Multilocus DNA sequencing of a widely distributed wood-inhabiting fungus reveals two main lineages with overlapping ranges in Europe: do hybrids occur?

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Forord/preface

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Abstract

Trichaptum fuscoviolaceum is a saprotrophic basidiomycete morphospecies growing on conifers. In the present study, the genetic structure in a circumboreal sample of *T. fuscoviolaceum* was analyzed using a multilocus sequencing approach. The analyses revealed a complex phylogeographic structure where two main lineages occurred; one European connected to *Pinus* and another circumboreal group connected to various coniferous hosts. The European group may have resided in South European refugia during the last glaciation followed by a northward range expansion. The circumboreal group probably have migrated into Europe from northeast. In Europe, in areas where the two main lineages occur in sympatry, some specimens possess heterozygous sequences, apparently because allelic versions from both lineages co-exist in the same dikaryons. The presence of alleles from both groups in the same individuals may reflect hybridization as a result of secondary contact between the two main groups. Additional data are needed to conclude whether the two main lineages represent cryptic species. The observed phylogeographic pattern resembles the pattern observed in other wood-inhabiting fungi, which could indicate that general phylogeographic trends exist across species.

Introduction

Phylogeography has become a well established research area during the last twenty years. Based on gene genealogies, the field aims to construct hypothesis about species distributions in connection with recent and historical events (Avise 2000). In comparison to plants and animals, rather few phylogeograpic studies have been undertaken on fungi. Most fungi are able to disperse over long distances by air-borne spores and as such, fungal species are generally thought to be widespread (Lumbsch *et al.* 2008). However, recent studies have demonstrated that many widely distributed fungal morphospecies include cryptic species with more restricted geographic distributions and ecology (Dettman *et al.* 2003a; Fischer & Binder 2004). For example, Geml et al. (2006) detected three distinct phylogenetic species in the cosmopolitan fungus *Amanita muscaria*, and that these groups are present in sympatry in Alaska. The identification of various phylogenetic species within the *Heterobasidion* complex is another example of cryptic speciation (Garbelotto *et al.* 1998; Karlsson & Stenlid 1991). This species complex was first delimited into different intersterility groups based on mating studies (Korhonen 1978), and subsequent genetic studies (Johannesson & Stenlid 2003) support the existence of three divergent species in Europe. Similarly, Carriconde et al. (2008) demonstrated that the morphospecies *Tricholoma scalpturatum* comprise two genetically distinct cosmopolitan lineages and suggested that this is best explained by allopatric speciation with secondary contact between the groups.

The distribution of fungi is, to a varying extent, dependent on their hosts. While some fungi, especially biotrophic pathogens and mutualists, are host specialists; others can associate with multiple hosts. Murat et al. (2004) discussed the colonization route of *Tuber melanosporum* after the last glaciation. The migration pattern was similar to *Quercus pubescens*, a host tree of this truffle. They suggested that the fungus followed the oak's range expansion northwards, and that the host distribution influenced the distribution of *Tuber melanosporum* populations in France. In addition to affecting distribution patterns, a strong host – fungal association can eventually lead to co-speciation. Dixon et al. (2009) discovered that phylogenetic diversity among the fungal pathogen *Corynespora cassiicola* corresponds with the origin of various plant hosts, and they suggested that the occurrence of identical haplotypes connected to the same host could indicate host specialization. On the other hand, many saprotrophic fungi are able to decompose different types of substrates, and these generalists do not necessarily exhibit evolutionary patterns closely tied to those of their hosts. For example, in a recent

phylogeographic study of the saprotrophic polypore *Gleoporus taxicola*, it was shown that some haplotypes had a wide distribution on various hosts (Seierstad 2009).

Fungal distributions have also been affected by human activities during the past several thousand years. This adds an extra layer of complexity to phylogeographic analysis of many fungi. When plants are transported around the world by humans and introduced to new habitats, fungi are often spread along with them (Vellinga *et al.* 2009). There are several examples of long-distance dispersal events of fungi with man as a vector. Pringle et al. (2009) discovered that the ectomycorrhizal fungus *Amanita phalloides* recently has been introduced from Europe to northwestern North America. The devastating root pathogen *Heterobasidium annosum* has also been introduced to new locations by man. A multilocus analysis revealed that Italian populations of this pathogen possessed genotypes originating from northeastern North America (Linzer *et al.* 2008). Similarly, there is evidence for the introduction of an *Armillaria* species into South Africa from the Netherlands (Coetzee *et al.* 2001).

The establishment of secondary contact between previously isolated lineages can also complicate fungal phylogeography. Without the development of full intersterility barriers, the potential for hybridization is present. Fungal hybrids may in many cases experience low survival due to competition with both parents and backcrossed individuals (Giraud *et al.* 2008). Garbelotto et al. (2007) tested hybrid virulence compared to parental virulence in the *Heterobasidion* species complex. They observed that in cases where the parents were host specialists, the hybrid often performed less successful compared to their parents. However, on neutral hosts no differences in virulence were detected between the hybrids and the parents. Such a scenario can lead to hybrid proliferation in novel environments. A few studies have detected the occurrence of fungal hybrids in nature. Newcombe et al. (2000) have described a hybrid in the rust fungus *Melampsora*, and there has also been detected natural hybrids between two *Flammulina* species. Both these examples are probably results of survival due to novel host adaptation (Hughes & Petersen 2001).

In this study, the phylogeography of the saprotrophic fungus *Trichaptum fuscoviolaceum* (Dicks.:Fr.) Ryvarden is analyzed. *Trichaptum* is a basidiomycete genus in the order Hymenochaetales (Hibbett *et al.* 2007) that includes saprotrophic white rot fungi. *Trichaptum* species are mainly associated with conifers, but some are adapted to hardwood substrates. *Trichaptum fuscoviolaceum* occurs mainly on *Pinus*, and produces small annual basidiocarps (Ryvarden & Gilbertson 1994) on standing or fallen trunks and branches (Ræstad 1940). It

has a worldwide distribution and it appears both in the Southern and the Northern Hemisphere. Like most other homobasidiomycetes, *T. fuscoviolaceum* has a tetrapolar heterothallic reproductive system (Ræstad 1940), which promotes high levels of outcrossing.

The morphology and mating compatibility between the three closely related *Trichaptum* species, *T. laricium*, *T. abietinum* and *T. fuscoviolaceum*, has been investigated by Macrae (1967). While *T. abietinum* and *T. laricinum* produce poroid and lamelloid hymenophores, respectively, *T. fuscoviolaceum* has a toothed hymenophore (Ryvarden & Gilbertson 1994). Despite the high level of similarity between them in both macro- and microscopic features, the conclusion has been that they belong to three different species (Macrae 1967). Ræstad (1940) observed no mating compatibility between the sister species *T. fuscoviolaceum* and *T. abietinum*. Results from a molecular-based phylogenetic study were consistent with the morphological studies in the division of these species (Ko & Jung 2002).

