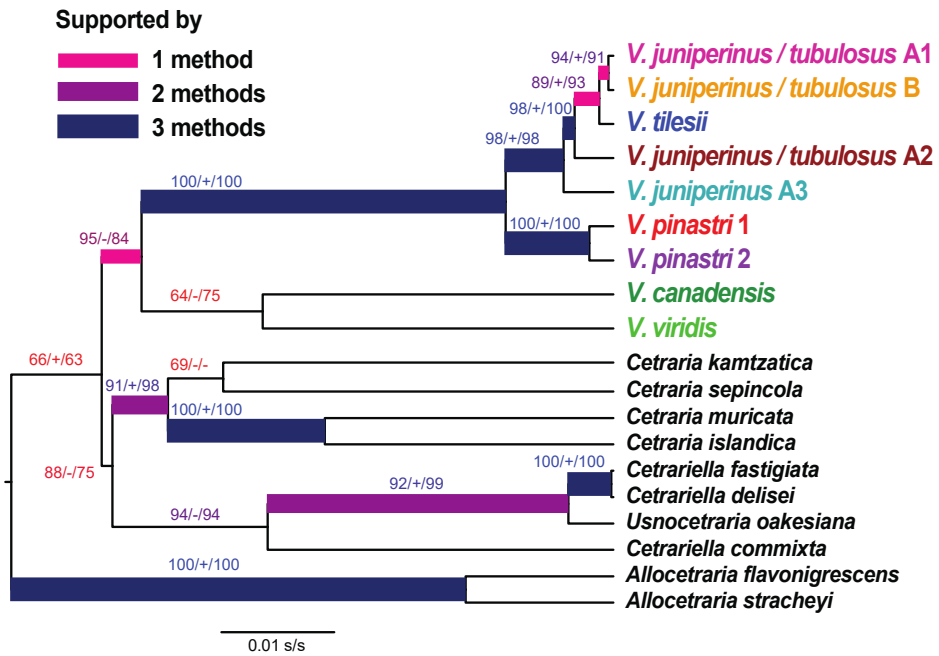

Figure 3. *Vulpicida* species tree from paper **II** based on five loci, inferred by *BEAST. Branch annotations indicate *BEAST posterior probability/presence of the branch in STEM analysis/BEST posterior probability. Branch thickness reflects the number of methods strongly in support, with thresholds ≥95% for *BEAST and BEST, and ≥70% for STEM. Scale bar shows the number of substitutions per site.

3.2. Species boundaries and evaluation of diagnostic characters in the genus *Usnea* sect. *Usnea* (III)

High quality sequences of six analysed loci for 144 specimens were generated, resulting in 864 sequences. The combined dataset for phylogenetic analyses included 3225 aligned nucleotides. In general, individual gene trees showed weak genetic structure and short branch lengths for all studied loci. The overall genetic variability in all loci was low considering that 19 species from different locations in Europe and North America were included. Combining loci allowed better differentiation among samples. Even though the backbone of the tree remained poorly resolved, several strongly supported groups were revealed: (1) the *Usnea cavernosa* clade; (2) the *U. silesiaca* clade; (3) A clade of newly described species, *U. parafloridana*; (4) the *U. wasmuthii* clade; (5) the fulvoreagens-glabrescens clade that included species *U. fulvoreagens*, *U. glabrescens* and *U. pacificana*; (6) the florida-subfloridana clade with *U. florida* and *U. subfloridana*; (7) the *U. praetervisa* clade; (8) the barbata-chaetophora-dasopoga-diplotypus clade that included specimens with the morphology of *U. barbata* (in part), *U. chaetophora*, *U. cylindrica*, *U. dasopoga* and *U. diplotypus*; and (9) the barbata-intermedia-lapponica-substerilis clade that included mainly specimens with the morphology of *U. barbata* (in part), *U. intermedia*, *U. lapponica* and *U. substerilis* (see Figure 1 in paper **III**). Thirteen clusters from STACEY species delimitation analyses, validated also in the BP&P analyses, were assigned as the putative species for the *BEAST species tree analyses (Fig. 4; illustration from paper **III**). In species tree analyses, section *Usnea* formed a strongly supported monophyletic group on a long branch. The backbone of the tree was mainly unresolved and the overall supports for the relationships among the putative species remained weak just as in the single-gene analyses and the concatenated multilocus phylogeny.

Seventeen morphologically circumscribed species from sect. *Usnea* were included in our study, but only four of them – *Usnea cavernosa*, *U. praetervisa*, *U. silesiaca*, and *U. wasmuthii* – were recovered as monophyletic in phylogenetic analyses, while others formed clusters of two or more species. Our study supported the view that several nominal species in sect. *Usnea* could merely represent intraspecific phenotypes. For example, the barbata-chaetophora-dasopoga-diplotypus clade included several morphospecies (i.e. *U. barbata*, *U. chaetophora*, *U. diplotypus*, *U. cylindrica*) that did not separate genetically or chemically. On the other hand, our analyses showed weak clustering of *U. intermedia* with *U. barbata* accessions and separation from *U. lapponica* and *U. substerilis* in the STACEY analyses (Fig. 4; illustration from paper **III**). When *U. intermedia* and *U. barbata* are easily distinguished based on their reproductive mode, the conspecific *Usnea lapponica* and *U. substerilis* are considered similar also morphologically, thus, a synonymization of *U. substerilis* under *U. lapponica* was proposed (see the Taxonomy section in **III**). At the same time, several distinct lineages that did not correspond to previously

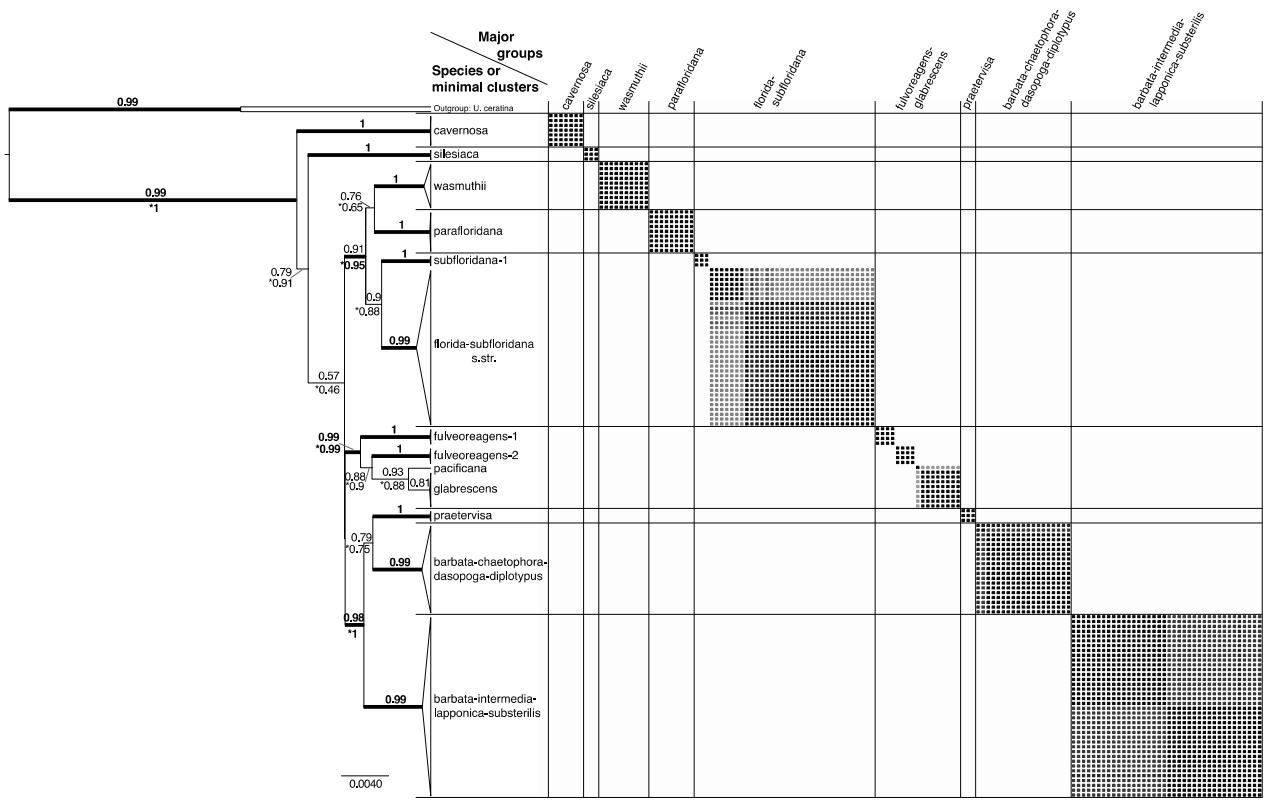

Figure 4. Maximum clade credibility SMC-tree with posterior probabilities (PP) from STACEY (above branches) and *BEAST (below) analyses together with similarity matrix for the sect. *Usnea* dataset (illustration from paper **III**). The squares represent posterior probabilities (white=0, black=1) for pairs of individuals to belong to the same cluster. The lines in the matrix separate major groups (named above matrix) while labels next to the cartooned clades of the SMC-tree represent the 13 species or minimal clusters delimited in STACEY analyses. Braches and probability scores with strong support (PP≥0.95) from STACEY and/or *BEAST analysis are marked in bold. Scale bar shows the number of substitutions per site.

described morphological taxa were detected within our data. The most notable of these was a group with distinct morphology and chemistry from Wisconsin, USA that seemed to represent an undescribed species, and proposed in this paper as *Usnea parafloridana* (see the Taxonomy section in **III**). This taxon is characterised by a shrubby thallus, with relatively few and thick branches, and soralia bearing many isidiomorphs (Fig. 5). It is closely related to *U. wasmuthii* that, however, differs in its medullary chemistry and soralium morphology.

