Towards a better understanding of the systematics and diversity of *Cortinarius*, with an emphasis on species growing in boreal and temperate zones of Europe and North America

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Academic dissertation

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Cover photograph: *Cortinarius* heaven in Fairbanks, Alaska, U.S.A. 2011. One of the still unstudied collections of *Cortinarius* section *Calochroi* we made during the extraordinary fruiting season in 2011.

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"If there is no sequence, it is a rumour"

This thesis is based on the following papers:

I Niskanen T, Kytövuori I, Liimatainen K 2011: *Cortinarius* sect. *Armillati* in northern Europe. – Mycologia 103(5): 1080–1101. doi:10.3852/10-350.

II Niskanen T, Kytövuori I, Liimatainen K, Lindström H 2013: *Cortinarius* section *Bovini* (Agaricales, Basidiomycota) in northern Europe, conifer associated species. – Mycologia 105(4): 977–993. doi: 10.3852/12-320.

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IV Niskanen T, Liimatainen K, Ammirati JF, Hughes K 2013: *Cortinarius* section *Sanguinei* in North America. – Mycologia 105(2): 344–356. doi: 10.3852/12-086.

V Niskanen T, Liimatainen K, Ammirati JF 2013: Five new *Telamonia* species (*Cortinarius*, Agaricales) from western North America. – Botany 91: 478–485. doi: 10.1139/cjb-2012-0292.

VI Liimatainen K, Niskanen T, Dima B, Kytövuori I, Ammirati JF, Frøslev T 2013: The largest type study of Agaricales species to date: bringing identification and nomenclature of *Phlegmacium* (*Cortinarius*, Agaricales) into the DNA era. – Persoonia (submitted).

These are referred to in the text by their Roman numerals.

The following table shows the main contributions (%) of authors to the original papers or manuscripts.

	I	**II**	**III**	**IV**	**V**	**VI**
Original idea	KL 33, TN 33, IK 33	KL 30, TN 30, IK 30, HL 10	KL 50, TN 50	KL 33, TN 33, JFA 33	KL 33, TN 33, JFA 33	KL 50, TN 50
Morphology	TN 60, IK 40	IK 57, TN 38, HL 5	TN 100	TN 90, JFA 10	TN 90, JFA 10	IK 70, TN 30
Molecular data	KL 100	KL 100	KL 100	KL 97, KH 3	KL 100	KL 70, BD 25, TF 4, AT 0.5, DB 0.5
Phylogenetic analyses	KL 100	KL 100	KL 100	KL 100	KL 100	KL 100
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Initials refer to authors of the paper in question:

KL = Kare Liimatainen
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JFA = Joseph Ammirati
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HL = Håkan Lindström
TF = Tobias Frøslev
KH = Karen Hughes
AT = Andy Taylor
DB = Dimitar Bojantchev

Introduction

The genus *Cortinarius*

Cortinarius (Pers.) Gray is the largest genus of Agaricales with a global distribution and thousands of species. *Cortinarius* species are important ectomycorrhizal fungi associated with different trees and shrubs, belonging to the order Fagales, families Pinaceae, Salicaceae, Myrtaceae, Dipterocarpaceae, Caesalpiniaceae, Cistaceae, Rhamnaceae, and Rosaceae as well as a few herbaceous plants in the Cyperaceae (Moser & Horak 1975, Moreno & Esteve-Raventós 1997, Garnica et al. 2005). Owing to their often narrow ecological preferences and sensitivity to environmental change, many *Cortinarius* species have been used as indicator species for valuable natural environments, e.g. in Sweden and Denmark (Vesterholt 1991, Hallingbäck and Aronsson 1998). Recently it also was suggested that they have a key role in the carbon cycling of boreal forests (Bödeker et al. 2011).

Cortinarius produces conspicuous, small to large basidiomata. Most species have a cobweb-like inner veil protecting the young lamellae – the cortina, from which the generic name is derived. They have brown ornamented spores giving a cinnamon brown to rusty brown spore deposit. The name *Cortinarius* was first used at the genus level by Fries (1836-1838). Since then many mycologists have contributed in the systematics of the genus. Most of the major studies in *Cortinarius* have dealt with North American and especially European species, while the species of southern hemisphere are less studied (Moser & Horak 1975, Cleland 1976, Garnica et al. 2002, Gasparini & Soop 2008). In Europe the most extensive studies have been done by Fries (e.g. 1821, 1836-38, 1851) from Sweden, Henry (e.g. 1958, 1981) and Bidaud et al. (e.g. 1992, 2010) from France, Moser (e.g. 1960, 1969-1970, 1983) mainly from Austria, Orton (1955, 1958, 1983) from Great Britain, Høiland (1984), Brandrud et al. (e.g. 1989, 2012), Frøslev et al. (e.g. 2006, 2007) and Niskanen et al. (e.g. 2009, 2012) mainly from Northern Europe, and Consiglio et al. (e.g. 2003, 2006), Ortega et al. (2008) and Suárez-Santiago et al. (2009) from mediterranean area. Selected papers of contributors to *Cortinarius* systematics in North America include Peck (1873; also see Gilbertson 1962), Kauffman (1918, 1923, 1932), Smith (1939, 1942, 1944), Ammirati (1972), Moser et al. (1995), Moser and Ammirati (1996, 1999), Liu et al. (1997), Garnica et al. (2009), Bojantchev (2011a,b), and Ammirati et al. (2013).

Several infrageneric classifications, based on morphology, have been proposed, i.e. Moser (1983) recognized the subgenera *Cortinarius*, *Leprocybe*, *Myxacium*, *Phlegmacium*, *Sericeocybe*, and *Telamonia*, but regarded *Dermocybe* (Fr.) Wünsche as a separate genus. Brandrud et al. (1989) divided the genus in four subgenera *Cortinarius*, *Myxacium*, *Phlegmacium*, and *Telamonia*. Bidaud et al. (1994) recognized *Cortinarius*, *Dermocybe*, *Myxacium*, *Phlegmacium*, *Telamonia* and *Hydrocybe*. From southern hemisphere also subgenera *Icterinula*, *Cystogenes* and *Paramyxacium* have been recognized (Moser and Horak 1975).

Phylogenetic classification of *Cortinarius*

Liu et al. (1995, 1997), Seidl and Liu (1998), Chambers et al. (1999), Høiland and Holst-Jensen (2000), and Seidl (2000) were the first to include DNA sequence data for phylogenetic studies in *Cortinarius*. Although, the focus of the study by Liu et al. (1995, 1997) was in subgenus *Dermocybe*, that of Chambers et al. (1999) in molecular identification of 10 co-occuring *Cortinarius* species in southeastern Australian sclerophyll forests, and that of Seidl and Liu (1998)

and Seidl (2000) in subgenus *Myxacium*, the results already suggested that the traditional infrageneric groups were at least partly artificial and should be reevaluated. Furthermore, Liu et al. (1997) proposed that *Dermocybe* should be treated as a separate genus and i.e. *Telamonia* and *Phlegmacium* could be monophyletic. The results of Chambers et al. (1999) and Høiland and Holst-Jensen (2000) supported the monophyly of subgenus *Telamonia* but all the other species including a monophyletic clade /Dermocybe were placed in /Cortinarius. In addition, Høiland and Holst-Jensen (2000) found that *Rozites caperatus* (Pers.) P. Karst. was included in /Cortinarius. The studies of Peinter et al. (2001, 2002) further showed that circumscription of *Cortinarius* needed to be emended. Their results suggested that the sequestrate taxa, *Thaxterogaster, Quadrispora, Protoglossum* and *Hymenogaster p.p.* as well as *Cuphocybe, Rapacea* and species of *Rozites* are not monophyletic and should be included in *Cortinarius*.

The studies of Peintner et al. (2004) and Garnica et al. (2005) are thus far the most extensive ones covering all classical groups of *Cortinarius*. The sampling is biased toward Northern hemisphere taxa but includes also species from South America, Australia, Tasmania and New Zealand. Results show that *Cortinarius* consists of many lineages, although some with low support, but the relationships among these clades could not be resolved. The lineages corresponded to some extent with classical groupings but mainly at the section rather than the subgeneric level highlighting the need for reevaluation of traditional groupings with denser taxon sampling and several gene regions.