The main aim of this study was to examine the geographic distribution of the genetic variation within *Trichaptum fuscoviolaceum*. I wanted to determine whether *T. fuscoviolaceum* can be considered a single species or whether multiple cryptic species occur in this morphotaxon. Furthermore, I wanted to postulate hypotheses about mechanisms which have contributed to the current genetic pattern within the species. To answer these questions I have performed multilocus sequencing of a worldwide sampling of *T. fuscoviolaceum* with three nuclear and two mitochondrial markers.

Materials and methods

Material

A total of 130 specimens and cultures of *Trichaptum fuscoviolaceum* and six of *T. abietinum* were included in this study (see Appendix). Of these, 15 were cultures obtained from fresh fruiting bodies collected in the Oslo area. From the living cultures, mycelia were scraped off with a sterile scalpel and added to a tube with 600 µL cetyltrimethyl ammonium bromide (CTAB) for DNA extraction.

Approximately 1 mm² hymenium of each herbarium specimen was added directly to eppendorf tubes with a sterile foreceps. Two sterile tungsten carbide beads were added to the tubes and the mycelia were crushed with a Retsch® MM301 machine (Andres Phil AAS, Dale, Norway) for four minutes with a frequency of 20 Hz. The tubes were then stored at -80ºC prior to DNA extraction.

Molecular methods

DNA was extracted using the 2% CTAB protocol (Murray & Thompson 1980), modified for fungi by Gardes and Bruns (1993), with two additional minor modifications: 60 µL of distilled H2O was added in the final step of the extraction, and template was diluted 10 X before PCR amplification. Three different nuclear ribosomal DNA regions and two mitochondrial regions were amplified and sequenced for each sample. The primer pairs ITS3/ITS4 (White *et al.* 1990), CNL12/5SA (Anderson & Stasovski 1992; White *et al.* 1990), and LR5/LR0R (Rehner & Samuels 1994; Vilgalys & Hester 1990) were used to target the nuclear ribosomal internal transcribed spacer 2 (nrITS), and parts of the nuclear ribosomal intergenic spacer (nrIGS), and the nuclear ribosomal large subunit (nrLSU) regions, respectively. A part of the mitochondrial ribosomal large subunit (mtLSU) region was amplified with the primer pairs ML5/ML6 (White *et al.* 1990), while a part of the mitochondrial ribosomal small subunit (mtSSU) region was amplified with MS1/MS4 (Bruns & Szaro 1992; White *et al.* 1990).

PCR was performed either using puRe *Taq*™Ready-To- Go™ PCR beads (GE Healthcare, Milwakee, Wisconsin) with 23 µL 10X diluted DNA and 1 µL of each 5 µM primer or performed in 40 µL reaction volumes containing 4µL 10X buffer, 4µL of 5 µM dNTP, 4µL

of each 5µM primer, 0.4 µL Dynazyme™II DNA polymerase (Finnzymes, Espoo, Finland) and 23.6 µL 10X diluted DNA. The following PCR program was used for the nrITS and the nrLSU regions: 4 min denaturation at 94ºC, followed by 35 cycles with 25s denaturation at 94ºC, 30s annealing at 50ºC and two minutes extension at 72ºC, and ending with 72ºC extension for 10 minutes and then storage at 4ºC. For the nrLSU region the penultimate extension step was run for 1 minute before the final extension step that lasted for 5 min before storage. For the nrIGS, mtLSU and mtSSU regions the PCR program started with a 4 min denaturation step at 94ºC, followed by 40 cycles of 20s denaturation at 94ºC, 20s annealing at 55ºC and 1 min extension at 72ºC, and ending with a final elongation step of 10 minutes at 72ºC before storage at 4ºC.

The PCR amplicons were sequenced in both directions with the corresponding PCR primers using the ABI BigDye v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and visualized on an ABI PRISM 3730 Genetic analyzer (Applied Biosystems). The 5SA primer was not suitable as a sequencing primer, so only CNL12 was used in the sequencing of the nrIGS region.

DNA sequences were edited manually and aligned using the program BIOEDIT Sequence Alignment Editor v.7.0.0 (Hall 1999). The mtSSU region was discarded from all further analyses due to the absence of intraspecific genetic variation in *T. fuscoviolaceum*. As both NeighborNet and Neighbor Joining analyses are sensitive to missing data, samples with more that 10% missing data were removed from the datasets used in these analyses.

Phylogenetic and statistical analyses

Phylogenetic analyses can be complex when dealing with data at the species and intraspecies level due to factors like incomplete lineage sorting, intra-locus recombination and the presence of heterozygous sites in diplophase (or dikaryophase) sequences. Traditional phylogenetic trees may give the wrong visualization of the intraspecific relationships because of conflict in the dataset due to e.g. intralocus recombination (Posada & Crandall 2001). To avoid the constraints of the tree-based analyses, the intraspecific genetic relationships in this study, were investigated using a NeighborNet analysis as implemented in the program SPLITS TREE4 (Huson 1998). The program is based on the idea that any dataset can be partitioned into a set of splits and the resulting networks visualize both compatible and incompatible

splits. In this way, possible conflict in the dataset will be visualized, leading to a more detailed presentation of the phylogenetic information at the population level (Posada & Crandall 2001). The NeighborNet analyses were performed using the Jukes Cantor model to generate distance measures on each DNA region independently, and on a concatenated dataset of the three nuclear markers. For the concatenated dataset 1000 bootstrap replicates were run. All datasets were also analyzed by Neighbor Joining (NJ) analyses to visualize the presence of heterozygote sites in the dataset, as heterozygous sequences tend to cluster intermediately between the homozygous alternatives. NJ analyses were conducted in PAUP* 4.0b10 (Swofford 2000) with 1000 bootstrap replicates were run.

The program PHASE as implemented in DNASP version 5 (Librado & Rozas 2009) was used to convert all the diplophase sequences into hypothetic haplophase data prior to haplotype network analysis. Haplotype networks were then calculated for each DNA region using ARLEQUIN version 3.5 (Excoffier *et al.* 2005), but the resulting haplotype networks were judged as too complex to be visualized and these results are therefore not shown.

Results

Characteristics of the four analyzed DNA regions are given in Table 1. Results from the NeighborNet and NJ analyses are given in Figs. 1 and 2, respectively. Results of a NeighborNet analysis of the concatenated dataset is given in the Appendix.

The mtLSU dataset

The NeighborNet analysis of the mtLSU dataset revealed four main groups, referred to as A-D in Fig. 1a. Out of the 17 individuals in group A, 14 are from North America, 1 is from Cuba, and 2 are from Europe. Group B includes specimens from Norway, Finland and Russia. It is noteworthy that all members of group D are of Norwegian origin. Group C primarily includes samples from Fennoscandia, with the exception of one specimen from Russia and one from North America. The NeighborNet analysis of this dataset demontrates the occurrence of conflicting splits. In the NJ analysis (Fig. 2) only three clusters were recognized, corresponding to group A, D and C. Specimens from group B form a paraphyletic group with the exclusion of groups A and C. The splitting of group B into two groups may be due to an artefact of the tree building method, as identical individuals are located at different positions in the tree. Nevertheless, the two analyses generally concurred.