The analysed species in sect. *Usnea* have wide distributional ranges, often occurring across the Northern Hemisphere, but they show a low degree of geographical structure within the phylogeny. Instead, secondary metabolites corroborated the phylogenetic clades (results in paper **III**). Therefore, a very high chemotypic variation within morphology-based entities may indicate the need for phylogenetic re-evaluation of species boundaries. Additionally, *Usnea* thallus anatomy (the parameters of the inner structure of a branch) seemed to support our genetic clusters. It came especially evident in the *U. barbata* species complex where two clades of intermixed species were statistically significantly different in the branch anatomy (see Figure 2 in papr **III**); one constituted species mostly with a relatively thick cortex and a thin, compact medulla (i.e. *Usnea chaetophora*, *U. cylindrica*, *U. dasopoga*), while specimens in the other had a relatively thin cortex with a thick and more lax medulla (*U. intermedia*, *U. lapponica*, *U. substerilis*).

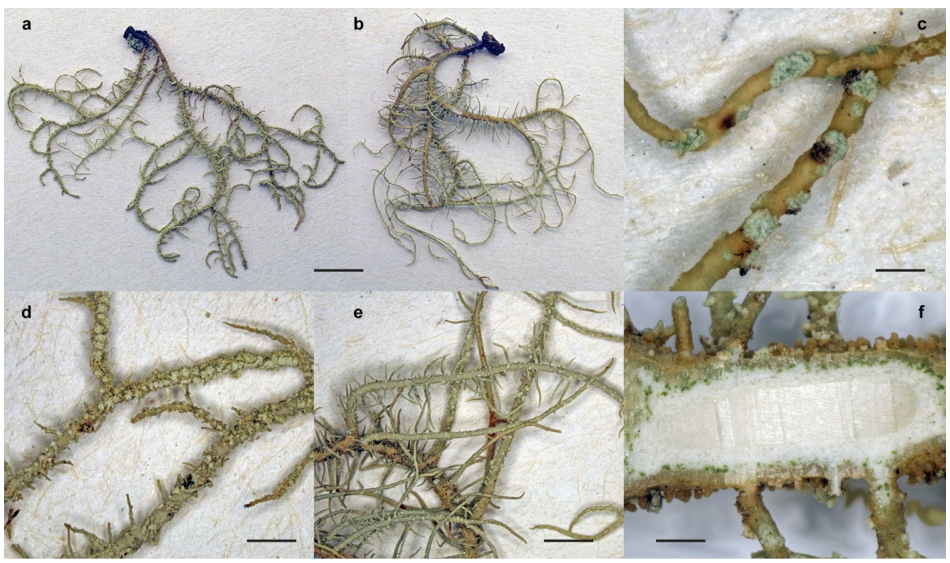

Figure 5. *Usnea parafloridana* K. Mark, Will-Wolf & Randlane – view of general habit (a, b), soralia (c), soralia with isidiomorphs (d), fibrils (e), branch anatomy (f). *Scale bars* 7 mm (a, b), 0.4 mm (c), 1.5 mm (d), 2 mm (e), and 0.3 mm (f). Photographed specimens WW14807 (holotype; a, c, d); WW14858 (b, e); WW14857 (f).

3.3. Species diversification history in the genus *Cetrelia* (IV)

Eleven *Cetrelia* species representing three morphotypes and all six chemotypes were investigated in this study (Table 4 in paper **IV**). For phylogenetic analyses, 114 new sequences for 47 specimens were generated and sequence data of 17 specimens with checked morphology and chemistry was mined from the NCBI nucleotide database. The full data matrix included 64 specimens and 2314 aligned nucleotides. In molecular analyses we recovered a relatively well-resolved phylogeny where the genus was monophyletic and the currently accepted species generally appeared justified. Our phylogenetic analyses suggested the monophyly of species *Cetrelia monachorum*, *C. alaskana*, *C. olivetorum*, *C. japonica*, and *C. braunsiana*. A few other taxa (*C. pseudolivetorum* and *C. chicitae*) appeared polyphyletic and sorediate *C. cetrarioides* was paraphyletic since sexually reproducing *C. delavayana* (however, with poorly developed morphological characters) clustered strongly within.

The species of the same chemotype formed monophyletic clades with subclades that mostly correlated to morphotypes, reflecting so the diversification history of *Cetrelia* species. Figure 6 illustrates the species phylogeny and their chemotype from character evolution perspective (illustrartion from paper **IV**). The imbricaric acid chemotype seems to have evolved earliest as the two taxa representing it – *C. alaskana* and *C. monachorum* – positioned at the base of the genus, while the rest of the species formed a monophyletic group within. The two species with olivetoric acid chemotype – *C. olivetorum* and *C. pseudolivetorum* – clustered together, and based on the four markers analysed, dispersed after the imbricaric acid chemosyndrome but before anziaic, perlatolic, microphyllinic, or alectoronic/α-collatolic chemosyndromes. The fact that anziaic and perlatolic acids are biogenetically closely related (perlatolic acid is the methylated derivate of anziaic acid) is reflected also by the multilocus gene genealogy – even though not strongly supported, the two chemotypes formed a monophyletic group. The phylogenetic position of microphyllinic acid chemotype remains unresolved in this study. The alectoronic & α-collatolic chemosyndrome formed a monophyletic group, represented by three species, *Cetrelia braunsiana*, *C. chicitae*, and *C. orientalis*. The development of a specific type of reproductive structures (soredia vs. isidia vs. lobulae vs. aptohecia or no structures) – according to which the morphotypes are mainly characterised – has evidently occurred independently multiple times during the evolution of the fungal lineages. For example, the distribution through soredia has developed in *Cetrelia* at least in four occasions according to the current dataset – in *C. monachorum*, *C. olivetorum*, *C. cetrarioides*, and in *C. chicitae*, and therefore treatment of taxa of the same morphotype as one species is not phylogenetically acceptable, although currently widely used.

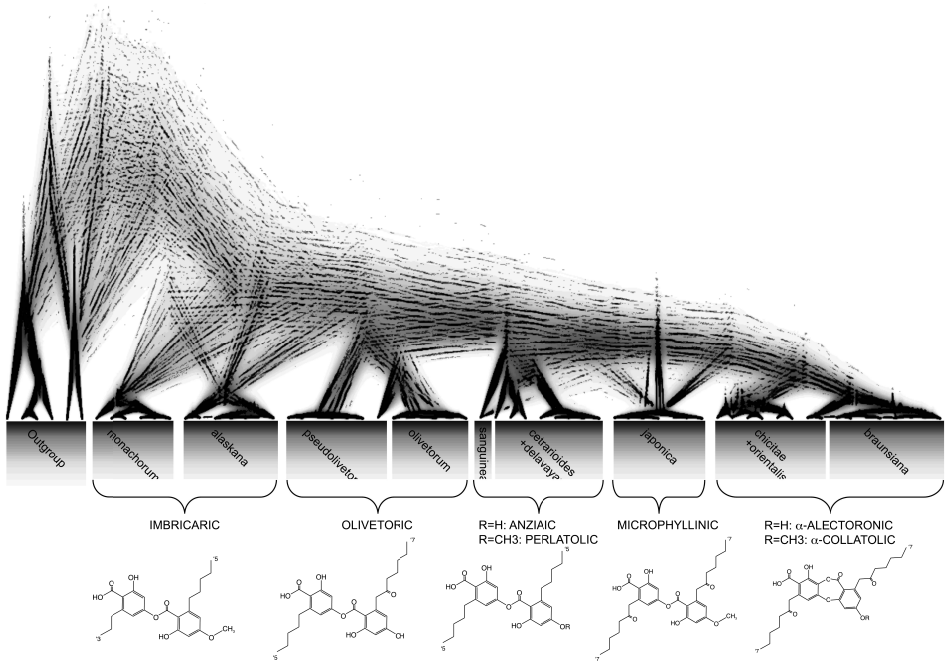

Figure 6. Densitree of 20,000 trees from B/MCMC analyses inferred from BEAST, together with the currently accepted species that are marked in boxes below the densitree and reflect the species' borders in the genus *Cetrelia* (**IV**). The species' chemotypical affiliation and the formula of the major compound in the chemotype are given below the species.