Most of the studies above are based on ITS sequences. In Peintner et al. (2002, 2004) and Garnica et al. (2005) LSU sequences also were used. Thus far, only Frøslev et al (2005) have tested other gene regions, RNA polymerase II genes *rpb1* and *rpb2*, for inferring the phylogeny of *Cortinarius*. Their study was focused on subgenus *Phlegmacium p.p*. Results showed that *rpb1* and *rpb2* increased resolution and nodal support in phylogenetic analyses and indicated that both genes have the potential for resolving phylogenetic problems at several taxonomical levels in *Cortinarius*. Frøslev et al (2005) also concluded that phylogenetic relationships based on analysis of ITS alone are only reliable for nodes receiving high support. It is important to realize that this statement only concerns relationships of species, sections, subgenera, etc., not the delimitations of species.

Species delimitation and barcoding

Until the beginning of the DNA era the identification and classification of *Cortinarius* species relied primarily on morphological, chemical, and ecological characteristics (morphological species concept, e.g. Kuyper 1988). Due to the relatively simple structure of fungus reproductive structures, the morphological characteristics suitable for classification are fewer than in most animals and plants. In addition, very few characters are discontinuous and a number are convergent, for example, basidiospore shape and size. Therefore, the application of the morphological species concept has led to very different results in the same groups by different authors.

Biological species concepts have not been developed for *Cortinarius*. Some species have been grown in culture, but basidiospores have not been germinated to date and no mating studies have been done (Liu et al. 1997). The strict phylogenetic species concept employing several hypervariable genetic markers as in e.g. (Taylor et al. 2000) also has not been applied to *Cortinarius*.

In the genus *Cortinarius* ITS regions are the only DNA regions used for species delimitation and identification (e.g. Kytövuori et al. 2005, Ammirati et al. 2007, Frøslev et al. 2007, Ortega et al. 2008, Garnica et al. 2009, Niskanen et al. 2009). RNA polymerase II genes, *rpb1* and *rpb2*, were

tested by Frøslev et al. (2005) for infrageneric classification in *Cortinarius*, but they concluded that the species level results were in concordance with the results from the ITS regions and provided no further resolution.

The classification of *Cortinarius* species based on ITS regions mostly has been supported by morphological data (e.g. Moser and Peintner 2002a, Kytövuori et al. 2005, Frøslev et al. 2007, Garnica et al. 2009, Ammirati et al. 2013). The amount of intraspecific variation reported has usually been fewer than six base pairs while the interspecific variation has been more than six base pairs, and in addition, specimens from different continents can have identical ITS sequences (e.g. Garnica et al. 2009). Frøslev et al. (2007) and Niskanen et al. (2009) reported that some morphologically distinguishable species were separated only by 3–5 nucleotides. On the other hand, some studies, have shown that species separated on the basis of morphology have identical or almost identical ITS sequences (Garnica et al. 2003, Ammirati et al. 2007, Frøslev et al. 2007, Peintner 2008). Also, morphologically indistinguishable subgroups have been found inside morphologically delimited species (e.g. Frøslev et al. 2007, Niskanen et al. 2009). However, to date, neither cryptic species without distinguishing morphological characteristics nor species with identical ITS sequences have been described in *Cortinarius*, where both morphological and molecular data were considered. Unlike most studies comparing taxa at species rank, Ortega et al. (2008) and Suárez-Santiago et al. (2009) also compared intraspecific varieties. In their study species differed by at least 10 diagnostic positions and varieties by 2–9 diagnostic positions. The distinction between species and varieties was made based on the number and usefulness of morphological characteristics.

The idea of species identification based on DNA characteristics was introduced by Hebert et al. (2003a). In DNA barcoding a short genetic marker in an organism's DNA is used for species identification. The main aim is not to determine classification but to identify an unknown sample by comparing the sequence to the reference DNA library. A desirable locus for DNA barcoding should be standardized, universal, easy to sequence without species-specific PCR primers, short enough to be easily sequenced with current technology, and provide enough variation to discriminate species (Hollingsworth et al. 2009, Schoch et al. 2012).

Ideally, the barcoding marker would be the same for all organisms but this is not the case. For animals the barcoding region is *CO1* (cytochrome *c* oxidase subunit 1) (Hebert et al. 2003a, 2003b). In plants *CO1* has limited value for differentiating species and a 2-locus system of chloroplast genes was recommended – *rbcL* (ribulose 1-5-biphosphate carboxylase/oxygenase large subunit gene) and *matK* (maturase-encoding gene from the intron of the trnK gene) (Hollingsworth et al. 2009). Schoch et al. (2012) tested the potential of nuclear ribosomal RNA regions ITS, LSU, and SSU, and protein coding genes *rpb1*, *rpb2* and *MCM7* (minichromosome maintenance protein) for fungal barcoding. They concluded that the protein-coding gene regions often had a higher percent of correct identifications compared with ribosomal markers, but low PCR amplification and sequencing success eliminated them as candidates for a universal fungal barcode. Among the ribosomal markers the ITS region had the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation. Therefore, it was suggested as a primary fungal barcode marker but with the possibility that supplementary barcodes may be developed for particular narrowly circumscribed taxonomic groups.

Following the selection of the barcode region focus has shifted towards the lack of a high-quality reference database of fungal sequences for the ITS region (e.g. Bates et al. 2012). At the moment, the most reliable database for identification of ectomycorrhizal fungi is the UNITE

(http://unite.ut.ee/). The creation of the database was initiated already in 2001 (Kõljalg 2005, Abarenkov et al. 2010). The Barcode of Life Database (BOLD) was initially mainly a platform for identification of animals, due to the lack of official barcode regions for fungi and plants, but currently the amount of data of the two latter groups in the database is growing. Also, in GenBank actions for making the data more suitable for identification have been initiated (Schoch Per. Comm.). In all these cases the reliability of the database will remain the responsibility of taxonomists.

Diversity, Distribution and Ecology

At the moment, any estimation of the true diversity of *Cortinarius* is impossible to determine. The molecular taxonomic studies done during the past 15 years have shown that many species are still undescribed and that the diversity is greater than previously thought (e.g. Ammirati et al. 2007, Frøslev et al. 2007, Niskanen et al. 2008, 2009, Garnica et al. 2011, Harrower et al. 2011). The number of species even in the best studied area of the world, Europe, is unknown, not to mention other continents that are far less extensively studied. Niskanen et al. (2012a) estimated that in Nordic countries alone there are at least 900 species. Therefore, the global diversity has to be thousands of species.

In addition,very little is known about the distribution of *Cortinarius* species on a larger scale or the differences in the species composition between continents. However, recent molecular studies on *Cortinarius* have shed some light on these questions. The studies of Peintner et al. (2004), Garnica et al. (2005), and Danks et al. (2010) indicate that the species in the Southern Hemisphere are distinct from those in the Northern Hemisphere. Some of the species, however, belong to the same clades as species of the Northern hemisphere, others seem to be isolated taxa, and certain ones represent lineages only known from Southern hemisphere, i.e. /Pseudotriumphantes and /Splendidi. Representatives of /Calochroi and /Dermocybe are so far only known from Northern hemisphere (Garnica et al. 2005). Preliminary studies on Cortinarii in Costa Rican oak forests revealed endemic species but with relationships to northern taxa, for example, *C. quercoarmillatus* Ammirati, Halling & Garnica with *Quercus* in the mountains of Costa Rica and *C. armillatus* (Fr.) Fr., a boreal species with *Betula* (Ammirati et al. 2007).

