Saprotrophy and lichenization as options for the same fungal species on different substrata: environmental plasticity and fungal lifestyles in the *Stictis–Conotrema* complex

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Summary

• Lichenization is one of the most important fungal lifestyles and lichen fungi are assumed to form obligate symbioses with their photobionts. Here we test if lichenized and non-lichenized samples in three cases of closely related lichenized *Conotrema* and saprotrophic *Stictis* (Stictidaceae, Ostropales, Ascomycota) in northern Scandinavia, form distinct monophyletic groups (= species).

• We applied phylogenetic species recognition, by analysing fungal DNA sequence data from four independent genomic markers.

• Separate parsimony and parsimony jack-knifing analyses of three independent genes are congruent and result in intermixed groups of lichenized and saprotrophic specimens. The sequence variation in an intron also supports this. Our results suggest that all three cases represent independent fungal phylogenetic species, which can undergo their whole sexual lifecycle either as lichens or as saprotrophs.

• The use of different nutritional modes – optional lichenization – allows separate individuals to exploit different niches during the forest succession. We suggest that this environmental plasticity may be common in the Stictidaceae, and propose that it is an overlooked strategy in fungi adapted to unpredictable successional ecosystems.

Key words: boreal forest succession, ecological niches, environmental plasticity, fungi, lichenization, nutritional mode, phylogenetic species recognition, symbiosis.

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Introduction

Fungi are heterotrophic organisms, depending on the supply of carbohydrates from other living or dead organisms. Lichenization, the partnership between photosynthesizing green algae or cyanobacteria and fungi, is one of the major sources of carbohydrates in fungi, with *c.* 20% of all known fungi being lichenized (Kirk *et al.*, 2001). It is clearly one of the most important types of symbiosis involving ascomycetes, where over 40% of the known species are lichenized (Kirk *et al.*, 2001). This mutualistic lifestyle has also been suggested as a possible way for the colonization of land in the course of evolution (Heckman *et al.*, 2001; Selosse, 2002). Not until recently, however, have the major patterns of lichen evolution become better understood (Gargas *et al.*, 1995; Lutzoni *et al.*, 2001; Wiklund & Wedin, 2003; Lumbsch *et al.*, 2004), and although most researchers agree that lichenization has evolved many times in the phylogeny of fungi, it has also been suggested that many non-lichenized ascomycete groups have lost the symbiotic connection with algae secondarily (Lutzoni *et al.*, 2001).

Although lichens are supposed to be obligate symbioses, there are scattered reports of fungi with a very casual lichenization, mainly involving genera well known as plant pathogens or saprotrophs (Hawksworth, 1988; Aptroot, 1991). There is also evidence that some lichen fungi may reproduce

asexually during the short time interval between germination and the lichenization event (Tschermak-Woess & Poelt, 1976), and it has been predicted – but not yet shown – that free-living asexual stages can be expected in certain pin-lichens (Tibell, 1997). The lichen fungi are facultative biotrophs in the sense that they can be cultivated separately from the algae (Ahmadjian, 1993; Stocker-Wörgötter, 2001), but they have not been observed to reproduce sexually under such non-lichenized conditions (Honegger, 1998). Furthermore, some familiar lichen genera include a few species that start their lives as non-lichenized lichen parasites, but eventually form independent lichens (Friedl, 1987; Hawksworth, 1988). Santesson (1967) suggested that some well-known lichens were closely related to saprotrophic fungi, and later Gilenstam (1969) classified both the lichenized genus *Conotrema* and the non-lichenized saprotrophic genus *Stictis* in the same family, the Stictidaceae (Ostropales, Ascomycota), based on detailed anatomical studies. The Stictidaceae is a large group of small, not easily noticeable and mainly saprotrophic discomycetes, where *Conotrema* and a few other genera are rather inconspicuous crustose lichen representatives (Sherwood, 1977). The generic delimitations in the Stictidaceae are very unclear, as indicated by Gilenstam (1969), and he also suggested that *Conotrema* and *Stictis* were probably congeneric.