3.4. Utilization of pyrosequencing in lichen barcoding and exploring intragenomic sequence variation in lichenized fungi (V)

Pyrosequencing of 100 lichens resulted in 128,449 fungal ITS reads. The number of reads per sample varied between 207 and 5,473. Despite conservative quality sorting, high nucleotide variation per sample was present in the data. While a high proportion of this variation resulted from contaminant fungi within samples, significant nucleotide variation in the target taxon reads was also present. The mean number of clusters per target species was 8 and the average nucleotide diversity within a target was 0.01128. Comparing centroid sequences with consensus barcodes revealed a mean difference of 1.46%. About 90% of the differences were insertions (69%) and deletions (21%) that were especially prominent towards the end of the sequences, while the rest were nucleotide substitutions and CAFIE errors.

From 100 samples we were able to recover the fungal ITS sequence of the lichen mycobiont for all sequenced samples except one. Good success in generating barcodes shows the high potential of this method, especially con-

cerning crustose lichens, which can be difficult to sequence with the Sanger technique. The targeted lichenized fungi were, in most cases, morphologically and molecularly identified to the same species (n=69) or at least to the same genus (n=18) using the NCBI nucleotide database as reference. Multiple species showed a low intraspecific ITS sequence similarity (<97%) to other available sequences in GenBank or a high similarity (≥97%) to a different taxon (results in Table S2 of the paper **V**). Therefore, before specimen identification via DNA barcoding can be confidently applied, taxonomic studies in several groups (see paper **V**) are needed to confirm the species monophyly or, in cases of non-monophyly, propose segregation into multiple species. However, our results also suggest that the NCBI nucleotide database, currently the most complete database for lichen-forming fungi, could be used as a reference database to identify common species, as the majority of the analysed lichens were identified correctly to the species or, at least, to the genus level.

We studied the nucleotide variation of samples where (a) less frequent version(s) of ITS represented more than 10% of the expected mycobiont reads (n=22; listed in Table 3 of the paper **V**). Such nucleotide variation can result from PCR/sequencing errors, or due to biological reasons, such as incomplete concerted evolution within the ITS. For 13 species, distinct nucleotide variation could not be explained by PCR or CAFIE errors only. By comparing the variation within the reads of the mycobiont with available Sanger sequences from GenBank of the same species (where available), we also found the same variable bases in Sanger sequences in eight species. Therefore, it seems probable that intragenomic and/or intra-mycelial (i.e. allelic heterozygosity) variation was present at least in the following studied samples: *Bryoria capillaris*, *Lepraria rigidula*, *Melanohalea exasperata*, *Parmelia sulcata*, *Physcia adscendens*, *Usnea intermedia*, and *Usnea lapponica*. For eight species, such comparisons could not be made due to a lack of Sanger sequences. Sequence variation possibly resulting from intragenomic or intra-mycelial variation was additionally detected in *Bacidina arnoldiana*, *Chaenotheca cf. stemonea*, *Fellhanera bouteillei*, *Lecania cf. cyrtella*, *Lecidella scabra*, and *Lecidella sp.* In three crustose species – *Bacidina arnoldiana aggr.*, *Chaenotheca cf. stemonea*, and *Fellhanera bouteillei* – more than one distinct lineage of the genus, probably representing different species and seemingly resulting from lichen chimeras or microcosms (diverse communities living mixed or alongside), were identified in their sequence pools (see phylogeny illustrations of the three genera in Figures 3.–5. in paper **V**).

4. DISCUSSION

4.1. Species concept and species delimitation in young species complexes

The prevailing species concept assumes that species represent independently evolving metapopulation lineages and usually monophyly criterion is applied on such lineages. However, species are constantly changing under different evolutionary processes. Diversification and extinction of lineages are continuously happening due to recombination and random mutations in the genome, and depending on the selection pressure, the lineages are evolving faster or slower (Coyne & Orr 2004). Older species have had time to accumulate apomorphies and gene-tree monophyly while young species often lack monophyly most often due to ILS (Maddison & Knowles 2006). Under the effects of ILS multiple gene versions can persist through speciation events. ILS can occur in any taxonomic group and with any molecular marker, but is especially common in closely related taxa (de Queiroz 2007; Hobolth *et al.* 2011).

The species can be defined by the collection of all its genes and the ancestral relationships inferred from a gene can differ considerably from the ones between species (Drummond & Bouckaert 2015). Therefore, using a single gene fragment is inappropriate to make claims about species evolution since it represents the history of the gene and not nessecarily of the species. Furthermore, concatenating nucleotide sequences from different conflicting loci can result in misleading estimates (Degnan & Rosenberg 2006; Kubatko & Degnan 2007; Heled & Drummond 2010). Newly developed methods that base on the multispecies coalescent model (Rannala & Yang 2003; Degnan & Rosenberg 2009) incorporate population genetic processes into phylogenetics and shift the focus of inference from gene trees to species trees (Edwards 2009; Fujita *et al.* 2012). These novel model-based frameworks are increasingly preferred for species phylogeny estimations in different organism groups (e.g. Wiens *et al.* 2010; Melo-Ferreira *et al.* 2011; Harrington & Near 2012; Tomasello *et al.* 2015), and also among fungi (e.g. (Henk *et al.* 2011; Blair *et al.* 2012; Silva *et al.* 2012; Leavitt *et al.* 2016).

Young species complexes are common in groups where rapid radiation takes place (Givnish 2015; Pease *et al.* 2016). They are characterized by conflicts between individual gene trees and gene trees vs. a species tree, few apomorphies compared to synapomorphic mutations, and fuzzy species boundaries that can reflect also in morphology. Such features are clearly evident in two of the study groups investigated in this thesis – the genus *Usnea*, sect. *Usnea,* and the genus *Vulpicida* (**I–III**).

By the example of *Vulpicida*, the advancements provided by the multispecies coalescent model are illustrated. We saw fuzzy species boundaries with morphological intermediates between two *Vulpicida* species growing on the western islands of Estonia. When analysing some of the more popular genes of

these “problematic” species, we found the morphospecies (and their intermediates) to be intermixed and divided between two clades, and concluded in the paper **I** that the two clades could represent cryptic species. Since strong conflicts between the genes were found, more markers and additional specimens were used, and the multispecies coalescent model in species tree analyses in different methods was incorporated for the paper **II**. The new analyses resulted in opposing conclusions, suggesting a young species complex in *Vulpicida* with varying thallus morphology, and clustered the clades representing the previously defined “cryptic species” into a single clade. Since none of the morphospecies (*Vulpicida juniperinus*, *V. tilesii*, or *V. tubulosus*) were supported by any of the analyses, the taxa were synonymized under a single species, *Vulpicida juniperinus*.

In young species complexes with recent and rapid diversification vast amounts of genetic data are required to recover species (Wagner *et al.* 2013) and appropriate analytical species tree and species delimitation methods are necessary to account for conflicting evolutionary signal from different genes. Integrative approaches, including morphological, chemical or geographical data, can also be effective in delimiting species (Edwards & Knowles 2014). Species delimitation in the sect. *Usnea* proved especially difficult (**III**), as commonly used species delimitation models, such as The Generalized Mixed Yule Coalescent (Pons *et al.* 2006), Poisson Tree Processes (Zhang *et al.* 2013), O’Meara’s heuristic method (O'Meara *et al.* 2006), that use gene trees to estimate speciation or branching events and identify putative species based on a threshold failed to find consensus in the number of species and grouping of the specimens. However, the recently developed coalescent-based species delimitation method STACEY, that estimates the species tree and groups specimens into minimal independent evolutionary lineages, showed a conclusive and stable clustering of the *Usnea* specimens (Fig. 4; illustration from paper **III**). The data structure was also supported by lichen chemistry and thallus anatomy, by thus furthermore assuring the justness of the results.