The majority of molecular studies covering larger geographical areas have been concentrated on Europe and North America with an emphasis on species from Europe and Western North America (Moser and Peintner 2002a,b, Matheny and Ammirati 2006, Garnica et al. 2009, 2011, Harrower et al. 2011, Niskanen et al. 2011, 2012b, Ammirati et al. 2013). These studies show several patterns of species distributions. There are species common to North America and Europe, especially those species from more northern and montane conifer forests, i.e. *Cortinarius angelesianus* A.H. Sm., *C. armeniacus* (Schaeff.) Fr., *C. napus* Fr. and *C. pinophilus* Soop, but also presumably endemic species occur both in Western North America, Eastern North America and Europe, i.e. *C. elegantio-occidentalis* Garnica & Ammirati and *C. californicus* A.H. Sm. in Western North America, *C. hesleri* Ammirati, Niskanen, Liimat. & Matheny and *C. grosmorneënsis* Liimatainen & Niskanen in Eastern North America, and *C. albogaudis* Kytöv., Niskanen & Liimat. and *C. puniceus* P.D. Orton in Europe. *Cortinarius* species composition is somewhat similar between Eastern North America and Europe but there appears to be less similarity between Europe and Western North America (Niskanen et al. 2011).

In general, the distribution patterns of fungi seem to follow to some extent the vegetation zones, i.e. boreal, temperate, and Mediterranean zones. The distribution of fungi, however, are often wider than those of plants due to their better dispersal potential. For example, several hemiboreal-boreal-oroboreal species like *C. adustorimosus* Rob. Henry (syn. *C. pseudorubricosus* Reumaux), *C. rusticus* P. Karst. (syn. *C. canabarba* M.M. Moser), and *C. pinophilus* Soop occur both in Europe and Western North America but with different coniferous trees (Niskanen et al. 2009, Harrower et al 2011, Ammirati et al. 2012a).

Obviously, there are a number of factors that have influenced the speciation and present day distribution patterns of *Cortinarius* species. These include topography, particularly major mountain building events, climate patterns, edaphic factors, seasons, and the history and patterns of ectotrophic forests and plant communities, including host/fungus migration patterns and host switching (Garnica et al. 2011). Most *Cortinarius* species are primarily found associated with only one or a few host trees and there is a general host preference for either coniferous or frondose trees, but there are exceptions. For example, *Cortinarius arcuatorum* Rob. Henry is associated with *Quercus, Corylus* and *Fagus* in Europe whereas in the Rocky Mountains it is associated with *Picea* (Garnica et al. 2011). The pH of the soil is important for *Cortinarius* and many species are known to be either acidophilous, calciphilous or calcicolous. There are certain groups of *Phlegmacia* where most of the species occur on calcareous soils, for example, *Calochroi* and *Fulvi*, whereas many species in *Scauri* and *Phlegmacioides* are found on acidic soils. In western mountains of North America several species occur only in the spring or are part of the snowbank mycota, i.e. *C. ahsii* McKnight and *C. parkeri* Ammirati, Seidl & Ceska (Ammirati et al. 2012b). From Europe e.g. *C. inexspectatus* Brandrud is known to fruit only in spring (Jeppesen et al. 2012). Sorting out the distribution patterns and ecological parameters of *Cortinarius* species over regional and broad geographical areas is still a work in progress, and will not be resolved until we have a more complete idea of the species that occur across the landscape, their patterns of migration, and how they function in the various forest ecosystems.

Nomenclature and type studies

The names for many species of macrofungi have been difficult or even impossible to interpret and apply to material collected from the field. The most difficult ones are the older names with brief descriptions and usually without type material. Also the species concept of individual authors is important to understand. For example, how many species actually represent groups of closely related or similar species, e.g. in paper II we delimit species in sect. *Bovini* based on ITS and *rpb2* sequences as well as macro- and micromorphological characters, but Fries's delimitation of *C. bovinus* Fr. is based on macroscopic characters and represent a broad morphological species concept. But even if type material exists it can be very difficult to be certain of the identification based only on morphology, especially in challenging genera like *Cortinarius* where there is considerable convergence in morphology, coloration and microscopic features. Furthermore, the literature is often difficult to obtain, making it hard to get information on available names and their application by earlier workers. Consequently, many names have not been used consistently and in some instances the same species has been described two or more times under separate names. In instances where there is no type material available, a neotype (or a lectotype if collections of the author are available) is required to stabilize the use of the name. Finally, old type collections that are considered historical materials, may not be available for study or DNA sequencing, requiring the selection of an epitype.

Molecular techniques have been in use for more than a decade and the sequencing of ITS regions even from older *Cortinarius* specimens is possible, even from type specimens over 100 year old , for example, *C. rusticus* (unpublished data). Furthermore, molecular type studies are essential for a stable and consistent application of names in *Cortinarius* where currently a large percentage of the *Cortinarius* sequences are incorrectly named or without a name in the public sequence databases (e.g. Niskanen et al. 2009). While several papers present sequences from type specimens for individual or groups of species, for example, Garnica et al. (2009), Niskanen et al. (2012c), the only large study is that of Frøslev et al. (2007) where 52 types of *Cortinarius* section *Calochroi* were sequenced.

Aims of the thesis

The focus of this thesis was to study the systematics and diversity of *Cortinarius* with an emphasis on species growing in boreal and temperate zones of Europe and North America. The aim of papers I and II was to study the species in section *Armillati* and *Bovini* in northern Europe and the delimitation of these sections based on morphology and molecular data. The molecular data of paper I included only ITS data but for the paper II sequence data from the *rpb2* region was also included. In papers III and IV the goals were to extend geographical sampling and study the diversity and delimitation of section *Sanguinei* and *C. bovinus* in North America and Europe. The aim of paper V was to describe and study the taxonomic placement of five new species from Western North America. Finally paper VI was constructed to bring the identification and nomenclature of *Phlegmacia* into the DNA era and stabilize the use of names by studying the type material of *Phlegmacium* species, choosing neotypes for those without type material, and describing species new to science, thus creating the ground work for a correctly identified ITS barcoding database for species of *Phlegmacium*.

Material and methods

Material of species was mainly collected by the authors of the papers from Europe and North America (Canada: AB, NL, NS, ON, QC; U.S.A: AK, CA, OR, WA). We also examined herbarium material, especially for the paper I. In addition, type specimens were studied for papers I, II, IV, V and VI.

For the molecular analysis the nuclear ribosomal RNA region ITS was chosen because of it common and effective use in the study of *Cortinarius* species. The region is present in several chromosomes and is arranged in tandem repeats that are thousands of copies long (Burnett 2003). Due to the high copy number the region usually is easy to amplify and sequence, even from very old specimens. Different alleles in one individual may, however, cause some problems in direct sequencing. They may originate from the heterozygotes or from the heterogeneity among the ribosomal repeat units of a single, haploid genotype. Usually the problem is due to an indel after which the subsequent bases will be shifted resulting in conflicting peaks spanning the remaining length of the region (Ammirati et al. 2012b). The problem can be overcome by sequencing the regions from both ends, but sometimes two indels in the same individual causes unreadable stretches when using direct sequencing.

The other locus sequenced for molecular taxonomy in papers II–V was the single-copy RNA polymerase II *rpb2* gene between conserved domains 6 and 7. In *Cortinarius* the region is about 750 base pairs long (Frøslev et al. 2005). The reason for choosing this locus as the second marker

was that it is not linked to the ITS region and it was tested in *Cortinarius* before with promising results (Frøslev et al. 2005). Frøslev et al (2005) also tested RNA polymerase II gene *rpb1* but it did not work well enough in our studies. In addition, we tested nuclear ribosomal RNA IGS1 region, but the data is not yet ready for publication.

Primers ITS 1F, ITS 2, ITS3 and ITS 4 (White et al. 1990, Gardes and Bruns 1993) were used to amplify ITS regions, and specific primers cort6F and b7.1R (Frøslev et al. 2005) for the *rpb*2 region. Sequences were assembled and edited with Sequencher 4.1 (Gene Codes, Ann Arbor, Mich., USA). The alignments were produced with the program Muscle (Edgar 2004) under default settings and the alignments were manually adjusted in BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html). For reconstructing the phylogeny Bayesian inference (BI) was performed with MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003). The reason for using Bayesian inference instead of Parsimony or Maximum Likelihood methods is purely practical. In our earlier papers (e.g. Kytövuori et al. 2005, Niskanen et al. 2009) also Parsimony was used, however, the results with both methods were consistent. Furthermore, reviewers and editors prefer phylograms instead of cladograms so we chose to use the Bayesian method. In addition, this method is popular among *Cortinarius* taxonomist and there are no known flaws in the method to date.