The nrITS dataset

Four clusters (named A-D in Fig. 1a) also appeared in the nrITS NeighborNet analysis, as seen in the mtLSU analysis. Two groups (B and C) were linked to the outgroup, while the remaining groups (A and D) protrude from groups B and C, respectively. Both groups A and B include specimens from the entire distribution area, while groups C and D are restricted to Europe. The NJ tree (Fig. 2) separated the specimens into two different groups that correspond to the geographic separation between groups A and B, and groups C and D depicted in the NeighborNet analysis. The various groups detected were not supported in the bootstrap analysis

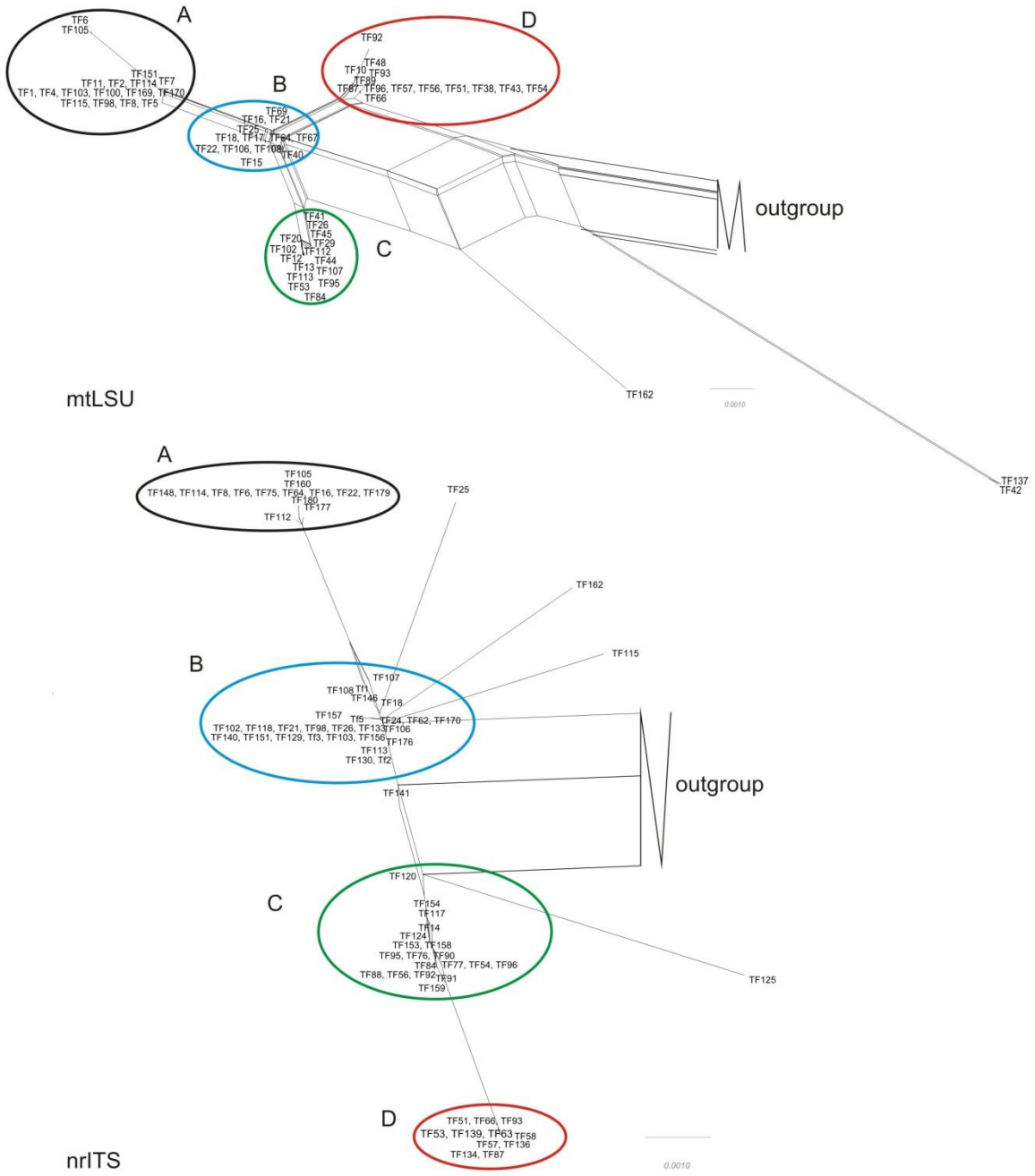

Fig. 1a.

Figure 1. Results from NeighborNet analyses. a. Splits trees of mtLSU and nrITS. Four groups were detected in both regions, named A-D. Groups A, B and mtLSU group C have a circumboreal distribution while groups C (ITS) and D are distributed in Europe. However, the mtLSU group C includes specimens that goes into all the four groups in the nrITS analysis. b. Splits trees for nrIGS and nrLSU. Three groups were detected in both regions, indicated with A-C. The group A are widely distributed in both regions, while group B and nrLSU group C are European. nrIGS group C has an Eurasian distribution.