4.2. Lichen identification – the utility of morphology and chemistry vs. DNA barcoding approach

Accurate identification of species presents an acute problem in many groups of lichens (Lumbsch & Leavitt 2011). Traditionally, lichens have been circumscribed using mainly morphological characters. Among clonally reproducing species, most often thallus morphology, medullary chemistry and the type of asexual reproductive structures are characterized. Various parameters of the sexual structures (apothecia and/or pycnidia) are additionally used in sexually reproducing species. Ecological preferences and distribution patterns can also help to discriminate between species. Nowadays species are advised to be described through combination of molecular and additional (i.e. morphological, chemical, ecological) data, while in many cases no discriminative pattern in

morphology is visible in genetically distinct lineages. Such phylogenetic species are frequently referred to as 'cryptic' (Funk & Omland 2003; Crespo & Pérez-Ortega 2009). Incongruence between the morphological circumscriptions of species and phylogenetic delimitation based on DNA sequence data is known from several groups of lichen-forming fungi. In some cases, overestimation of the species diversity based on morphology and chemistry has been demonstrated (Leavitt *et al.* 2011b; Velmala *et al.* 2014), while in others, new lineages, representing cryptic or undescribed species with newly discovered diagnostic characters, have also been found (Lumbsch *et al.* 2011; Kraichak *et al.* 2015b; Singh *et al.* 2015). Finding and applying the appropriate character sets is crucially important for both species delimitation and accurate species identification.

The genus *Usnea* is considered taxonomically one of the most difficult macrolichen genera, as many of its species are highly variable in morphology and chemistry, and some traditionally used characters have proven to be homoplasious (Clerc 1998; Wirtz *et al.* 2012). We included seventeen morphologically circumscribed species from sect. *Usnea* in our study but only four of them were recovered as monophyletic in phylogenetic analyses, while others formed clusters of two or more morphospecies. Our data suggest that using most diagnostic morphological characters together with branch anatomy and thallus chemistry are useful for delimiting some genetic lineages in sect. *Usnea*, while other clades (e.g. 'barbata-chaetophora-dasopoga-diplotypus' and 'barbata-intermedia-lapponica-substerilis') have very wide morphological variation and some currently accepted diagnostic morphological characters are not useful for delimiting these clades.

The general appearance of specimens (e.g. whether being pendulous or shrubby in *Usnea*) and their reproductive mode (whether bearing apothecia or reproducing clonally) has played an important role in the circumscription of lichen taxa. However, thallus gross morphology can greatly vary with age and environmental conditions. Also, reproductive mode can vary between lineages and transitions between sexuality and asexuality may occur (Scherrer *et al.* 2005; Tehler & Irestedt 2007; Cornejo *et al.* 2009). Our results from paper **I** show that *Vulpicida juniperinus* thallus can vary from foliose with adenate flat lobes to sub-fruticose with flat or terete lobes, and sexual reproduction can be induced or not (apothecia present or not). Asexual lineages have been reported co-occurring with fertile species also in the genus *Usnea* (Articus *et al.* 2002; Wirtz *et al.* 2008; Saag *et al.* 2011), as also demonstrated in paper **III**.

In *Cetrelia*, the species are discriminated in combining lichen chemistry with morphology (Culberson & Culberson 1968; Randlane & Saag 1991). The latter is mainly characterized by type of reproductive structures. We found that the analysed *Cetrelia* species are relatively well circumscribed and justified – chemotypes formed monophyletic clades and included subclades that mostly correlated to morphotypes (paper **IV**). While the composition of lichen medullary substances proved to be a valuable diagnostic character in identifiying *Cetrelia* species, the reproductive mode of a species might not

always be sufficient in delimiting genetically, and possibly reproductively, isolated fungal lineages.

Genotyping specimens can be useful for separating morphologically similar taxa, and identifying morphological intermediates, juvenile forms, cryptic species, or sub-groups within clades. Specimen identification via DNA barcoding is increasingly popular and has proven successful also in some groups of lichens (Kelly *et al.* 2011; Divakar *et al.* 2016). DNA-based specimen identification to a species level is useful in a system of well-circumscribed taxa and a high-quality reference database (Seifert 2009; Begerow *et al.* 2010). DNA barcoding approach is promising for identifying macrolichens in areas where lichen diversity has been thoroughly investigated (results of paper **V**), while accurate, purely DNA-based identification of majority of crustose lichens – without considering morphological and chemical characters – seems far from reach in practically any area.

The official barcoding marker for fungi includes two highly variable spacer regions ITS1 and ITS2 with many mutations, but being present in a genome in multiple copies can potentially raise problems in its usage. Mechanisms, such as horizontal gene transfer (HGT), gene duplication, and hybridization between species can lead to paralogous copies of genes. Paralogous ITS copies distorting phylogenetic trees have repeatedly been reported in different organism groups (Harris & Crandall 2000; Hartmann *et al.* 2001; Wang & Yao 2005; Lindner *et al.* 2013) and recently, Xu *et al.* (2009) demonstrated a high level of intra-individual polymorphism in rDNA, including multiple functional genes, putative pseudo genes, and recombinants. They also found a systematic bias in the efficiency with which different sequences were amplified. In paper **I**, the two cryptic species in *Vulpicida* from ITS were not supported by other loci. The possibility of paralogous ITS copies in this group was investigated using cloning but no trace of paralogous copies was found and the hypothesis was rejected (**II**). However, in pyrosequencing the fungal ITS region of *Physcia adscendens,* a second ITS version with unidirectional transitional mutations all over the marker region was detected (**V**). We hypothesized that it could represent a rare divergent ITS allele or a pseudogene, propagated to a higher frequency due to a PCR bias.

While the ITS is considered a marker with great genetic variation and is most widely used to discriminate between fungal species, it might not be sufficient in separating species in young diverging complexes using the suggested 97% similarity threshold (see Blaalid *et al.* 2013). For example, in the genus *Usnea* sect. *Usnea* with limited number of mutations, pairwise distances of 135 ITS sequences, representing 17 *Usnea* morphospecies analysed in the paper **III**, fell approximately within the 3% dissimilarity limit. Therefore, a higher similarity threshold is recommended in this group, but the higher the expected similarity is set, the more sequencing mistakes distort the results. More than one barcoding marker could be used to better delimit the genetic clusters in young species complexes, and tree-based identification of the sequences or using Bayesian model comparison under the multispecies

coalescent model (implemented in the BPP program; Yang & Rannala 2016) could give more accurate results than the simple threshold-based identification method.

4.3. Lichen diversification and evolution on species, character, and genome level

Following the discussion in the section 4.1., it is likely that two of the lichen groups studied in this thesis – *Usnea* sect. *Usnea* and *Vulpicida* – represent recently diverged young species complexes. Such complexes are common in groups where rapid radiation takes place (Givnish 2015; Pease *et al.* 2016), and are characterized by conflicts between individual gene trees and gene trees vs. a species tree, few apomorphies compared to synapomorphic mutations, and fuzzy species boundaries that can reflect also in morphology. Such features are clearly evident in the genus *Usnea*, sect. *Usnea*, where weak phylogenetic structure, short branch lengths within trees, and conflicts between single-locus topologies were found (paper **III**). This presumption on the evolutionary history of the sect. *Usnea* is supported by the study from Kraichak *et al.* (2015a), who proposed that the genus *Usnea* is a hyper-diverse group by showing exceptionally high speciation rates compared with many other genera in the family Parmeliaceae. They explained the success of these lichens by key innovations (Sanderson & Donoghue 1996), such as the development of pendulous thallus with a central axis, and the production of usnic acid in the cortex to better exploit habitats and protect the photobiont from high radiation (McEvoy *et al.* 2006; Trest *et al.* 2015).

While in the *Usnea* dataset most of the conflicts were only weakly supported, within the delimited clades, and much of the incongruence between genes could come from gene tree estimation error due to mutational homoplasy rather than real conflicting phylogenetic signal, then in the genus *Vulpicida*, with many more accumulated apomorphies, the conflicts between single-gene tree topologies were more evident. Comparison of the branch lengths in the coalescent-based species tree inferences (Fig. 3; illustration from paper **II**) indicated that the diversification in the *Vulpicida* core group (*V. juniperinus*, *V. pinastri*, *V. tilesii*, and *V. tubulosus*) has been relatively recent compared to the evolution of *V. canadensis* and *V. viridis,* the endemics of North America from *Vulpicida*, or several other taxa in the cetrarioid core group.

The object of the third case study (paper **IV**) – *Cetrelia* – represents a different, lengthier evolutionary history. Here, long diverging times have allowed accumulation of apomorphies and genetic differentiation of species, and concerted evolution of lineages seems likely. *Cetrelia* is, however, especially interesting subject from a character evolution aspect. The genus has been a system for discussions on chemical and morphological evolution in lichens. Poelt (1970) proposed in his concept of "species pairs" the development of largely asexual "secondary species" from exclusively sexual "primary species".