All detailed morphological studies of the papers I–VI of this thesis have been done by I. Kytövuori, T. Niskanen and J.F. Ammirati (see details in page 2). K. Liimatainen was responsible for photographing many of the specimens in fresh condition. Introduction to the use of morphological characteristics in the identification of *Cortinarius* can be found from Niskanen (2008).

Main results and discussion

Phylogenetic classification and evolution of *Cortinarius*

Our focus was primarily on species level taxonomy and therefore no nomenclatural changes were made at the section or subgeneric levels. Our results (I–II, IV, VI) confirm the findings of earlier molecular studies that the traditional infra-generic groupings are at least partly artificial, e.g. paper I and II, and should be reevaluated. Many morphological characteristics used for classification have evolved several times in *Cortinarius*, i.e. color of the universal veil, membranous veils, and color of the basidiomata (Peintner et al. 2002, Garnica et al. 2005). It is important to notice, however, that the newly prosed classifications based on molecular data are not in conflict with morphological data as also noticed by Garnica et al. (2005). Often the reevaluation of morphological data reveals characteristics suitable for delimitation of monophyletic clades which might have previously been ignored or omitted and helps in distinguishing between apomorphic and plesiomorphic characteristics.

The earlier classifications also have been hampered by an insufficient knowledge on species diversity and distribution. Many distinct or isolated species have not been placed in monotypic sections but rather in larger groups with other species. Our studies indicate (I–VI) that when more species are known and sampled a more natural classification will be achieved and species previously regarded as isolated will in reality be representatives of larger clades.

In studies II–V *rpb2* was used in addition to ITS to improve the resolution of the phylogenies. In the study VI *rpb2* sequences were not produced because many type specimens are old and the focus of the study was mainly at the species level rather than at the section or subgeneric level. As in Frøslev

et al. (2005) our clades that received support from ITS regions in paper I and in our earlier studies (Niskanen et al. 2008, Niskanen et al. 2012c) were supported by the combined ITS and *rpb2* data in papers II, IV and V. However, several relationships remained unresolved. In some instances, as with *C. alboambitus* Niskanen, Liimat. & Ammirati and *C. politus* Niskanen, Liimat. & Ammirati in paper V, this may be due to an insufficient sampling of taxa, since the diversity of *Cortinarius* species, especially in subgenus *Telamonia*, even in better studied areas, is still largely unexplored. Also, more data from other gene regions will be needed. For example, in section *Bovini* the interspecific differences between the species in *rpb2* region were smaller than with ITS which most likely did not provide sufficient phylogenetic signal. In other words, more species and more DNA regions will be needed for achieving a more complete view of species relationships.

Our results also provide some insights on the evolution of certain groups of *Cortinarius.* In paper I, the species of section *Armillati* were divided in two clades, one containing all the species associated with deciduous trees and the other including all the species associated mainly with conifers. The results of papers II and III show that all the species of *Bovini s. str.* are calcicolous. The results for section *Sanguinei* in paper IV suggests that the origin of certain species might be in the New World instead of the Old World. Also, in the study by Garnica et al. (2011) North America was proposed as the center of the origin for two *Phlegmacium* species, *C. arcuatorum* Rob. Henry and *C. elegantior* (Fr.) Fr. When more data on other groups of *Cortinarius* is in hand it will help us to better understand the current distribution of the species and the patterns of speciation in *Cortinarius.*

Species delimitation

Our current way of delimiting species in papers I–VI is certainly practical and acceptable for this transitional period from a morphological species concept to a molecular based delimitation. Our ITS data (papers I–VI, and the complete, partly unpublished dataset of over 3000 ITS sequences from over 500 *Cortinarius* species) correlates well with our very narrow morphological species concept and convinces us of the usefulness of ITS in species delimitation in *Cortinarius.* The first step in the process of delimitating species is to find a barcoding gap; the intra- and interspecific variation should be discontinuous. This can easily be observed from a simple pairwise alignment without a phylogenetic analysis. In more than 90% of the cases the intraspecific variation is less than a few substitutions and indel positions and the interspecific variation more than five substitutions and indel positions. The final step is to confirm, or find in the re-evaluation of the specimens, at least one morphological or ecological character which supports the delimitation of a species based on ITS data. I would like to emphasize that the species concept presented here is not a final one. It is not a perfect method, it will not detect all the species and some of the species it detects might turn out to be species groups or complexes. However, based on our data, it is the best available one we have in use at the moment.

The species concept presented by Frøslev (2007) in his PhD thesis was a "morfospecies concept with the extra criterion of monophyly added". Unfortunately the criterion of monophyly does not dependent only on the primary sequence data but in some cases is heavily affected by artifacts in the analysis methods. For example, in the study of sect. *Brunnei* (Niskanen et al. 2009) the monophyly of *C. glandicolor* (Fr.) Fr. was dependent on the number of species included in the analysis. The problem is related mainly to the length variation of the ITS region which causes ambiguity in the alignments of larger datasets and leads to the exclusion of diagnostic

characteristics. Also, in many phylogenetic analyses the gaps are ignored which can greatly affect the outcome. When using only one ITS region it is better to use the barcoding gap criterion than monophyly, because the former is a more precise method, it has a better repeatability, and it does not lose the resolution of ITS region.

The majority of species described in papers I–VI are well delimited based on molecular and morphological data, and correlate well with the results of e.g. Frøslev et al. (2007) and Niskanen et al (2009). In studies I and II two species pairs, *C. paragaudis* Fr. / *C. pinigaudis* Niskanen, Kytöv. & Liimat. and *C. fuscobovinus* Kytöv., Niskanen & Liimat. / *fuscobovinaster* Kytöv., Liimat., Niskanen & H. Lindstr., have morphological and ecological differences supported by the study of multiple collections but differ only by one base pair in the ITS1 region. As stated in paper II it is important to realize that similar ITS sequences are not necessarily in conflict with the idea of distinct taxa. We currently lack sufficient data to confirm that ITS regions can separate all *Cortinarius* species. Using more variable regions might resolve the problem.

In studies II and V the potential of *rpb2* in species delimitation was tested. The variation in the *rpb2* region was comparable to the ITS region and the former did not provide additional support, e.g. to the delimit species pair *C. fuscobovinus/fuscobovinaster*. The studies by Aanen et al. (2000) in *Hebeloma* indicate that IGS1 has more informative characteristics than ITS regions. Furthermore, species delimitation based on IGS1 was more congruent with results gained by using the biological species concept. Our unpublished studies support the results of Aanen et al. (2000); the comparison of IGS1, *rpb2*, and ITS regions showed that the IGS1 was the only locus containing enough variation for separating the species pair *C. paragaudis/C. pinigaudis* (Liimatainen & Niskanen 2011: http://www.dnabarcodes2011.org/conference/program/abstract_page.php?uniqid=Idee5X). Thus, it seems that the IGS1 locus would have the most potential for futher evaluating species in *Cortinarius*.

Cryptic species, species indistinguishable from one another based on morphological characteristics, remain an unresolved question in the current study. There are cases where we strongly suspect cryptic species, i.e. *C. sp23* Kytöv., Liimat. & Niskanen in paper VI and *C. carabus* Kytöv., Niskanen & Liimat. and *C. gentilis* (Fr.) Fr. in Niskanen et al. (2009). In these species the ”intraspecific” variation in ITS regions is rather high and even in the phylogenetic analysis subgroups inside the species are formed. Similarly, morphologically indistinguishable subgroups were also detected e.g. by Frøslev et al. (2007) in section *Calochroi*. It is highly likely that cryptic species exist in *Cortinarius*, since so many of them have already been found in other genera of fungi.

No varieties or subspecies were recognized in our studies. It is not that we don’t believe that intraspecific taxa exist, it is more than we lack a good definition for these intraspecific taxa which could be applied for units based on morphological and molecular data. Certainly there is small morphological and sequence variation within species. The problem is how to separate the normal variation inside populations from intraspecific variation which already has evolved and isolated enough to be considered as variety or subspecies.