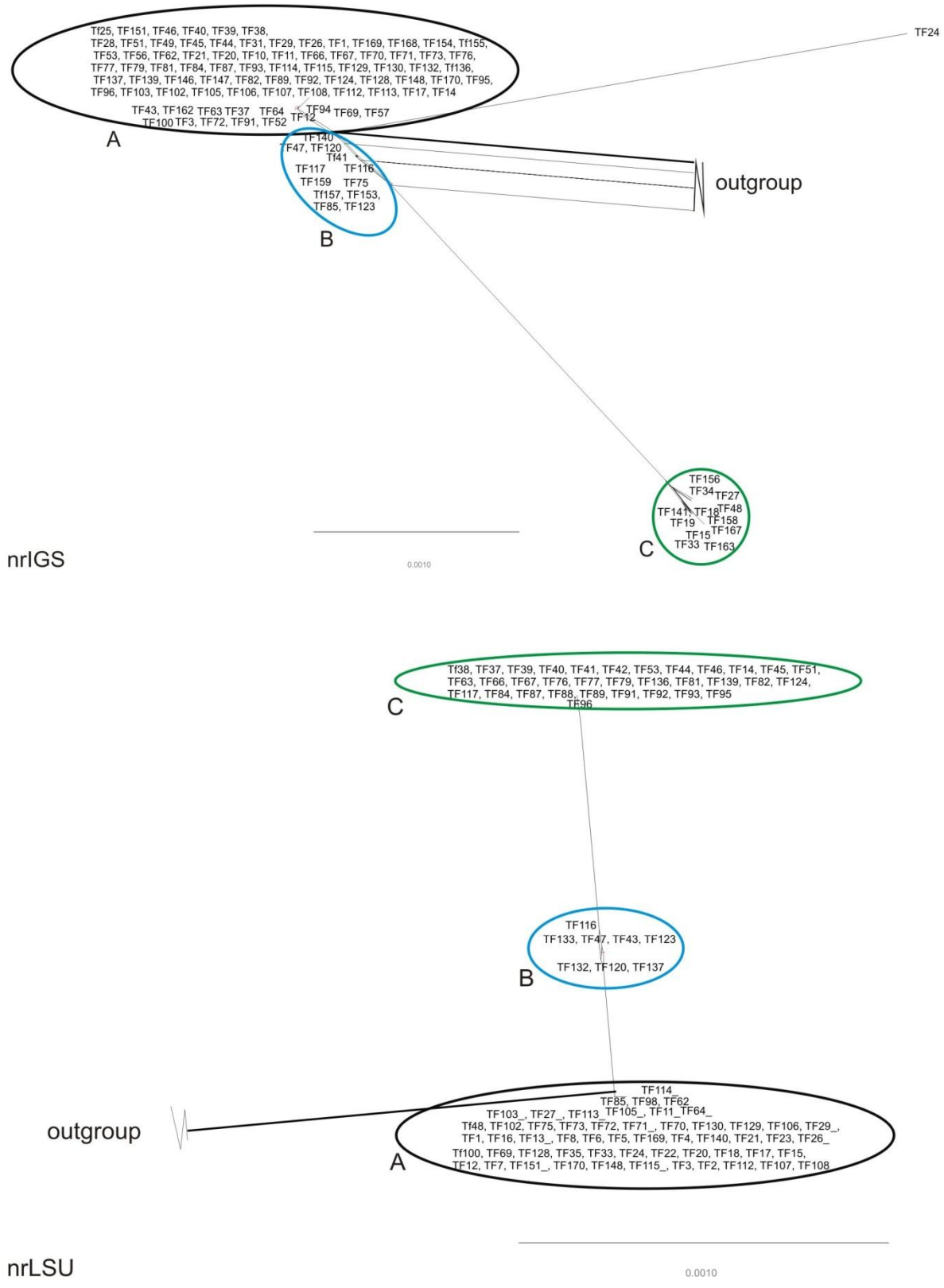
Tf25, TF151, TF46, TF40, TF39, TF38,
TF28, TF51, TF49, TF45, TF44, TF31, TF29, TF26, TF1, TF169, TF168, TF154, Tf155,
TF53, TF56, TF62, TF21, TF20, TF10, TF11, TF66, TF67, TF70, TF71, TF73, TF76,
TF77, TF79, TF81, TF84, TF87, TF93, TF114, TF115, TF129, TF130, TF132, Tf136,
TF137, TF139, TF146, TF147, TF82, TF89, TF92, TF124, TF128, TF148, TF170, TF95,
TF96, TF103, TF102, TF105, TF106, TF107, TF108, TF112, TF113, TF17, TF14
TF43, TF162 TF63 TF37 TF64 TF94 TF69, TF57
TF100 TF3, TF72, TF91, TF52 TF12
A
TF24
TF140
TF47, TF120
Tf41
TF117 TF116
TF159 TF75
Tf157, TF153,
TF85, TF123
B
outgroup
TF156
TF34 TF27
TF141 TF18 TF48
TF19 TF158
TF15 TF167
TF33 TF163
C
nrIGS
0.0010
Tf38, TF37, TF39, TF40, TF41, TF42, TF53, TF44, TF46, TF14, TF45, TF51,
TF63, TF66, TF67, TF76, TF77, TF79, TF136, TF81, TF139, TF82, TF124,
TF117, TF84, TF87, TF88, TF89, TF91, TF92, TF93, TF95
TF96
C
TF116
TF133, TF47, TF43, TF123
TF132, TF120, TF137
B
TF114_
TF85, TF98, TF62
TF103_, TF27_, TF113_TF105_, TF11_TF64_
Tf48, TF102, TF75, TF73, TF72, TF71_, TF70, TF130, TF129, TF106, TF29_,
TF1, TF16, TF13_, TF8, TF6, TF5, TF169, TF4, TF140, TF21, TF23, TF26_
Tf100, TF69, TF128, TF35, TF33, TF24, TF22, TF20, TF18, TF17, TF15,
TF12, TF7, TF151_, TF170, TF148, TF115_, TF3, TF2, TF112, TF107, TF108
A
outgroup
nrLSU
0.0010

Fig.1b.

The nrLSU dataset

Three distinct groups (referred to as A-C in Fig. 1b) appeared in the nrLSU NeighborNet analysis. The majority of the specimens were included in the widely distributed group A, which is connected to the outgroup. Both groups B and C are limited to Europe. Group B is united due to the presence of one heterozygote site which included the allelic versions from the A and C groups. The same three groups were also present in the NJ analysis with low bootstrap support (Fig. 2). Again, the delimiting of specimens into pure European groups and a wider distributed group is consistent with the nrITS dataset and partly with the mtLSU dataset.

The nrIGS dataset

Three main groups (referred to as A-C in Fig. 1b) appeared in the nrIGS NeighborNet analysis. The two distinct clusters A and C appeared distantly related, with group B in an intermediate position and most closely connected to the outgroup. The B group comprises a European cluster with 12 specimens, while the majority of the individuals belong to the widely distributed group A. The well-defined C group includes specimens from Eurasia. The NJ-analysis (Fig. 2) is consistent with the NeighborNet analysis. The NJ analysis splits group B into two clusters, which is again likely an artefact due to the tree building method. Sequences in group B are placed in-between group A and C due to the presence of a heterozygote site, which seems to be due to a mixture of haplotypes from group A and C. The nrIGS marker is not fully consistent with the delimitation between Europe and the widely distributed circumboreal groups and the presence of a separate Eurasian cluster is also unique.

A NeighborNet analysis of the concatenated dataset gave four subgroups similar to those obtained in the ITS analysis (see Appendix).

Host affinities

The majority of specimens in the European group were derived from *Pinus* (>90%). Specimens included in the widely distributed circumboreal groups were also collected on *Abies*, *Picea* and *Tsuga*.