Bowler & Rundel (1975) developed this theory by suggesting that chemical diversification occurred prior to the development of secondary, mostly asexual (sorediate and isidiate) reproductive mechanisms. According to this theory, the chemical differentiation in *Cetrelia* took place in hypothetical – and perhaps extinct – sexual species that gave rise to asexual species of corresponding chemistries through parallel morphological evolution. Randlane & Saag (1991) seconded to this theory and proposed an evolutionary scheme alternative to the Poelt's "species pairs" with species triplets and quartets. Culberson & Culberson (1976), however, did not see the necessity of sexual gene exchange for chemical diversification in *Cetrelia*, and presented a theory of parallel morphological and chemical evolution. They suggested that chemical diversification in *Cetrelia* can be explained by somatic loss mutations resulting in a progressive chemical evolution towards shorter side chains. Our results from paper **IV** contradict the earlier findings and suggest that the chemical evolution in *Cetrelia* occurred *prior* to the development of reproductive mechanisms, and the direction of chemical evolution seems to be, in contrary, towards more complex substances (Fig. 6; illustration from paper **IV**).

The chemotypes in *Cetrelia* are strongly concordant with species phylogeny (**IV**) and chemistry showed some correlation to genetic data also in the *Usnea* study (**III**). In *Usnea* sect. *Usnea* the chemotype with salazinic acid was distributed over the phylogeny of the studied species, while the thamnolic and squamatic acid chemotypes were restricted to a single clade (the 'florida-subfloridana' clade), suggesting a single evolutionary event, and norstictic acid appeared to have evolved independently multiple times (distributed in three distinct clades: 'parafloridana', 'fulvoreagens-glabrescens', and 'praetervisa'). Such patterns have been found in previous studies (e.g. Schmitt & Lumbsch 2004), while in some other groups lichen chemistry shows little correlation to genes (e.g. in *Thamnolia* Ach. ex Schaer.; Nelsen & Gargas 2009).

It is evident that development of a specific type of reproductive structures (soredia vs. isidia vs. lobulae vs. aptohecia or no structures) has occurred independently multiple times during the evolution of the fungal lineages in *Cetrelia* (**IV**), suggesting character parallel evolution. For example, distribution through soredia has developed in *Cetrelia* at least in four occasions according to the analysed dataset – in *C. monachorum*, *C. olivetorum*, *C. cetrarioides*, and in *C. chicitae*. Since our species set included mostly only secondary species with vegetative reproductive structures, and primary, i.e. apotheciate species were represented only by one specimen from two species, it remains to be investigated whether Poelt's theory (1970), that secondary species are derived from primary species, is valid in the genus *Cetrelia* or not.

Usnea florida and *U. subfloridana* are genetically indistinguishable species (at least in the loci studied in paper **III**) but differ in reproduction mode and ecological requirements. The apotheciate, *U. florida* morphotype (so-called "primary species") prefers old deciduous trees in areas with high humidity, and is considered threatened or near threatened in many European countries, while the sorediate, *U. subfloridana* morphotype ("secondary species") is less

ecologically-demanding and distributed widely over the northern hemisphere. Following the discussion in the chapter 4.2. on the utility of morphology and chemistry, it could be hypothesised that the development of sexual reproduction, that theoretically rapidly increases genetic diversity, is dependent on ecological conditions at least for some species (e.g. species complexes *Vulpicida juniperinus – V. tubulosus*, *Usnea florida – U. subfloridana*, *U. barbata – U. intermedia*), while other species, that do not show switching between sexual and vegetive modes, could reflect extreme tolerance or adaptiveness to different conditions. Alternatively, the selectivity between reproduction modes could be constrained due to evolutionary history of the respective genes. Many fungal populations seem to utilize mixed reproductive strategies with different proportions of sexuality and clonality (reviewed in Milgroom 1996). It has been suggested that sexual reproduction serves as a 'conservative' mechanism to preserve genome flexibility by increasing the probability of survival in a competitive and/or changing environment (Elliott & Harborne 1994; Otto & Gerstein 2006), while asexual propagation is considered successful evolutionary strategy for well-adapted genotypes in stable environments (Murtagh *et al.* 2000).

Ongoing evolutionary processes can be seen also on the genome level. In paper **V** we identified 12 specimens where nucleotide variation could represent intragenomic variation within ITS, or alternatively, allelic heterozygosity caused by differing nuclei within a single mycelium (Huang *et al.* 2010; Hyde *et al.* 2013). The latter is more likely when only two primary allele variants in approximatly equal ratios are observed (Lindner *et al.* 2013). Two more or less equally represented ITS paralogs were found in five, while one dominant ITS version together with relatively rare haplotypes was found in eleven species (see Table S3 in paper **V**). The latter includes representatives from a closely related species group in the genus *Usnea* (sect. *Usnea*), where low genetic variation, conflicting gene topologies resulting from ILS, and possible rapid radiation of species have been characterized (Truong *et al.* 2013; Kraichak *et al.* 2015a; **III**). Seven to eight variable sites were detected in the ITS reads of *Usnea intermedia* and *U. lapponica*, and four of these nucleotide positions were found variable also in Sanger sequences of these species. Thus, it could be that ILS is reflected in *Usnea* sect. *Usnea* not only in differing histories of genes (**III**), but also on a multi-copy gene level in the ITS sequences. High morphological divergence with low genetic variation has been demonstrated also in another lichenized fungal genus with pendulous thallus, in *Bryoria* Brodo & D. Hawksw., section *Implexae* (Velmala *et al.* 2014). Here, similarily, intragenomic variation with two alternate ITS versions was detected in pyrosequncing reads of *Bryoria capillaris* (**V**). It is possible that both groups, *Bryoria* sect. *Implexae* and *Usnea* sect. *Usnea*, are currently undergoing a rapid diversification process in response to a new ecological opportunity.

CONCLUSIONS

The main results and conclusions from this study are:

- Many groups and taxa of lichens are still in need of taxonomic revision. Two synonymizations were proposed in the publications of this thesis (*Vulpicida juniperinus* = *V. tilesii* = *V. tubulosus*, and *Usnea lapponica* = *U. substerilis*) and one new species (*Usnea parafloridana* K. Mark, Will-Wolf & Randlane) was described. However, further investigations are needed, as several other evolutionarily independent lineages appeared as cryptic species in the species delimitation analyses and multiple other nominal species probably represent intraspecific phenotypical variation.

- With conflicting information from different genes, application of inference methods incorporating multispecies coalescent model is suggested. In *Vulpicida* study, concatenating three conflicting genes resulted in erroneous results (**I**), but using the same 3-locus data in multispecies coalescent model suggested species history agreeing to the results of 5-locus data (**II**). Similarly, the multispecies coalescent method showed conclusive and stable clustering of the *Usnea* specimens while the commonly used species delimitation models failed to find consensus in the number of species and grouping of specimens (**III**).

- In the genus *Vulpicida*, four instead of six species are suggested. The current phenotype-based species *V. juniperinus*, *V. tubulosus* and *V. tilesii* could not be separated by any locus or any analysis, while the two species restricted to North America were distinct (**I** and **II**). The monophyly of the genus and the relationships between some cetrarioid species and genera is in need of further investigations.

- Only four out of seventeen morphologically circumscribed species from *Usnea* sect. *Usnea* were recovered monophyletic in genetic analyses (**III**), suggesting that several traditional, morphology-based species are in need of re-evaluation to specify their actual species boundaries. At the same time, the data suggest that using most diagnostic morphological characters together with branch anatomy and thallus chemistry are useful for delimiting some genetic lineages in sect. *Usnea*, while other clades have wide morphological variation, and many currently accepted diagnostic characters do not prove useful for delimiting these clades.

- The genus *Vulpicida* core group (*V. juniperinus* complex and *V. pinastri*; **I** and **II**) and the genus *Usnea* sect. *Usnea* (**III**) seem to represent recently diverged young species complexes. The genes and species evolving under rapid radiation show a high degree of gene incongruence resulting from

incomplete lineage sorting in these groups. Within pyrosequencing reads of two *Usnea* specimens (*Usnea intermedia* and *U. lapponica*), intragenomic variation of ITS sequences was detected (**V**) and thus, most likely, incomplete lineage sorting is reflecting in *Usnea* sect. *Usnea* not only in differing histories of genes (**III**), but also on a multi-copy gene level in the ITS sequences.