In the study of sect. *Brunnei* (Niskanen et al. 2009) four different classes of intraspecific variation were separated based on ITS regions: 1) no genetic variation, 2) all the intraspecific variation is intragenomic polymorphism, i.e. no characteristic sites exist where two sequences would have different character states, 3) different sequences occur within the species, but in all the characteristic sites that differ, intermediates (intragenomic polymorphism) also appear, 4) one or more characteristic sites with discontinuous variation (no intragenomic polymorphisms). In the fourth

case we most likely are dealing with species, but in the second and third instances there is potential for intraspecific taxa. However, there was not any correlation between morphological and sequence variation and also the sequence variation did not match with the distribution patterns. Therefore, no further limitations were made. One possibility also is that with a more variable DNA-region (e.g. IGS1) two clearly separated groups with no intermediates would be formed. Then in cases three and four you could consider them as separate species with unfixed ITS alleles, but some additional data would be needed to support this conclusion.

In paper VI varieties and subspecies described by Bidaud et al. (e.g. *C. rufoallutus* var. *caesiolamellatus* Bidaud), Brandrud et al. (e.g. *C. patibilis* var. *scoticus* Brandrud), and A.H. Smith (e.g. *C. orichalceus* var. *olympianus* f. *luteifolius* A.H. Sm.) were studied. Their concept for ranks below the species level was not stable based on either morphological or molecular data. In some cases the intraspecific taxa were not even sister species of the original species but something completely different. In paper I some of the intraspecific taxa described by Bidaud et al. had identical ITS sequences with the main variety. Since we did not find any morphological characteristics, supported by several specimens, to separate them, the names were presented as synonyms of the main variety. In these cases we did not have any good argument for rejecting the taxa, except for the lack of characteristics to support their possible delimitation. Also, in the overall work of the authors they did not provide a stable concept or grounds for delimiting intraspecific taxa that we could have follow.

In papers I and VI the species names have been synonymized when both molecular and morphological data have supported it, although there is a risk of synonymizing species with morphological differences that we have not observed by studying only a couple of specimens. Based on the overall data we have on *Cortinarius* it would seem probable, however, that in majority of cases the synonymy is correct, and that the cases like *C. paragaudis/C. pinigaudis* are not very common. In the doubtful cases we have left the original names, i.e., *C. volvatus* A.H. Sm. and *C. gentianeus* Bidaud (paper VI). The former is from North America and the latter from Europe and in ITS region they differ by a couple of bases.

Morphological vs. molecular characteristics in the study of *Cortinarius* taxonomy

For a long time, morphological characteristics and ecology were the main and best available data for the identification and classification of species. In general they have provided a rather solid basis for the classification of many animals and plants but have been less reliable for the majority of fungi.

Problems related to the morphological taxonomy of fungi can be explained by a combination of up to four factors. First, the number of species is relatively high in comparison to other eukaryotic organisms. Second, the number of morphological characteristics available for their classification is relatively few and mainly come from the reproductive structures. Third, most of the morphological characters are continuous and those rare characters that seem to be discontinuous, i.e.odor, KOH reactions, and color changes in MLZ preparations of lamellae and pileipellis, are usually only useful in the classification of a minority of the species. For example, thousands of *Cortinarius* species have basidiospore measurements somewhere between 5–15 × 3–8 µm so in the majority of cases sister or similar species will have overlapping basidiospore measurements. Theoretically there should be more homologous than homoplastic morphological characteristics on the strength of which we should be able to achieve the correct classification. This is not so evident in fungi. Since characters are so few, it is possible to find some homologous characteristics that link e.g. *C. bovinus*

with sect. *Bovini* and *C. cumatilis* Fr. in sect. *Claricolores* but other characters may point to other sections or do not connect the species to any particular section. Finally, learning the skills needed for morphological taxonomy takes a long time and passing on all known morphological data unambiguously is challenging if not impossible.

Some classifications of *Cortinarius* based on morphology are easier to adapt and use, and might seem more logically or stable than others, but there is no way to select the best classification based on morphological data alone. It is striking that only with some of the easiest *Cortinarius* species, like *C. triumphans* Fr. and *C. pholideus* (Lilj.) Fr., most of the *Cortinarius* taxonomists have had an agreement on species limits, and the limits also seem to be true based on molecular studies. In majority of the cases, which includes thousands of species, with numerous sections and several subgenera, it is hard to find any consensus in morphological studies of certain groups and even harder to find molecular data to support those conclusions. For example, in section *Sanguinei*, which was well studied and seemed easy based on morphology, the outcome with molecular data (paper IV, Niskanen et al. 2012c) was something that no taxonomist was able to achieve based on morphology only, concerning both species and section limits. As Peinter et al. (2004) already stated almost ten years ago "morphology alone is insufficient for recognizing natural units in this group of fungi", but still the weight of morphology in taxonomical studies is very strong – species and other ranks can be validly described based on morphology alone although no recent studies support using this more traditional approach.

Molecular data is more objective and has a better repeatability than morphological data. Thus, while it is common for researchers to disagree on classifications based on morphology these disagreements are infrequent in the sequence-based classifications using the same DNA regions. In addition, the whole process of obtaining DNA sequence data is easier to automate, more effective and much less time consuming

The four points discussed above in relation to morphological taxonomy are not a problem or at least much less of a problem in molecular taxonomy. . If the selected DNA region is suitable for species identification it does not matter how many species the genus includes. At the moment, the number of characters in one DNA region, in our case ITS, is not enough to separate all of the species or to define all of the higher taxonomic levels. One major advantage in molecular taxonomy is that the risk of unnatural grouping is minimal compared to classification based on morphology. The question is more about where to draw the taxonomic limits, and in which rank (e.g. species, section, subgenus) each monophyletic unit should be placed. The studies I–VI, however, show that the results gained so far already are much better than those achieved during the past 200 years by using only morphology. Furthermore, the molecular characteristics used, base changes, insertions and deletions, are discontinuous and therefore more suitable for classification. Also, learning the skills needed for molecular work takes less time, especially when thinking that the same skills can be applied to many genera, whereas in morphological taxonomy one needs much background work to be even in theory able to identify e.g. all the species of Agaricales. Also passing on molecular data and comparing the sequences is easy and unambiguous. In addition, more suitable DNA regions will most likely be found in the near future to augment existing molecular data.

It is evident that using a suitable DNA region is crucial, e.g. using LSU or SSU region for species level taxonomy of *Cortinarius* does not provide enough information to separate many of the species. In addition to a current lack of the most suitable DNA regions for classification, the use of the current locus ITS is not always completely unambiguous. Since the length of the region varies producing an accurate alignment is challenging. With protein coding genes, like *rpb2*, this problem is avoided. Finally, there is one minor pitfall in using molecular data and that is using an incorrect

sequence. This might be due to a contamination or errors in the laboratory work. The rate of incorrect sequences can vary enormously, with recent collections it can be less than 1 % but with old, moldy collection, like Henry's types, it can be even 30–50 %. However, in more than 90 % of the cases the incorrect sequences are the result of some mold on the *Cortinarius* species so the error is easy to detect.

Using morphology in taxonomic studies of *Cortinarius*

In the beginning of this project it was extremely important to use independent, morphological data to test the suitability of ITS for species delimitation, and compare the results of these two approaches. As the usefulness and value of molecular data has been confirmed the role of morphology in delimitating species is much reduced. However, morphology is still used for species descriptions, which relates to the rules of taxonomical nomenclature, but is not needed for limiting the species itself.

At the moment, it is still faster and cheaper to use morphology to pre-select collections for sequencing rather than just sequencing all collections; but this might not be true for long since the costs of sequencing will most likely continue to decrease. The same goes for studying species distributions based on morphological studies. We have also used morphology for tracing errors in ITS results caused by contaminations. This is mainly relevant in type studies and the problem could be overcome by sequencing the type specimens multiple times. Morphological studies have also revealed mixed collections, but this of course can also be also be detected using molecular methods.