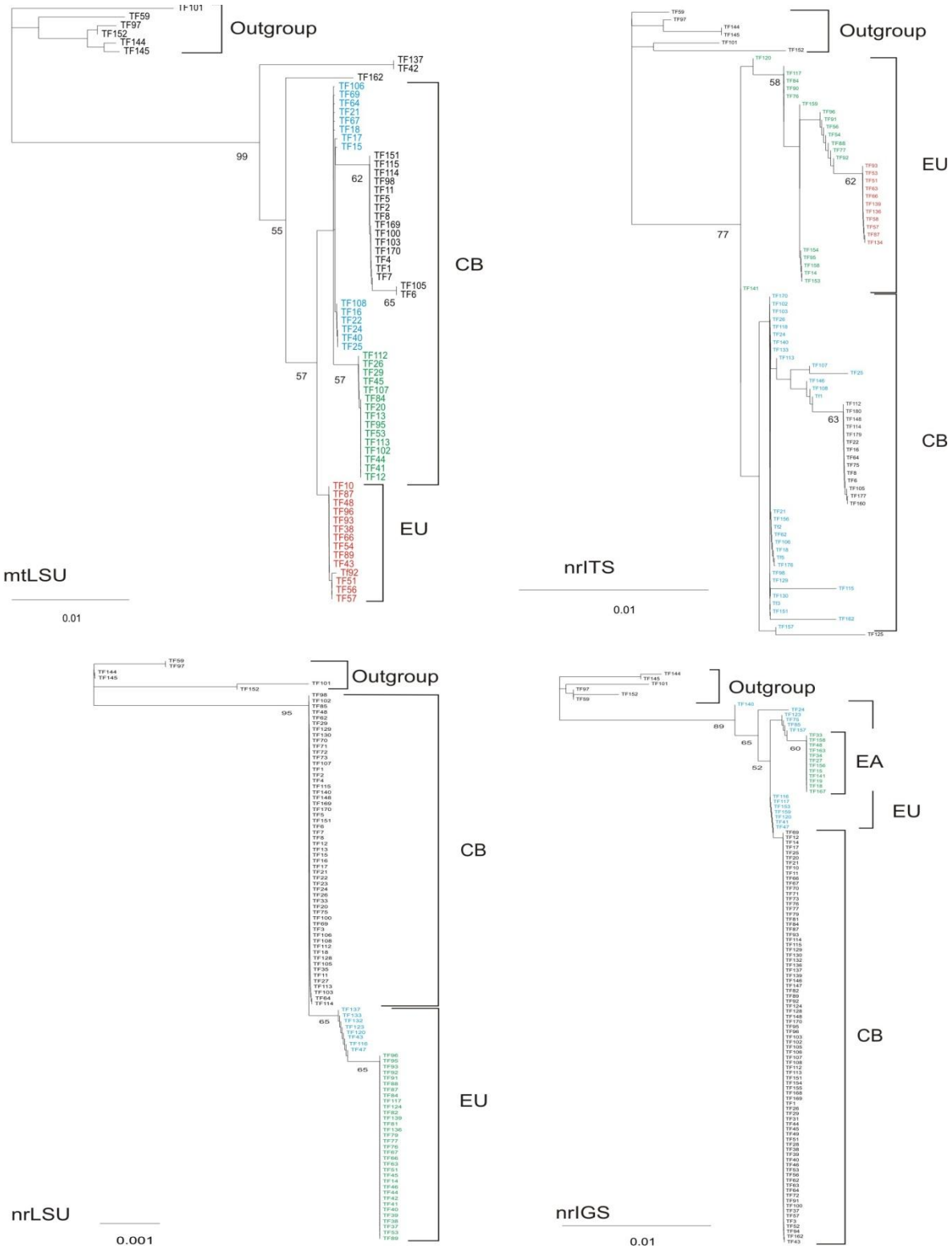

Figure 2. Neighbor-joining (NJ) trees of the mtLSU, nrITS, nrLSU and nrIGS regions. Bootstrap values >50 are given below branches. The same colors as in Fig. 1 are used to indicate subgroups (black = group A, blue = group B, green = group C, and red = group D). CB = circumboreal, EU = European, EA = Eurasian.

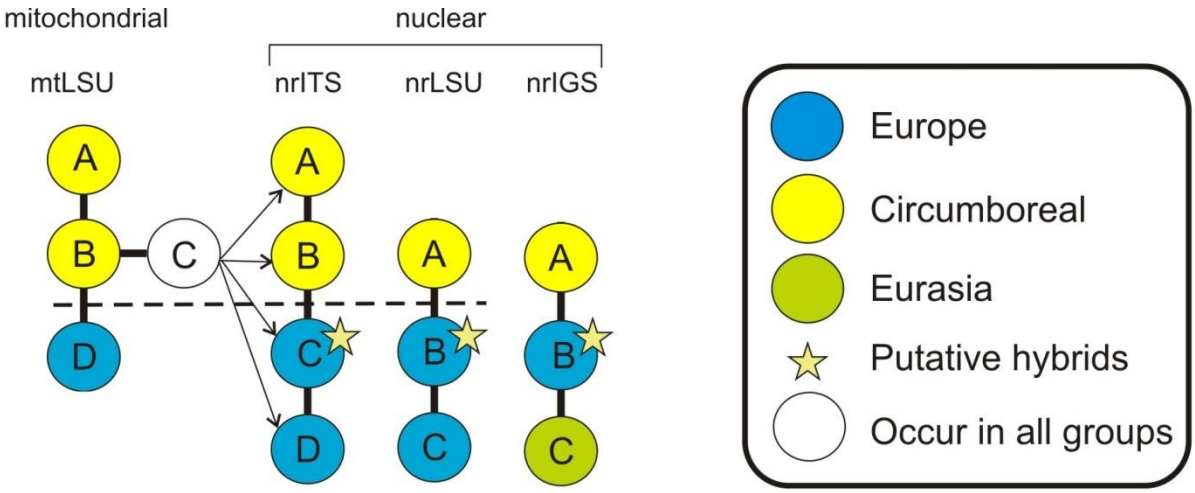

Figure 3. A rough summary of the main patterns revealed by the NeighborNet analyses. Each circle represents the different sub-groups detected by the four regions and the lines indicate the genetic relationship among them. The dotted line represent the delimitation between the European (EU) and the circumboreal (CB) main group. The arrows indicates that mtLSU group C is present in all the four groups in the nrITS region. The different colors reflect the geographic distribution of the groups.

Table 1. Characteristics of the four analyzed DNA regions

Locus	ntaxa	nchar	var.char.	var.inform.
nrITS	76	291	7	3
nrLSU	96	840	1	1
nrIGS	106	329	2	1
mtLSU	63	564	12	10

Discussion

In this study, the phylogeographic pattern in the morphotaxon *T. fuscoviolaceum* has been investigated using sequencing of four different DNA regions. However, it must be emphasized that three of the regions, nrIGS, nrITS and nrLSU, are physically linked in the nrDNA cistron and do not segregate independently.

Overall, a complex phylogeographic pattern appears in *T. fuscoviolaceum.* The main patterns in the data are summarized in Fig. 3. Two of the regions (nrIGS and nrLSU) identified three main groups while in the two other regions (mtLSU and nrITS) the sequences clustered into four main groups. Both nrITS and nrLSU identified two European groups, while mtLSU identified one European group. The nrLSU region identified one circumboreal group, while this group was split into two circumboreal groups in the nrITS analyses. One group (C) in the mtLSU region belonged to both the European and the circumboreal group defined by nrLSU and nrITS, so its origin is not that evident, but it seems to belong to the circumboreal group based on the NJ analysis. Furthermore, one circumboreal group and one European group were identified with nrIGS, while another group had a Eurasian distribution. Hence, although the resolution varied somewhat, the nrITS, nrLSU and mtLSU regions were to a great extent congruent, segregating into a European (hereafter referred to as EU) and a circumboreal group (hereafter referred to as CB), with nrIGS deviating somewhat from this pattern. The nrIGS region is closely linked to nrITS and nrLSU and it is therefore somewhat surprising that nrIGS gives a divergent phylogenetic pattern.

Noteworthy, only the mitochondrial region show alternative connections between the subgroups in the NeighborNet analysis. Intra-locus recombination may give rise to this kind of pattern, but recombination is not expected to occur in the mitochondrial genome, due to uniparental inheritance. However, Saville *et al.* (1998) have shown that in natural populations of *Armillaria gallica* mitochondrial recombination may occur. Alternatively, but less likely, the observed pattern may be due to reoccurrency mutations within this region leading to homoplasy.