- The genus *Cetrelia*, on the other hand, represents a lengthier evolutionary history: the species are relatively well circumscribed and their diagnostic characters are justified – chemotypes form monophyletic clades with subclades corresponding generally to morphotypes (**IV**). Thus, treatment of taxa in *Cetrelia* merely by their morphotype is not phylogenetically acceptable. However, some of the studied species were polyphyletic, suggesting that reproductive mode of a species might not always be sufficient in delimiting genetically, and possibly reproductively, isolated fungal lineages.

- Species identification via DNA barcoding is useful in a system of well-circumscribed taxa and a high-quality reference database (**V**). For accurate species identification and phylogeny estimation it is necessary to be aware of the factors that can distort phylogenetic trees and bias DNA barcoding results, such as sequencing errors, genetic variation in population (=appropriate identification threshold), multiple ITS versions in a genome, and precence of mixed lichen-forming fungi when sequencing environmental samples. The advantages of next generation sequencing can be used to investigate these factors and help to accelerate reference database construction when species are difficult to sequence in Sanger technique.

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SUMMARY IN ESTONIAN

Liikide lahknemine ja nende piiritlemine valitud lihheniseerunud seente rühmades perekonnas Parmeliaceae (Ascomycota)

Süstemaatika kui teadusharu kaks peamist tegevusala on elusloodu ühikuteks jagamine ja neist süsteemi koostamine (kitsamalt taksonoomia) ning süsteemi ühikute vaheliste evolutsiooniliste suhtete uurimine (fülogeneetika). Elusloodu süsteemi põhiühik on liik, kuid seni pole õnnestunud rakendada ühesuguseid standardiseeritud liigikriteeriume kogu elusloodu mitmekesisusele. Liikide piiritlemise kriteeriumid erinevad tihti suurel määral, sõltudes neid rakendava teadlase hinnangust tunnuste diagnostilisusele. Püsiva taksonoomia arendamine on oluline paljudes valdkondades, kus uuringute objektiks on liigid või teised taksonid (sh ökoloogias ja looduskaitses) või kus tegeletakse tunnuste evolutsiooni väljaselgitamisega (sh võrdlevas bioloogias ja genoomikas). Ebapüsiv või ekslik taksonoomia võib põhjustada isegi mittevajalikke rahalisi kulutusi, nt kui kaitsealuste, invasiivsete või patogeensete liikide piiritlemine või määramine on väär. Pärilikkusaine ehk DNA uuringud võimaldavad liigikontseptsioonide ühtlustamist erinevates rühmades, rakendades liikide monofüleetilisuse kriteeriumit metapopulatsioonide liinidele. Liigid ei ole fikseeritud, nad on pidevas muutumises erinevate evolutsiooniliste protsesside tõttu. Liinides toimub pidev uute alleelide tekkimine ning kadumine, ning sõltudes valiku survest, evolutsioneeruvad erinevad liinid eri kiirusega. See on ka üks põhjus, miks ühetaolist liikide monofüleetilisuse ning geneetilise erisuse piirmäära ei saa rakendada. Loomulike monofüleetiliste liikide piiritlemine sõltub mitmetest erinevatest teguritest, sh uuritavale rühmale mõjuvatest evolutsiooniprotsessidest. Vanad liigid on geneetiliselt hästi eristunud, kuna neis on aja jooksul kogunenud rohkelt apomorfseid tunnuseid, samas kui noored liigid võivad isegi pikema aja jooksul olla mittemonofüleetilised mittetäieliku liinide sorteerumise tõttu (*incomplete lineage sorting, ILS*). Mittetäielik liinide sorteerumine tähendab, et ühe eellase erinevate järglaste erinevad geeniversioonid püsivad järglastes liikide eristumise protsessi vältel. See tuleneb asjaolust, et kui liikide vahel on väike geenivoog, siis sugulasliikide geenide paar eristub juba enne vastavat liigiteket. ILS on eriti tavaline lähedalt suguluses olevate liikide rühmades, sest geenide monofüleetilisus on liigitekke üks viimaseid faase. Kuna alleelide koalestseerumine ei toimu erinevates geenides üheaegselt, siis mittetäielik liinide sorteerumine on tuvastatav sellistes rühmades vastuoluliste geenipuude näol. Selliste geenide signaal, mis ei peegelda liikide tegelikku evolutsiooni, võib anda valesid tulemusi nii liikide määramisel kui ka liikide sugulussuhete hindamisel. Mitme erineva geeni infot kasutades vähendame juhusliku vea riski. Praegu arendatakse uusi analüütilisi meetodeid, mis põhinevad liikide koalestseerumise mudelil, ning mis rekonstrueerivad liigipuu, võttes aluseks ka eri kujuga geenipuid. Liigipuu meetod võimaldab arvesse võtta lookuste erine-

vaid evolutsioonikiirusi ning populatsioonigeneetikaga seotud protsesse (sh liinide sorteerumist).

Kuigi samblikud on sümbiootilised organismid, koosnedes vähemalt kahest komponendist – saprotroofsest seenest (mükobiont) ja fotosünteesivast rohevetikast ja/või tsüanobakterist (fotobiont), siis süstemaatiliselt käsitletakse samblikke seentena. Lihheniseerunud seened on mittemonofüleetiline rühm seeni, millest suur enamus on kottseened (Ascomycota, 98%) ning vaid väike osa kuulub kandseente hulka (Basidiomycota; 2%). Hinnanguliselt enam kui 20% kõigist seeneliikidest on võimelised moodustama samblikutallust. Samblike hulgas on mitmeid bioindikaatoreid keskkonna kvaliteedi hindamiseks, kuid liikide eristamine mitmetes samblikurühmades on jätkuvalt keeruline. Traditsiooniliselt on samblikke piiritletud nende morfoloogiliste tunnuste alusel. Lisaks on kasutatud ka infot samblike sekundaarainetest, nende levikust ja ökoloogilistest eelistustest (nt substraadieelistusest). Molekulaarsete meetodite kasutuselevõtuga on samblike määramine ja taksonoomia uurimine liikunud paljuski nö mikroskoobi ja binokulaari alt molekulaarlaborisse, kus seente DNA järjestuste fragmente kasutatakse näiteks samblike määramiseks (DNA triipkoodistamise meetod), monofüleetiliste liikide piiritlemiseks, evolutsiooniliste suhete uurimiseks eri tasandite taksonitel, ja üldisemate nähtuste (nt lihheniseerumise kui eluviisi evolutsiooni) selgitamiseks. Mükobiondi sekveneerimine Sangeri meetodil võib osutuda problemaatiliseks, kui erinevad samblikku moodustavad seened või mitmesugused saprofüütsed, endofüütsed ning parasiitsed seened elavad uuritava samblikuga koos. Pürosekveneerimine võimaldab kergesti tuvastada seeneliikide mitmekesisust keskkonnaproovidest, kuid seni vaid vähesed tööd on rakendanud seda samblike triipkoodistamisel.

Käesoleva doktoritöö uuringute keskmes olid kolme samblikke moodustava seeneperekonna evolutsioonilised suhted ning liikide piiritlemine, kasutades selleks morfoloogilisi, keemilisi, anatoomilisi ning geneetilisi tunnuseid. Läbiviidud fülogeneetiliste uuringute objektid – rebasesamblikud (perek. *Vulpicida*), habesamblikud (perek. *Usnea*) ning helksamblikud (perek. *Cetrelia*) – kuuluvad kõik kottseente sugukonda Parmeliaceae. See sugukond on tõenäoliselt tuntuim ning enim uuritud samblikke moodustavate seente sugukond, olles ühtlasi ka kõige suurem, sisaldades üle 2700 liigi ligikaudu 80-s erinevas perekonnas. Perekonnad *Cetrelia*, *Usnea* ja *Vulpicida* valiti uuringuobjektideks hägusate liigipiiride või ebaselge liigikontseptsiooni tõttu.

Helksamblikud on võrdlemisi suure lehtja tallusega ning neid iseloomustab mitmete keemiliselt lähedaste samblikuainete sisaldus. Helksamblikud on levinud üle põhjapoolkera, kuid enamus liike, millest mitmed on väga haruldased, kasvab ainult Ida- ja Kagu-Aasias. Taksonoomiliselt pakub huvi see, et liigid on perekonnas kirjeldatud peamiselt nende kemo- ja morfotüüpide kombinatsioonina. Jätkuvalt arutatakse, kas sama morfotüübi erinevaid kemotüüpe võib nimetada eri liikideks või peaks neid käsitlema varieteedi või muu alama taksoni tasemel. *Cetrelia* on huvitav ka tunnuste evolutsiooni seisukohast. On leitud, et helksamblikes toodetavad sekundaarsed samblikuained on kõik omavahel lähedalt seotud orsinoolsed depsiidid, mis moodustavad

omavahel liigispetsiifilisi samblikuainete rühmi ehk kemosündroome. Umbes nelikümmend aastat tagasi pakuti välja hüpotees, et samblikuained helk-samblikes on evolutsioneerunud paralleelselt morfoloogiliste tunnustega ning sealjuures on samblikuainete keemiline areng toimunud ainete lihtsustumise suunas.