In summary, the main problem in using morphology in taxonomical work is that the problems related to it are not temporary and will not be easily solved if at all. Molecular data is free from most of the problems of morphological taxonomy and it is likely the molecular data will be better and more reliable in the future. With morphology alone, in some rare cases, we can achieve the correct result and also agreement among taxonomists, but most of the time we will be lost in the morphological wilderness without DNA sequences or some other additional relevant data. Thus, although it sounds odd, in the future there might not be a need to use morphology to reveal the diversity of *Cortinarius* and to delimit the species and higher ranks in the near future.

When we discover new species that are easy to identify based on morphology (e.g. *C. pholideus*) it will be important to make high quality morphological descriptions with photographs that will enable a wider audience to identify species. Unfortunately in the more difficult groups like sect. *Bovini* there will be very few workers who will be able to use morphology to identify species.

DNA-based taxonomy might exclude some amateurs who do not have access to DNA data for their studies. Unfortunately the use of DNA in taxonomy is not an option any longer and it should be used in all fungal genera in which the sequencing of the material is possible and by all taxonomists who have sufficient resources for conducting the study. We would also have made a number of errors in nomenclature and species delimitations in the papers in this thesis without the use of DNA sequence data.

Species diversity, distribution and ecology

The *Cortinarius* species of Europe are best studied in the world but still our knowledge of species is far from complete. In paper I which concentrated on sect. *Armillati*, one of the most studied groups of subgenus *Telamonia*, two of the six species were unknown (33%). In section *Bovini* (paper II) the

proportion of unknown species was 86 % and in section *Brunnei* 50% (Niskanen et al. 2009). Also, the study of subgenus *Phlegmacium* (paper VI) revealed over 20 new species. Based on these figures it might well be that as much as 30–50 % of the European species are still unknown and highlights the fact that revealing the true diversity based on morphology only, has been an overwhelming task.

Our studies III–VI support the findings of e.g. Ammirati et al. (2012b, 2013), Bojantchev et al. (2011a,b), Garnica et al. (2011), Harrower et al. (2011), and Niskanen et al. (2011, 2012b), that there is a lot of diversity to discover in North America as well. If the identification and distribution of species in Europe and North America are still poorly known, then even less, and in some cases nothing, is known of the diversity for other areas of the world. The species are so poorly known that any reliable estimate of species richness and diversity are not possible at this time. The number of species has to be thousands but the actual number of species remains unknown at this time.

The results for species distributions (papers I–VI) are largely in concordance with the results of other recent studies (e.g. Garnica et al. 2009, 2011, Harrower et al. 2011, Niskanen et al. 2011, 2012b, Ammirati et al. 2013). Species with a wide distribution, covering at least two continents, were detected in papers I, II, IV and VI, i.e. *C. armillatus*, *C. oulankaënsis* Kytöv., Niskanen, Liimat. & H. Lindstr., *C. vitiosus* (M.M. Moser) Niskanen, Kytöv., Liimat. & S. Laine, and *C. cupreorufus* Brandrud. As indicated by earlier studies, these mainly represent boreal or conifer associated species; one of the rare exceptions is *C. triumphans* (= *C. ophiopus* Peck from Maryland). This may partly be due to the lack of information for species from eastern North American temperate forests. It may be that there will be more similarity between boreal than temperate forests of North America and Europe, but further field and molecular studies, including species from forested area of Mexico, will be necessary to determine whether or not this is correct.

Papers I, II, IV and VI include many species so far only known from Europe i.e. *C. roseoarmillatus* Niskanen, Kytöv. & Liimat., *C. bovinus*, *C. puniceus*, and *C. varius* (Schaeff.) Fr., but it is very likely that in the future many of these species will be found in Asia (e.g. Russia) once more data is available from those areas; currently data from Asia is almost completely lacking. For example, the collections of Sesli et al. revealed that *C. sp24*, a sister species of *C. multiformis* Fr. that occurs commonly in hemiboreal to boreal, mesic coniferous forests of North and Central Europe, also is found in the mountains of East Black Sea Region, Turkey (paper VI).

The extensive studies of sect. *Armillati* (paper I) and sect. *Bovini* (paper II) in northern Europe revealed more detailed information on distributions of the species in different vegetation zones. For example, *C. bovinaster* Niskanen, Kytöv. & Liimat. seems to represent a truly boreal species and *C. pinigaudis* and *C. suboenochelis* Kytöv., Liimat. & Niskanen have the centre of their distribution in the boreal zone and they become less common or absent in the southern parts of the hemiboreal zone. On the other hand, *Cortinarius anisochrous* Kytöv., Liimat., Niskanen & H. Lindstr. and *C. fuscobovinaster* are more southern species and only fruit in hemiboreal and southern boreal zones. Also, two species with presumably continental distribution were detected, *C. roseoarmillatus* and *C. pinigaudis*.

Species only known from eastern North America, i.e. *C. harrisonii* Ammirati, Niskanen & Liimat. and *C. subsolitarius* A.H. Sm., were reported in papers IV and VI. The sampling of section *Sanguinei* is rather good from Europe and the northern parts of western North America and appears that *C. harrisonii* does not occur in those areas. But what is currently unknown is how far South and South-West this and other occur, since data from those areas is almost completely lacking. For example, *C. acystidiosus* Thiers (paper VI) is described from Texas but also recorded from

Tennessee (pine-hardwood forest). In addition, a new report of *C. marylandensis* (Ammirati) Ammirati, Niskanen & Liimat. from Costa Rica (paper IV) shows an interesting connection between the mountainous *Quercus* forests of Central America and the deciduous forests of southern and eastern North America.

Papers III, IV, V, and VI include species only known from western North America, i.e., *C. bovarius* Liimat. & Niskanen, *C. neosanguineus* Ammirati, Liimat. & Niskanen, *C. albofragrans* Ammirati & M.M. Moser, and *C. brunneovernus* Niskanen, Liimat. & Ammirati. They most likely have very different evolutionary histories since for example, *C. bovarius* occurs in coniferous forests on calcareous soil in Alaska and on the eastern side of the Rocky Mountains in Alberta, *C. neosanguineus* grows in mesic coniferous forests extending from California to British Columbia, *C. albofragrans* occurs in *Quercus* from California to Washington, and *C. brunneovernus* is representative of spring and snowbank mycota of the western mountains of North America. Even though we can currently be sure that different distribution patterns occur on a larger scale, the coverage of the current data is still far from perfect and does not allow us to evaluate how common each pattern is at this time.

Cortinarius species are usually associated either with coniferous or deciduous trees. This was also supported by the studies I–VI. The only exception found was in section *Armillati*; in the boreal zone *C. paragaudis* and *C. luteo-ornatus* (M.M. Moser) Bidaud, Moënne-Locc. & Reumaux associate with conifers but in the subalpine zone with *Betula* spp. The pH of the soil is also important for *Cortinarius*. Certain groups of subgenus *Phlegmacium* are known to be calcicolous but studies II and III showed that also in subgenus *Telamonia* such a group, *Bovini s. str.*, exists. Therefore, the species of section *Bovini* could be used as indicators of valuable forest sites as can the calcicolous *Phlegmacium* species (Vesterholt 1991, Hallingbäck and Aronsson 1998).

Even though *Cortinarius* sequences in public databases are still relatively few, and geographically restricted, they provide valuable additions to the knowledge of species. In all the studies I–VI the sequences retrieved from the public databases added some information on species distribution and ecology which otherwise would not be known. For example, the sequences deposited in the GenBank revealed that *C. oulankaënsis* (II) and *C. sp2* (VI), two species we described from northern Europe, also occur in western North America. To gain this knowledge on our own would have taken multiple field excursions. These results also show that our knowledge of the distribution of species is patchy and that considerable work remains to be done on *Cortinarius* biogeography.

The nomenclatural problems arising from a man-made system

Taxonomic studies have two parts. The first, the biological part, is when the limits of species are studied and determined using available data. The second, the man-made part, is naming the taxonomic units and using the rules related to this process, which are presented in the International Code of Nomenclature for algae, fungi, and plants (previously International Code of Botanical Nomenclature). Names are given so that the taxonomic units have unique names which facilitate passing on knowledge of the species and to prevent confusion with other species. In theory, this should work well, but in practice it is a very time consuming and difficult to achieve.