The origin of various geographic groups

The EU group is distributed in Norway and Sweden, in eastern Europe and Spain. As *T. fuscoviolaceum* is a saprotrophic basidiomycete, it is dependent on its hosts. The changing climate throughout the Quaternary (last 2.4 million years) (Hewitt 2001; Hubberten *et al.* 2004; Svendsen *et al.* 2004) has affected host tree distributions. *Pinus sylvestris* is the main host for the EU group and a phylogeographic analysis supports the hypothesis of a recent common origin of *P. sylvestris* in northern Europe from a southern European refugium (Soranzo *et al.* 2000). As pointed out by Bennett et al. (1991), *P. sylvestris* was probably present close to the ice margin. The northward migration of the host made it possible for *T. fuscoviolaceum* to also extend its range northwards. The presence of a unique Norwegian *T. fuscoviolaceum* mtLSU haplotype indicates that a founder event may have happened during the range expansion into this region.

A sub-structuring of the EU group was detected in two of the nuclear regions (nrITS and nrLSU). Substructure within the nrLSU marker and the presence of the nrIGS EU sub-group can be fully explained by introgression of alleles from the CB group leading to heterozygous individuals clustering into a sub-group (see below). However, in the nrITS region, one of the EU sub-groups (D) is apparently derived from the other and was only sampled from *P. sylvestris*. The other sub-group (C) is also collected from *P. pinaster* and *P. halepensis* and includes specimens from Southern Europe (including Spain). In theory, this group could represent the ancestral population from which group D spread northwards. Alternatively, the two groups could reflect origin from different refugia. Notably, some specimens included alleles from both EU sub-groups, which could be a result of secondary contact between them.

The CB group is distributed over Central and North America and throughout Asia to Europe. Some substructure is also found within the CB group, but it is interesting that there is no distinct separation between Eurasian and North American samples. The CB group occurs on various hosts, including both North American conifers such as *Abies balsamea* and Asian conifers such as *Pinus sibirica*. This indicates that the CB group survived on both continents during the ice ages. This hypothesis is supported by the mtLSU marker as one mitochondrial CB sub-group is mainly North American (A), one is Eurasian (B) and the last (C) is mainly distributed in Fennoscandia. Some specimens included alleles from both CB sub-groups, indicating secondary contact between the two sub-groups. The presence of multiple rare

genotypes associated with one of the nrITS CB sub-groups (B), indicates additional genetic diversity in this widely distributed group.

The observation of two geographic groups in *T. fuscoviolaceum*, one mainly European and the other circumboreal, parallels the phylogeographic pattern observed in the wood-inhabiting fungus *Gloeoporus taxicola*, where a similar structure appeared (Seierstad 2009). A wide distribution pattern is also seen in the basidiomycete *Heterobasidion annosum* complex where the intersterility group connected to *Pinus* is genetically differentiated between North America and Eurasia, and further substructuring within North America is detected (Linzer *et al.* 2008). Similarly, the ectomycorrhizal morphotaxon *Amanita muscaria* is widely distributed, and includes two cryptic species with an Eurasian distribution and one cryptic species distributed in North America (Geml *et al.* 2006).

Secondary contact and hybridization

Range expansions of the two evolutionary groups of *T. fuscoviolaceum* have apparently led to secondary contact between them and sympatric occurrence in parts of Europe. Since *T. fuscoviolaceum* is an outcrossing fungus, there is possibility for hybridization in these areas. The nrIGS, nrITS and nrLSU regions indicate that hybridization occurs, as both EU and CB alleles appeared admixed in some specimens. It is noteworthy that the presence of these heterozygous individuals were the only reason why two European sub-groups appeared in nrLSU and nrIGS analyses. The sub-structuring in these two regions can fully be explained by introgression between the CB and EU groups. However, the potential hybrid specimens in *T. fuscoviolaceum* were not heterozygous across all DNA regions; several of the specimens clustered either in CB or EU in other regions. This inconsistency could be a result of repeated backcrossing and recombination, implying that the investigated specimens are persistent hybrids and do not represent first generation hybrids. Although hybridization is a common phenomenon in plants, it has rarely been recognized in fungi. However, hybridization has been detected in the root rot pathogen *Heterobasidion annosum* (Garbelotto *et al.* 1996), while Hughes and Petersen (2001) discovered a putative interspecific hybrid between *Flammulina velutipes* and *F. rossica*. In a study of *Ophistoma ulmi* and *O. novo-ulmi*, hybrids appeared very infrequently, but nevertheless functioned as a genetic link between the two taxa (Brasier *et al.* 1998). Also in *Gloeoporus taxicola*, putative hybrids were observed in Europe where the two sub-groups had overlapping ranges (Seierstad 2009).

Alternatively, the observed pattern is also compatible with a recent speciation event in Europe leading to the EU and CB groups, followed by rapid worldwide range expansion of the CB group.

A further step to separate between the different evolutionary scenarios could be to conduct mating studies between the different populations. A higher interfertility rate between allopatric compared to sympatric populations of recently diverged lineages has been reported in several fungi (Le Gac & Giraud 2008). Using an experimental approach, Dettman et al. (2003b) observed higher fertility between allopatric compared to sympatric species of *Neurospora*. In *Trichaptum abietinum* it was demonstrated that two intersterility groups in North America were partly interfertile with a European group (Macrae 1967). The same pattern also appeared in crossings between cryptic lineages in the *Heterobasidion annosum* complex (Korhonen 1978). Whether the same also holds true for *T. fuscoviolaceum*, remains to be investigated.

Substrate

The CB group is connected to several substrates while *Pinus* species are the main hosts for the EU group. In a field survey of wood-inhabiting basidiomycetes, generalists on *Pinus* and *Picea* in China had a tendency of substrate specialization in Fennoscandia (Dai & Penttila 2006). At least in the EU group there seems to be a host specialisation in Europe, which could lower the viability of CB x EU hybrids through reduced fitness (i.e. reinforcement of barriers). However, the greater host range in North American and Asian CB populations could also just be a consequence of wider host availability compared to Europe. Interestingly, the very same pattern as observed in *T. fuscoviolaceum* was seen in the saptrotrophic basidiomycete *Gloeoporus taxicola*; one cirumboreal lineage was found on a number of hosts, while the European lineage were mostly connected to *Pinus* (Seierstad 2009).

Concluding remarks

Based on current data, there seem to be two main evolutionary groups present within *T. fuscoviolaceum*: one European and another widely distributed circumboreal group. The EU group has apparently recolonized northern Europe from one or several southern European

refugia, while the CB group may have expanded into Europe from the northeast, leading to secondary contact in Europe. Lack of complete intersterility barriers has apparently led to hybridization and subsequent introgression between these groups. Although judged less likely, a recent speciation event in Europe could also have given rise to a similar genetic pattern. Mating experiments in combination with additional genetic markers with higher resolution (e.g. microsatellites or AFLPs) might be valuable tools to discern between the different scenarios. Analyses of additional genetic markers that segregate independently from the nrDNA markers used herein are also needed to fully conclude whether the two main groups (CB and EU) represent two cryptic species.