Habesamblikud on ühed tuntuimad ning perekonna tasemel suhteliselt lihtsasti tuvastatavad oma rohekaskollase habet-meenutava tallusega, mille peened harud sisaldavad keskjuhet. Samas on see üks suurimatest perekondadest sugukonnas Parmeliaceae, sisaldades umbes 350 liiki ja väga paljude *Usnea* liikide üksteisest eristamine on keeruline isegi spetsialistidele nende varieeruva morfoloogia, keemia ning raskesti määratavate diagnostiliste tunnuste tõttu. Keeruline liikide määramine peegeldub ka taksonoomias – üle maailma on kirjeldatud ligikaudu 770 erinevat *Usnea* taksonit, millest umbes pooled on tõenäoliselt sünonüümid. Seevastu rebasesambliku perekonda kuulub vaid kuus varasemalt kirjeldatud liiki, millest kolm kasvavad ka Eestis. *Vulpicida* liigid on kergesti määratavad oma kollase, valdavalt lehtja talluse ja erkkollase südamikukihi värvuse järgi, kuid just Eesti läänesaartelt leitud morfoloogilised hübriidid tekitasid autoris huvi nende liikide geneetilise erisuse testimiseks.

Käesoleva doktoritöö peamisteks eesmärkideks oli (1) hinnata fülogeneetilisi suhteid ja liikide piire perekondades *Cetrelia*, *Usnea* (sektsioonis *Usnea*) ja *Vulpicida* kasutades selleks DNA järjestuse andmeid mitmest lookusest ning uudseid koalestsentsil põhinevaid liigipuu konstrueerimise ja liikide piiritlemise mudeleid lisaks tavalistele geenipuu ja liidetud geenide (*gene concatenation*) puu meetoditele, (2) võrrelda geeniandmestikul põhinevaid tulemusi praeguste morfoloogial põhinevate taksonitega ning hinnata kasutatavate diagnostiliste tunnuste praktilistust, (3) hinnata ITS markeril põhinevat lihheniseerunud seente liikide määramise täpsust, ning (4) selgitada mõningaid uuritavate liikide lahknemisega seotud protsesse liigi, tunnuse ning genoomi tasandil. Uuring püstitati neljaetapilisena – fülogeneetilsed uuringud perekondades *Vulpicida*, *Usnea* ja *Cetrelia* ning pürosekveneerimise rakendamine ning testimine samblike triipkoodistamisel.

Uuringu tulemused näitasid, et paljud lihheniseerunud seente liigid ei esinda jätkuvalt loomulikke, evolutsiooniliselt eristunud liine ning seetõttu vajavad taksonoomilist korrastamist. Rebasesambliku perekonnas leidsime kuue liigi asemel neli geneetiliselt eristunud rühma. Samblikutalluse üldise haabituse ja substraadieelistuste alusel kirjeldatud *Vulpicida juniperinus*, *V. tubulosus* ja *V. tilesii* osutusid uuringute järgi üheks liigiks, samas kui ülejäänud kolm liiki esindasid geneetiliselt eristunud liine (**I** ja **II**). Pakkusime välja, et rebasesambliku seniseid iseseivaid liike *Vulpicida tubulosus* ja *V. tilesii* tuleb käsitleda liigi *V. juniperinus* sünonüümidena (**II**). Uuritud 17-st sektsiooni *Usnea* liigist vaid neli osutusid geenianalüüsides monofüleetilisteks (**III**). Selles töös leidsime, et laik-habesamblik *Usnea substerilis* on lapi-habesambliku *U. lapponica* sünonüüm (**III**). Mitmed teised morfoloogia alusel kirjeldatud habesamblikud on tõenäoliselt samuti liigisisesed fenotüübid, kuid taksonoomiliste

muudatuste soovitamiseks on vajalikud edasised uuringud enamate eksemplaridega. Selles töös kirjeldasime uue habesambliku liigi – *Usnea parafloridana* – mille eristamine sarnastest liikidest on võimalik morfoloogiliste, keemiliste ja geneetiliste andmete kombineerimisel. Hetkel teadaolevate andmete põhjal on see liik teada Põhja-Ameerikast, vaid ühest USA osariigist (Wisconsinist), kuid kuna tegemist on semikrüptilise liigiga ehk pelgalt morfoloogia põhjal on teda sarnastest liikidest keeruline eristada, siis võib edaspidi selguda, et ta on levinud ka suuremal alal. Seega, mitmed klaadid sektsioonis *Usnea* on eristatavad kombineerides teatud morfoloogilisi, anatoomilisi ja keemilisi tunnuseid, samas kui osad tunnused (nt üldine haabitus ja paljunemisviis) ei ole klaadide eristamiseks kasutatavad. Helksambliku perekonnas on liigid seevastu hästi eristunud ja nende tunnused asjakohased. Perekonna *Cetrelia* kemotüübid moodustasid geneetiliste andmete alusel monofüleetilisi klaade, mis sisaldasid enamasti morfotüüpidele vastavaid alamklaade (**IV**). Tunnuse evolutsiooni seisukohast on antud tulemus huvitav selle poolest, et kemotüübid perekonnas *Cetrelia* lahknesid enne morfotüüpide arengut ning selgus, et paljunemisviisi areng on toimunud paralleelselt erinevates rühmades. Seega, kemotüüpide ühendamine ühe morfoliigi alla – praegu kohati kasutusel – ei ole perekonnas *Cetrelia* põhjendatud. Lisaks, mõned senised liigid osutusid ka polüfüleetilisteks, viidates, et alati ei pruugi kemo- ja morfotüüpide määramine olla piisav tuvastamaks geneetiliselt eristunud populatsioone.

Perekonna *Vulpicida* tuumikrühm (st. *V. juniperinus* liikide kompleks ja *V. pinastri*) ning perekonna *Usnea* sektsioon *Usnea* on tõenäoliselt hiljuti lahknenud noorte liikide kompleksid (**II** ja **III**), samas kui perekonda *Cetrelia* iseloomustavad liikide pikem ajalugu ning liinide selge lahknevus (**IV**). Geene ja liike, mis arenevad liinide suure lahknevuse all (*rapid radiation*), iseloomustab liinide mittetäielik sorteerumine. See nähtus avaldus neis rühmades konfliktsete geenipuudena ning geenipuude ja liigipuu erinevates topoloogiates (**I**, **II** ja **III**). Uuringutest järeldus, et liikide koalestseerumise mudelil põhinevad liikide piiritlemise ja liigipuude analüüsid peaksid olema eelistatud konfliktse geeniinfoga rühmades. Lisaks tuvastasime genoomisisese varieerumise ITS järjestustes kahel pürosekveneerimise abil analüüsitud *Usnea* eksemplaril (**V**). See viitab, et suure tõenäosusega ei peegeldu liinide mittetäielik sorteerumine mitte ainult konfliktsete geenide tasemel populatsioonis, vaid ka ühe isendi genoomi tasandil mitmekoopialises geenis.

Liikide määramine DNA triipkoodistamise meetodil on praktiline siis, kui on selgelt piiritletud taksonid ja olemas kõrge kvaliteediga referentsandmebaas (**V**). Sekveneerimisvead, liigi geneetiline varieeruvus uuritavas markeris, millest sõltub otseselt liigi identifitseerimiseks sobiv sarnasusmäär, ühe geeni genoomisisene varieeruvus (nt paraloogsed koopiad) ning sugulasliikide võimalik kooselu kimäärse organismina – need kõik võivad moonutada korrektse puu rekonstrueerimist ja mõjutada DNA triipoodistamisel põhinevate uuringute tulemusi. Pürosekveneerimine võimaldab neid nähtusi uurida ning kiirendab DNA järjestuste referentsandmestiku koostamist, viimast eelkõige liikide puhul, mille sekveneerimine Sangeri meetodil osutub problemaatiliseks.