The nomenclatural part of the taxonomic work has become overwhelming for two reasons, and this is particularly evident in *Cortinarius*. First, there are numerous species and many researchers studying the same genus over a broad geographical range. Secondly, morphology alone is insufficient for recognizing natural units and passing the knowledge of species in the form of

descriptions is difficult, misinterpretations are common, often resulting in chaos. For example, paper VI showed that all authors who have described more than five *Phlegmacium* species have described synonyms, and 35 % of the published names were synonyms of earlier described species. Furthermore, the nomenclatural work is difficult to do because there is no complete list of type specimens, although you can find the literature reference of the description from *Index Fungorum*. Even then it is not enough to download all the major papers in your field of study from the last ten years, to solve a problem, but in addition you soon find yourself in a library in the basement of a herbariums seeking papers published 50 years ago in small local mushroom journals written in languages you cannot read – if you are so lucky as to find that the journal is there.

Furthermore, it is not uncommon to find that after revealing the species, you must search the literature multiple times, and order and study the relevant type collections to find the correct name for your species – or to discover that it is undescribed. What we have attempted in paper VI is a first step toward resolving this problem. We must stabilize the nomenclature and make it to work better for us. It is clear that all the type specimens should be sequenced as soon as possible and the names without type specimens should be typified, e.g. by choosing a lecto-, neo- or epitype depending on the situation. After this process is completed all the names that lack DNA sequences from type collections should be rejected. The publication of new names should not be allowed without an ITS sequence of the species which will then serve as an unambiguous reference for the future work. Unfortunately this is not self-evident in our community, i.e. sequences can be found only for about 25 % of newly describe species (Hibbett et al. 2011). Basically, our aim should be to have all the valid names in the form of sequences from type specimens in the public databases (GenBank, UNITE) and other names should no longer be applied to collections. This is something that should be accomplished sooner rather than later. Taxonomists should not continue to interpret old or forgotten names without typification and providing DNA sequence data. There is no point in spending valuable research time puzzling over names that cannot be resolved because of an antiquated approach, something that in reality derives from a man-made system that has nothing to do with the nature of science itself.

Barcoding

Although many suitable morphological characteristics for the identification of species can be found in the re-evaluation of the data following molecular studies (like in papers I–VI), it still does not make morphology a perfect way to identify them. The reliability of morphological identification in *Cortinarius* can vary dramatically between the species. For example, most basidiomata of *C. armillatus* are quite easy to identify already in the forest with moderate expertise in *Cortinarius*. With other species in sect. *Armillati* you need a microscope if you are not already very familiar with this group. *Cortinarius roseoarmillatus* you might be able to identify rather simply when you have a key which includes all the known species (e.g. *Funga Nordica*), and you have the basic skills to observe the microscopical characteristics. But for distinguishing *C. paragaudis* from *C. pinigaudis* or *C. luteo-ornatus* from *C. suboenochelis*, one needs to evaluate this group for several years, collect fresh material, and send that to the authors for confirmation or sequence the material to confirm the identification. It would not be an exaggeration to state that the correct identification of more than half of the Nordic *Cortinarius* species requires knowledge far beyond the skills of just using taxonomic keys and a microscope. Also, one should be aware that even for the best experts the morphological identification of species is far from perfect. There is no individual who can identify all the *Cortinarius* species presented in the *Funga Nordica* or other major taxonomic papers.

Many workers believe that the current problems of identifying fungus species using morphological characteristics are a result of incomplete and incorrect keys and therefore something that can be resolved in the near future. The larger problem is understanding the overwhelming diversity and size of the genus and the resulting impact on identification species. For example, one might feel that species like *C. obtusus* (Fr.) Fr., *C. fulvescens* Fr. and *C. hinnuleus* Fr. are quite easy to recognize in the Nordic countries, because there are not or only few similar looking species in our keys, i.e., in *Funga Nordica*. Unfortunately, in reality all of those species represent clusters of species and will go through the same transformation than we have witness in e.g. *C. calochrous* (Pers.) Gray, *C. cyanites* Fr. (paper VI) or *C. bovinus* (paper II). Thus, better knowledge does not guarantee easier morphological identification.

When you have a taxonomic group, like birds, with a reasonable number of species and species which are easy to find, observe and identify, there is the possibility of getting reliable data on the distribution, ecology and conservation needs of those organisms based on morphological identification alone. But none of these data can be acquired for fungi using morphology. Therefore, the only rational choice is molecular identification and barcoding, which is faster and more reliable than the traditional, morphological methods.

For the correct molecular identification of species three things are needed. Molecular data should be available for all of the species, and the species should be unambiguously and correctly named. These requirements are not unique to a barcoding database but also apply to identification books and keys based on morphology and ecology. Finally the region selected for DNA sequencing must be suitable for the identification of all species. Paper I shows that currently with ITS this is not always the case, but how extensive this problem is in *Cortinarius* is unknown to date. A more variable region, however, will be needed in the future for the reliable barcoding of all Cortinarii.

The coverage of *Cortinarius* species in the barcoding databases is still poor. In section *Armillati* only 50 % of the species were represented in public sequence databases. A similar situation was observed for sequences of sect. *Brunnei* by Niskanen et al. (2009). For section *Bovini* the coverage was only 30 %. This lack of data will be corrected in the course of time since the number of molecular studies is increasing and more and more data is being deposited each year.

Also, the reliability of the identifications of species in the databases is still very poor. For example, in study I 65% of the sequences of *Armillati* species were deposited in GenBank under an incorrect name or as *Cortinarius* sp. The errors in identification are mainly due to the fact that the names in the public sequence databases are not based on type studies, but are identifications made from the interpretations of species descriptions based on morphology. This emphasizes the importance of type studies and the role of taxonomists in the creation of an identification database. The aim of the studies I–VI, and especially the VI, has been to produce sound basic data on *Cortinarius* species.

When identification databases are still incomplete it is important to use correct similarity values for determinating if the BLAST-result really represents your species. In fungi a 97% similarity value has commonly been used for delimiting species (e.g. Hughes et al. 2009). ITS-region on *Cortinarius* is about 600 bases long which means 18 substitutions and single indel positions of intraspecific variation with a 97% similarity values. That is clearly too much. Proper value should be at least 99% (about 6 substitutions and single indel positions) or even 99.5%. This 1 % cutoff value has already been used for *Cortinarius* and *Lactarius* e.g. by Lim and Berbee (2013).

Current problems and future perspectives

The development and use of molecular methods in taxonomic studies of fungi has revolutionized our field of science. We are currently in a new era, one that requires a critical evaluation of how we should move forward, and what changes should be made in the way we do fungus taxonomy. The aim, however, still remains the same, study the limits and relationships of the species as well as their evolution, ecology and distribution.

Revealing the diversity, and the study of ecology and distribution

To date, our study of *Cortinarius* and other fungi has almost completely been based on reproductive structures (basidiomata, ascomata). Although many herbaria around the world have preserved fungus collection for at least 100 years, the amount and coverage of certain genera in individual herbaria is still completely based on a single artificial factor, the activity of a few collectors. This phenomenon can be seen in Nordic countries where there has most likely been more collections made per area than anywhere else. For example, half of all the Nordic herbarium specimens of section *Armillati* in paper I were collected during the last two decades by Kytövuori and colleagues. Thus, herbarium material is still so sparse that single contributions made by one or few authors can easily have a great affect on the amount and diversity of collections. But even if the volume of collecting increased significantly, one problem would still remain. Reproductive structures are produced only during a short period of the year, particularly in the autumn season. Some species do not reproduce every year and it may well be that some species do not reproduce at all. Therefore, sampling based on reproductive structures is far from ideal.

Currently 40 % of ITS fungus sequences deposited to GenBank each year originate from environmental sampling with Sanger sequencing (Hibbett et al. 2011). This amount will likely increase during the coming years as soil sampling with the next generation sequencing methods becomes more common. Sequences from environmental samples most likely will turn out to be the major resource also for part of the taxonomical studies in the near future. The disadvantage of the latter method, however, is that information from only one gene region is produced. Thus at the moment reproductive or other sources of single species are still needed for the study of the evolutionary history of the group for which several gene regions need to be sequenced. Still studying diversity, distribution and ecology with soil samplings method has a great potential.