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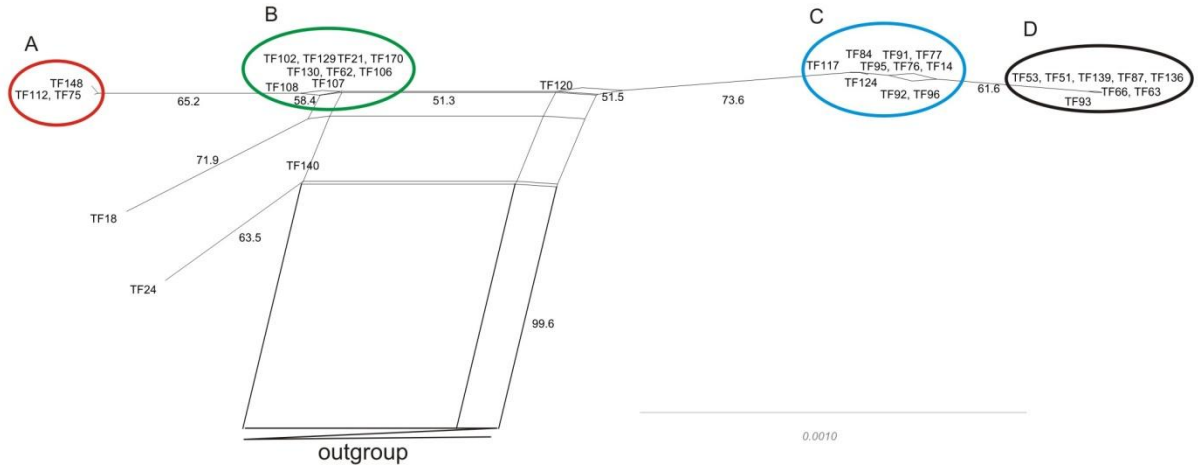
Appendix



Splits tree of a concatenated nrDNA dataset including ITS, IGS and LSU. Four main groups were detected named A-D. Groups A and B belong to a circumboreal (CB) group, and groups C and D belong to a European (EU) group. Bootstrap values >50 are given next to the connections.

Appendix. List of specimens included in the study. Crosses indicate sequences obtained for each individual. Each individual has a lab code number that can be recognized in the phylogenetic analyses.

Lab code	Isolate code	Herbarium	Origin	Substrate	nrITS	nrIGS	nrLSU	mtLSU
TF1	DAOM53076	Culture	Canada	*Abies balsamea*	x	x	x	x
TF2	DAOM53117	Culture	Canada	*Abies balsamea*	x		x	x
TF3	DAOM72244B	Culture	Canada	*Tsuga canadensis*	x	x	x	
TF4	DAOM53116	Culture	Canada	*Abies balsamea*			x	x
TF5	DAOM53118	Culture	Canada	*Abies balsamea*	x		x	x
TF6	F7521	Culture	Estonia	*Pinus sylvestris*	x		x	x
TF7	DAOM72244A	Culture	Canada	*Tsuga canadensis*			x	x
TF8	DAOM53300	Culture	Canada	*Tsuga canadensis*	x		x	x
TF10	DAOM53127	Culture	Norway	*Pinus sylvestris*		x		x
TF11	DAOM9517	Culture	Canada	*Abies balsamea*		x	x	x
TF12	10943.1	Culture	Finland	Unknown		x	x	x
TF13	OM10944.1	Culture	Finland	Unknown			x	x
TF14	DOOM2788.2	Culture	Sweden	Unknown	x	x	x	
TF15	HK11	Culture	Russia	Unknown		x	x	x
TF16	OM10946.2	Culture	Finland	Unknown	x		x	x

Lab code	Isolate code	Herbarium	Origin	Substrate	nrITS	nrIGS	nrLSU	mtLSU
TF17	OM10816	Culture	Russia	Unknown		x	x	x
TF18	HK12	Culture	Russia	Unknown	x	x	x	x
TF19	HK10	Culture	Russia	Unknown		x		
TF20	OM10813	Culture	Russia	Unknown		x	x	x
TF21	HK1	Culture	Russia	Unknown	x	x	x	x
TF22	M10942.1	Culture	Finland	Unknown	x		x	x
TF23	OM10812	Culture	Russia	Unknown			x	
TF24	OM10912.1	Culture	Finland	Unknown	x	x	x	x
TF25	OM10817	Culture	Russia	Unknown	x	x		x
TF26	HK7	Culture	Russia	Unknown	x	x	x	x
TF27	M10808.2	Culture	Russia	Unknown		x	x	
TF28	niemelä 6568	Culture	Finland	Unknown		x		
TF29	niemelä 6694	Culture	Finland	Unknown		x	x	x
TF31	om10416	Culture	China	Unknown		x		
TF33	oht17	Culture	Japan	Unknown		x	x	
TF34	oht19	Culture	Japan	*Abies firma*		x		
TF35		Culture	Norway	*Pinus sylvestris*			x	

Lab code	Isolate code	Herbarium	Origin	Substrate	nrITS	nrIGS	nrLSU	mtLSU
TF37		Culture	Norway	*Pinus sylvestris*		x	x	
TF38		Culture	Norway	*Pinus sylvestris*		x	x	x
TF39		Culture	Norway	*Pinus sylvestris*		x	x	
TF40		Culture	Norway	*Pinus sylvestris*		x	x	x
TF41		Culture	Norway	*Pinus sylvestris*		x	x	x
TF42		Culture	Norway	*Pinus sylvestris*			x	x
TF43		Culture	Norway	*Pinus sylvestris*		x	x	x
TF44		Culture	Norway	*Pinus sylvestris*		x	x	x
TF45		Culture	Norway	*Pinus sylvestris*		x	x	x
TF46		Culture	Norway	*Pinus sylvestris*		x	x	
TF47		Culture	Norway	*Pinus sylvestris*		x	x	
TF48		Culture	Norway	*Pinus sylvestris*		x	x	x
TF49	OM12537	Culture	Bulgaria	Unknown		x		
TF51	O 145859	O	Norway	*Pinus* sp.	x	x	x	x
TF52	O 242064	O	Norway	*Pinus* sp.		x		
TF53	O 284408	O	Norway	*Pinus* sp.	x	x	x	x
TF54	O 105837	O	Norway	*Pinus* sp.	x			x

Lab code	Isolate code	Herbarium	Origin	Substrate	nrITS	nrIGS	nrLSU	mtLSU
TF56	O 230777	O	Norway	*Pinus sylvestris*	x	x		x
TF57	O 67021	O	Norway	*Pinus sylvestris*	x	x		x
TF58	O 283649	O	Norway	*Pinus sylvestris*	x			
TF62	O 83535	O	Norway	*Pinus* sp.	x	x	x	
TF63	O 60068	O	Norway	Unknown	x	x	x	
TF64	O 223518	O	Norway	*Pinus* sp.	x	x	x	x
TF66	O 171137	O	Norway	*Pinus* sp.	x	x	x	x
TF67	O 159382	O	Norway	*Pinus* sp.		x	x	x
TF69	O 283714	O	Norway	*Pinus* sp.		x	x	x
TF70	O 145861	O	Norway	*Pinus* sp.		x	x	
TF71	O 145875	O	Norway	*Pinus sylvestris*		x	x	
TF72	O145870	O	Norway	*Pinus* sp.		x	x	
TF73	O145871	O	Norway	*Pinus* sp.		x	x	
TF75	O 145874	O	Norway	*Pinus* sp.	x	x	x	
TF76	O 145782	O	Norway	*Pinus* sp.	x	x	x	
TF77	O 82585	O	Norway	*Pinus* sp.	x	x	x	
TF79	O 145887	O	Sweden	Unknown		x	x	