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PUBLICATIONS

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Mark, K., Cornejo, C., Keller, C., Flück, D., & Scheidegger, C. (2016) Barcoding lichen-forming fungi using 454 pyrosequencing is challenged by artifactual and biological sequence variation. *Genome* (in press); doi: 10.1139/gen-2015-0189

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2011–2016 Doktoriõpingud botaanikas ja mükoloogias, Tartu Ülikool
Dissertatsioon: *Liikide lahknemine ja nende piiritlemine valitud lihheniseerunud seente rühmades perekonnas Parmeliaceae (Ascomycota)*
Juhendajad: Tiina Randlane ja Lauri Saag

2009–2011 Magistriõpinugud bioloogias, Tartu Ülikool
MSc, juuni 2011; magistritöö: *Lihheniseerunud seeneliikide Vulpicida juniperinus ja V. tubulosus (Parmeliaceae, Ascomycota) eristamine DNA tunnuste alusel*; juhendaja: Lauri Saag

2006–2009 Bakalaureuseõpingud ökoloogias ja elustiku kaitses, Tartu Ülikool
BSc, juuni 2009; bakalaureusetöö: *Liigikontseptsioonid ning -kriteeriumid mittesuguliste lihheniseerunud seente süstemaatikas perekonna Lepraria näitel*; juhendajad: Andres Saag ja Lauri Saag

Erialane töökogemus:

2011–2015 Tudengite osaline juhendamine TÜ kursustel Fungistiks välipraktika ja Mükoloogia ja lihhenoloogia praktikum

2011–2012 Spetsialist (0.70): Tartu Ülikool, Ökoloogia ja maateaduste instituut

Keelteoskus: eesti (emakeel), inglise (väga hea), saksa (baasteadmised)

Peamised uurimisvaldkonnad: bio- ja keskkonnateadused; biosüstemaatika ja süstemaatiline bioloogia; evolutsiooniline bioloogia; seente bioloogia ja mitmekesisus; lihheniseerunud seente bioloogia, molekulaarne süstemaatika, taksonoomia, kemotaksonoomia, morfoloogia ja mitmekesisus

Teadusartiklid:

Mark, K., Cornejo, C., Keller, C., Flück, D., ja Scheidegger, C. (2016) Barcoding lichen-forming fungi using 454 pyrosequencing is challenged by

artifactual and biological sequence variation. *Genome* (trükis); doi: 10.1139/gen-2015-0189

Mark, K., Saag, L., Leavitt, S. D., Will-Wolf, S., Nelsen, M. P., Tõrra, T., Saag, A., Randlane, T., ja Lumbsch, H. T. (2016) Evaluation of traditionally circumscribed species in the lichen-forming genus *Usnea* (Parmeliacea, Ascomycota) using six-locus dataset. *Organisms Diversity & Evolution* (trükis); doi:10.1007/s13127-016-0273-7

Divakar, P.K., Crespo, A., Wedin, M., Leavitt, S.D., Hawksworth, D.L., Myllys, L., McCune, B., Randlane, T., Bjerke, J.W., Ohmura, Y., Schmitt, I., Boluda, C.G., Alors, D., Roca-Valiente, B., Del-Prado, R., Ruibal, C., Buaruang, K., Núñez-Zapata, J., Amo de Paz, G., Rico, V.J., Molina, M.C., Elix, J.A., Esslinger, T.L., Tronstad, I.K.K., Lindgren, H., Ertz, D., Gueidan, C., Saag, L., **Mark, K.**, Singh, G., Dal Grande, F., Parnmen, S., Beck, A., Benatti, M.N., Blanchon, D., Candan, M., Clerc, P., Goward, T., Grube, M., Hodkinson, B.P., Hur, J.-S., Kantvilas, G., Kirika, P.M., Lendemer, J., Mattsson, J.-E., Messuti, M.I., Miadlikowska, J., Nelsen, M.P., Ohlson, J.I., Pérez-Ortega, S., Saag, A., Sipman, H.J.M., Sohrabi, M., Thell, A., Thor, G., Truong, C., Yahr, R., Upreti, D.K., Cubas, P., ja Lumbsch, H. T. (2015) Evolution of complex symbiotic relationships in a morphologically derived family of lichen-forming fungi. *New Phytologist* **11** (208): 1217–1226; doi: 10.1111/nph.13553

Saag, L., **Mark, K.**, Saag, A., ja Randlane, T. (2014) Species delimitation in the lichenized fungal genus *Vulpicida* (Parmeliaceae, Ascomycota) using gene concatenation and coalescent-based species tree approaches. *American Journal of Botany* **101** (12): 2169–2182; doi: 10.3732/ajb.1400439

Mark, K., Saag, L., Saag, A., Thell, A., ja Randlane, T. (2012) Testing morphology-based delimitation of *Vulpicida juniperinus* and *V. tubulosus* (Parmeliaceae) using three molecular markers. *The Lichenologist* **44** (6): 752–772; doi: 10.1017/S0024282912000448

Konverentsiettekanded:

IAL8 Helsinkis, Soomes, augustis 2016 – (1) *Untangling Usnea: Multi-locus concatenated and coalescent-based analyses reveal recent diversification history and clusters of mixed morphospecies in the section Usnea*" (suuline), (2) "*Barcoding lichen-forming fungi using 454 pyrosequencing*" (poster), (3) "*Phylogeny and species delimitation in the lichen genus Cetrelia*" (kaas-autor)

iBOL6 Guelphis, Kanadas, augustis 2015 – "*Barcoding Swiss lichens and associated fungal communities using 454 pyrosequencing*" (suuline)

SwissBOL konverents Neuchatêl'is, Šveitsis, oktoobris 2014 – "*Bar-coded amplicon 454 pyrosequencing for barcoding the mycobiont in Swiss lichens*" (suuline)

IMC10 Bangkokis, Tais, augustis 2014 – (1) "*Bar-coded amplicon 454 pyrosequencing for barcoding mycobiont – photobiont interactions in Swiss lichens*" (suuline), (2) "*Evaluation of traditionally circumscribed species in*

the lichen-forming genus Usnea (Parmeliaceae, Ascomycota) using a six-locus dataset" (poster)

XIX Balti mükoloogide ja lihhenoloogide sümpoosion Šķēdes, Lätis, septembris 2014 – "*Phylogeny and species delimitation of Vulpicida (Parmeliaceae, Ascomycota)*" (suuline)

20. Põhjamaade lihhenoloogia ühingu (NLF) kokkutulek Vadstenas, Rootsis, augustis 2013 – "*Phylogeny and species delimitation of Vulpicida (Parmeliaceae, Ascomycota)*" (suuline)

Down to Earth konverents Tallinnas, Eestis, mais 2013 – "*The tricky lichen genus Vulpicida: phylogeny and species delimitation*" (poster)

IAL7 Bangkokis, Tais, jaanuaris 2012 – "*Phylogeny of the genus Vulpicida and delimitation of the species*" (kaasautor)

Eesti mükoloogiaühingu koosolek Actiones Tartus, Eestis, detsembris 2011 – "*Morfoloogia alusel kirjeldatud liikide Vulpicida juniperinus ja V. tubulosus eristamine kolme DNA lookuse põhjal*" (suuline)

Teaduspreemiad, uurimistoetused ja stipendiumid:

2016 Sihtasutus Archimedes Kristjan-Jaagu reisistipendium 8. Rahvusvalise Lihhenoloogia Assotsiatsiooni sümpoosionil osalemiseks, Helsinkis

2015 Ajakirja Genome tudengi suulise ettekande auhind kõrgetasemelise teadustöö eest 6. Rahvusvahelisel elusllooduse triipkoodistamise konverentsil (iBOL6) Guelph'is, Kanadas

2015 Reisipreemia Ontario bioloogilise mitmekesisuses instituutilt iBOL6 konverentsil osalemiseks

2014 Parimate tudengite postri auhind 10. Rahvusvahelisel mükoloogia kongressil (IMC10), Bangkokis, Tais

2013 Sciex-NMSch stipendium 12 kuu jooksul teadustöö läbiviimiseks Sciex doktorandina Šveitsi Föderaalses Metsa-, Lume- ja Maastiku-uurimise Instituudis (WSL) professor Christoph Scheideggeri juhendamisel, teemal "Samblike sümbiontide triipkoodistamine: varieeruvus mükobiondi ja fotobiondi suhetes geograafilisel ning ökoloogilisel gradiendil"

2012 Sihtasutus Archimedes DoRa 6 programmi tudengi stipendium kolmekuuliseks uurimistegevuse väljaõppeks teadusasutuses The Field Museum of Natural History, Chicagos, Dr. H. Thorsten Lumbsch'i juhendamisel

2012 Tudengi reisiauhind Encyclopedia of Life (EOL) Biosünteesi keskuse pool EOL Parmeliaceae kokkusaamisel osalemiseks, Tais

2011 Sihtasutus Archimedes Kristjan-Jaagu reisistipendium 7. Rahvusvalise Lihhenoloogia Assotsiatsiooni sümpoosionil osalemiseks, Tais

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