Describing and naming species, is the current process too slow?

It is obvious that we should accelerate the process of naming species. More potential new species are found every year in ecological studies than the number of new species formally described annually (Hibbett et al. 2011). Hibbett et al. (2011) estimated that at the current rate of naming new species, which is about 1200 new species of fungi per year, it will take about 4000 years to formally name the all of the fungus diversity. The situation in *Cortinarius* is not remarkably better. At the moment about 2000 *Cortinarius* species have been described and about half of them are synonyms or names than can not be correctly interpreted. Thus, the number of valid names is about the same as the number of species that grow in the Nordic countries alone. After over 200 years of naming *Cortinarius* species we are not even close to the halfway point.

We certainly need to give every species a unique name to make the communication and passing on of knowledge possible. But should this name be Latin and should the naming process follow the current International Code of Nomenclature for algae, fungi, and plants and include morphological

characters? If we want a fast and reliable system for providing a unique "ID" for every species and information on how these species can be identified, then most likely the current system is not an optimal one.

Fungus taxonomists are overwhelmed by the diversity that needs to be described and named. In addition they tend to be traditional, cautious and conservative. It is already clear that they are unable to name all the species revealed by ecologists, who consequently have started to create a parallel system to stabilize the nomenclature of molecular operational taxonomic units (MOTUs) (Hibbett et al. 2011). The same idea of unambiguous naming of potential species discovered from molecular data lies behind giving unique, stable names for the accession number type for "species hypothesis" currently developed in UNITE (Kõljalg et al. 2013). If taxonomists are not willing to improve and accelerate the process of naming species, we will soon have two or several parallel systems; most researchers will communicate with the nomenclatural system created for molecular identification of fungi and the binominal system will be mainly left for already existing names and specimen based phylogenies.

The recent changes in the International Code of Nomenclature, i.e. removing the requirement of Latin in species description, do not remarkably change the work of naming species. An important question to ask is how much we need to know about the morphology of a species before we can describe it? Currently, the descriptions are thorough and morphological comparisons to sister or similar species are provided. Making this kind of descriptions is time consuming, although we would have found the same result much faster by using molecular data. For example, in paper III, where we describe a new species in sect. *Bovini* from Alaska and Alberta, it was clear already based on molecular data that we had found a novel species which we did not know from Europe (paper II) and for which there was no available name. Thus, only based on ITS sequences we already had confidence about the species status and characters for reliable identification, all that is needed for describing a species. Still we allocated time to do formal morphological descriptions. Why, I'm not completely sure. Therefore, we might want to explore the possibility of describing species based on molecular data only, as for example, in Index Fungorum (http://www.indexfungorum.org /names/IndexFungorumRegister.htm).

But this approach will solve only part of the problem. We have a growing number of species that have only been found using sequence data produced from environmental samples. Therefore we need to expand the criteria for type specimens, from a specimen or an illustration, to include soil and other substrate samples, DNA samples, and sequence chromatograms or alignments paving the way for the description of MOTUs or corresponding units formally. One may think that formally naming MOTUs will lead to a nomenclatural chaos because the naming process is hard to control. In fact, there are many possibilities to create quality standards for MOTU species, as for example suggested by Hibbett et al. (2011). Clearly in the coming years the majority of data for new species, diversity, distribution and ecology will be based on MOTUs and therefore we should be open-minded and objective in our thinking about the most informative and convenient way to move forward with the delimitation of species.

Citius, Altius, Fortius – effective ways to carry out taxonomy

One of the striking differences between taxonomy and many other fields of biology is that research is carried out not only by research groups and universities staff but also by citizen scientists. Clearly it has helped us to gather lots of information with a low amount of funding, but it also has a downside. Many taxonomists do not have any pressure to change or upgrade their methods to be able to receive funding for their studies. When considering how much molecular methods have

revolutionized the field of taxonomy, in the same way that carbon dating modernized archeology, you would think that it would already be a basic standard for all taxonomical studies. Most likely a formal research group would use DNA-data in their studies, but for the majority of amateur researchers it has just started to become available. Furthermore, it is not enough to have DNA-data from your specimens you also must interpret it correctly. It is not rare to find instances where researchers who have a strong morphological background and lack a clear understanding of DNA-information try to force and bend the DNA-data to fit their idea of a known "correct" classification.

To make taxonomy more reliable and efficient we need to develop criteria that are at the same level as those found in other natural sciences. We should only accept results that have been published in scientific papers with a peer review policy. It is the best and the most efficient way to communicate inside the scientific community, provide a rigorous critic, evaluate the quality of the findings, prove your methods, and in the long run improve the whole field of research. This kind of approach would benefit us all. If we think e.g. about the *Atlas des Cortinaires* project (Bidaud et al. 1992, 2010), which is the biggest single project in *Cortinarius* taxonomy and contains an exceedingly large number of novel species, the execution and rigor have not been ideal. All of the DNA-studies so far have shown that about half of their work is invalid and therefore a lot of hard work has been done with no real outcome (e.g. paper VI, Frøslev et al. 2007). If only the project members would have communicated with the scientific community through scientific papers, most likely their species concept would have been corrected and it would have saved a lot of time and energy.

Taxonomists have to become more cooperative and not just work alone or in small research groups. At moment there are about 30 *Cortinarius* taxonomists who publish actively. Thus, it is not an impossible task to create a worldwide collaboration network where exchanging data and ideas would be open and easy. In this way we could more quickly achieve an understanding of species concepts, develop a sound nomenclatural system and develop new methods. For this collaboration to be successful basic knowledge and application of both morphological and molecular methods in taxonomy from all participants would be needed. In the same way as one language is used for communicating in natural sciences, for a more rapid and uniform dialog, the sequence data would be an optimal "language" for communicating about species inside the network. At the same time, all fungal taxonomists should consider at least from time to time combine their knowledge and data in projects such as choosing the best barcode region to fungi (Schoch et al. 2011) or contributing to the barcoding databases (e.g. Kõljalg et al. 2013) as well as participating in ecological studies.

Conclusions

Species level taxonomy is the foundation of biological studies, without knowing the species and their boundaries it is very difficult to do ecological, applied or other research. Our aims as taxonomists are to discover and describe all species, define relationships, and provide tools for unambiguous identification. It is clear, also based on this thesis, that with morphology alone we are not able to achieve that goal. Is our current method of using molecular and morphology taxonomy based on fungus reproductive structures, specimen related nomenclature, and morphological keys adequate for discovering and naming the diversity of fungi in a timely manner.

The results of this thesis project have improved our understanding of the genus *Cortinarius* in several ways. No doubt this has been the biggest effort to stabilize the nomenclature of *Cortinarius* so far and also is an example of using only reliable names in taxonomic studies by excluding names without sequences from type material. Also, our view on the amount of diversity has changed dramatically, e.g. estimation of diversity of *Cortinarius* in Finland is now about same than it was

estimated to be in Europe (Brandrud et al. 1998). Our studies have revealed much needed information about species composition and have allowed for comparisons between North-America and Europe. New information about potential cryptic species in *Cortinarius* and limitations of ITS for species level taxonomy has been gained. Data from genus *Cortinarius*, paper I, was also included in the study for finding a suitable barcode region for fungi. Our studies, especially paper VI, could also be seen as an encouraging example of how reliable taxonomy does not always require focusing on small taxonomic groups in a relatively small restricted area for your entire life if you are using correct methods in a proper way. Finally, the sequences of type specimens published in papers I–VI create thus far the largest, reliable ITS identification database for *Cortinarius* containing over 200 species.

In recent years fungal taxonomy has moved rapidly forward; from using morphological characters to using DNA sequence data and from using specimens to environmental samples. Most likely the majority of taxonomical findings will soon come from ecological studies based on sequences from environmental samples. Scientist generating this data will be at the center of fungus taxonomy, with or without traditional taxonomists.

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