Lab code	Isolate code	Herbarium	Origin	Substrate	nrITS	nrIGS	nrLSU	mtLSU
TF81	O 145884	O	Sweden	Unknown		x	x	
TF82	O 145785	O	Norway	*Pinus* sp.		x	x	
TF84	O 159867	O	Norway	*Pinus* sp.	x	x	x	x
TF85		O	Spain	Unknown		x	x	
TF87	O 170121	O	Norway	*Pinus* sp.	x	x	x	x
TF88	O 145786	O	Norway	*Pinus* sp.	x		x	
TF89	O 145789	O	Norway	*Pinus* sp.		x	x	x
TF90	O 145812	O	Norway	*Pinus* sp.	x			
TF91	O 145813	O	Norway	*Pinus* sp.	x	x	x	
TF92	O 82589	O	Norway	*Pinus* sp.	x	x	x	x
TF93	O 82588	O	Norway	*Pinus* sp.	x	x	x	x
TF94	O 145774	O	Norway	*Picea* sp.		x		
TF95		Culture	Norway	*Pinus* sp.	x	x	x	x
TF96		Culture	Norway	*Pinus* sp.	x	x	x	x
TF98	FP-103470-Sp	Culture	USA	Unknown	x		x	x
TF100	HHB-245-Sp	Culture	USA	*Pinus* sp.		x	x	x
TF102	L-15382-Sp	Culture	USA	*Abies balsamea*	x	x	x	x

Lab code	Isolate code	Herbarium	Origin	Substrate	nrITS	nrIGS	nrLSU	mtLSU
TF103	MJL-3547-Sp	Culture	Canada	*Abies* sp.	x	x	x	x
TF105	B73,OMC505	Culture	Norway	Unknown	x	x	x	x
TF106	B7,OMC503	Culture	Norway	Unknown	x	x	x	x
TF107	C18,OMC504	Culture	Norway	Unknown	x	x	x	x
TF108	13284,OMC508	Culture	Finland	Unknown	x	x	x	x
TF112	1699	Culture	Finland	*Pinus* sp.	x	x	x	x
TF113	1894	Culture	Sweden	*Pinus* sp.	x	x	x	x
TF114	TJV-94-24	CFMR	USA	*Abies fraseri*	x	x	x	x
TF115	DLF95-26	CFMR	USA	*Abies balsamea*	x	x	x	x
TF116	MA-Fungi 1034	MA	Spain	*Pinus* sp.		x	x	
TF117	MA-Fungi 7516	MA	Spain	*Pinus* sp.	x	x	x	
TF118	MA-Fungi 23444	MA	Spain	*Pinus pinaster*	x			
TF120	MA-Fungi 33486	MA	Spain	*Pinus pinaster*	x	x	x	
TF123	MA-Fungi 69221	MA	Spain	*Pinus halepensis*		x	x	
TF124	MA-Fungi 44345	MA	Spain	*Pinus halepensis*	x	x	x	
TF125	MA-Fungi 44793	MA	Spain	*Pinus* sp.	x			
TF128	DAOM-53116	NY	Canada	*Abies balsamea*		x	x	

Lab code	Isolate code	Herbarium	Origin	Substrate	nrITS	nrIGS	nrLSU	mtLSU
TF129	NY-00520148	NY	USA	*Pinus* sp.	x	x	x	
TF130	NY-00520147	NY	USA	*Pinus* sp.	x	x	x	
TF132	WU 3133	WU	Austria	*Pinus* sp.		x	x	
TF133	WU 7475	WU	Austria	*Pinus* sp.	x		x	
TF134	WU 9093	WU	Austria	*Pinus* sp.	x			
TF136	WU 13561	WU	Austria	*Pinus* sp.	x	x	x	
TF137	WU 13795	WU	Austria	*Pinus* sp.		x	x	x
TF139	M-0147770	M	Sweden	*Pinus sylvestris*	x	x	x	
TF140	M-0147771	M	Yugoslavia	*Pinus halepensis*	x	x	x	
TF141	M-0147772	M	Spain	*Pinus halepensis*	x	x		
TF146	M-0147777	M	Slovakia	*Picea abies*	x	x		
TF147	M-0147778	M	Germany	*Pinus sylvestris*		x		
TF148	M-0147779	M	Germany	Unknown	x	x	x	
TF151	MUCL44123	Culture	Cuba	Unknown	x	x	x	x
TF153	CNF 6/350	CNF	Croatia	*Pinus* sp.	x	x		
TF154	CNF 6/351	CNF	Croatia	*Pinus* sp.	x	x		
TF155	CNF 6/352	CNF	Croatia	*Pinus* sp.		x		

Lab code	Isolate code	Herbarium	Origin	Substrate	nrITS	nrIGS	nrLSU	mtLSU
TF156	CNF 6/353	CNF	Croatia	*Pinus halepensis*	x	x		
TF157	CNF 6/354	CNF	Croatia	*Pinus halepensis*	x	x		
TF158	CNF 6/355	CNF	Croatia	*Pinus halepensis*	x	x		
TF159	CNF 6/356	CNF	Croatia	*Pinus* sp.	x	x		
TF160	K(M) 20548	K	Slovakia	*Picea abies*	x			
TF162	K(M) 109409	K	Belize	*Pinus caribaea*	x	x		x
TF163	K(M) 98043	K	Japan	*Pinus densiflora*		x		
TF167	BPI 841892	BPI	Japan	Unknown		x		
TF168	DAOM 197633	DAOM	Canada	*Abies balsamea*		x		
TF169	DAOM 232297	DAOM	Canada	Unknown		x	x	x
TF170	DAOM 231501	DAOM	Canada	Unknown	x	x	x	x
TF176	LE235538	LE	Russia	*Pinus sibirica*	x			
TF177	LE203863	LE	Russia	Unknown	x			
TF179	LE206239	LE	Russia	Unknown	x			
TF180	LE234499	LE	Russia	Unknown	x			

Lab code	Isolate code	Herbarium	Origin	Substrate	nrITS	nrIGS	nrLSU	mtLSU
Outgroup								
TF59		O 65466	Norway	*Picea* sp.	x	x	x	x
TF97	ECS-3133	CFMR	Unknown	Unknown	x	x	x	x
TF101	L-15378-Sp	Culture	USA	*Abies balsamea*	x	x	x	x
TF144	M-0147775	M	Spain	*Pinus* sp.	x	x	x	x
TF145	M-0147776	M	Spain	*Pinus* sp.	x	x	x	x
TF152	MUCL51615	Culture	China	Unknown	x	x	x	x

(O) - University of Oslo, (CFMR) - Forest Service, Northern Research Station,USA, (MA) - Madrid herbarium, (NY) - New York Botanical Garden, (WU) - University of Wien, (M) - München herbarium, (CNF) - Croatian Mycological Society, (K) - Royal Botanical Garden, Kew, (BPI) - National Fungus Collections, USA, (DAOM) - Ottawa herbarium, (LE) - Komarov Botanical Institute, St. Petersburg.