When investigating recently collected Stictidaceae-material from aspen-stands (*Populus tremula*) in the boreal forest region, we discovered several potentially undescribed taxa, both lichens and saprotrophs, reflecting our poor knowledge of the northern Scandinavian microfungi. The only comparatively well-known species among those studied is *Conotrema populorum*, a rare and red-listed lichen (Thor & Arvidsson, 1999) growing in bark crevices of old aspen trunks in old-growth forests. Utilizing a molecular phylogeny to clarify the generic positions of our samples within the Stictidaceae (Wedin *et al.*, 2004), we were amazed to find that several sexually reproducing non-lichenized and lichenized taxa could not be distinguished when using variable parts of the nuclear and mitochondrial ribosomal DNA. If these taxa would indeed be different expressions of the same fungal species, then these fungi would apparently have two optional ways of life.

It has to our knowledge never been suggested that fungal species may have the potential to complete their entire sexual lifecycle either as saprotrophs or as distinct lichens. Here, we test whether three cases of closely related lichenized and non-lichenized taxa constitute distinct species, by using several independent gene genealogies for the purpose of phylogenetic species recognition/circumscription (Grube & Kroken, 2000; Taylor *et al.*, 2000). If different gene genealogies would result in congruent monophyletic groups corresponding to nutritional mode, these groups would be interpreted as distinct lichenized and non-lichenized phylogenetic species. If phylogenetically circumscribed species contain specimens of both nutritional modes, however, this would show that the nutritional mode is optional within a species.

Materials and Methods

Examined material and study site

We examined specimens of *Conotrema* growing on bark, and *Stictis*-colonies occurring on bark-free branches (i.e. decorticated wood), of *Populus tremula* in northern Scandinavia (Fig. 1). Fertile samples of *Conotrema* and *Stictis* were collected in the field, and in addition sampled from the herbarium at the Museum of Evolution, Uppsala University (UPS), Uppsala, Sweden. All our own collections are deposited in UPS (Table 1).

DNA extraction, PCR and sequencing

DNA was extracted and four genomic regions were amplified by PCR with fungal-specific primers and sequenced, following the general procedures given in Wiklund & Wedin (2003). Partial sequences of four independent genetic markers were generated: (i) the internal transcribed spacers and the adjacent 5′ part of the large subunit of the nuclear ribosomal RNA gene cluster (nITS-LSU rDNA) using the primers ITS1F, ITS3, ITS4 and LR3 (White *et al.*, 1990; Gardes & Bruns, 1993; Vilgalys, 1990, http://www.biology.duke.edu/fungi/mycolab/primers.htm) (ii) the mitochondrial small subunit ribosomal RNA gene (mtSSU rDNA) using the primers mrSSU1, mrSSU2, mrSSU2R and mrSSU3R (Zoller *et al.*, 1999) (iii) the glyceraldehyde-3-phosphate dehydrogenase gene (GPD; EC 1.2.1.12) using the primers gpd1, gpd1-LM and gpd2-LM (Berbee *et al.*, 1999; Myllys *et al.*, 2002), and (iv) the eukaryotic translation elongation factor 1-alpha gene (EF-1a) using the primers EF1–728F and EF1-986R (Carbone & Kohn, 1999). The nITS-LSU and the mtSSU rDNA were amplified with the following PCR cycle parameters: initial denaturation at 94°C for 5 min, then five cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 60 s. For the EF-1a the annealing temperature was raised to 58°C in the five starting cycles, followed by 35 cycles with 53°C. GPD was amplified with a touch-down profile: initial denaturation at 95°C for 4 min, followed by 10 cycles of 95°C for 30 s, annealing temperature starting with 65°C decreased by 1°C per cycle for 40 s decreased by 1 s per cycle, and 72°C for 90 s, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 90 s. All generated sequences are deposited in GenBank (Table 1).

Data analysis

The sequences were aligned by pairwise alignment in MacClade 4.06 OSX (Maddison & Maddison, 2003) and the alignments were optimized manually. The data sets were analysed separately with maximum parsimony and parsimony jack-knifing (Farris *et al.*, 1997) using PAUP* 4.0b10 (Swofford, 2002). All gaps were treated as missing data. Constant sites were excluded from phylogenetic analyses, and tree and branch lengths were

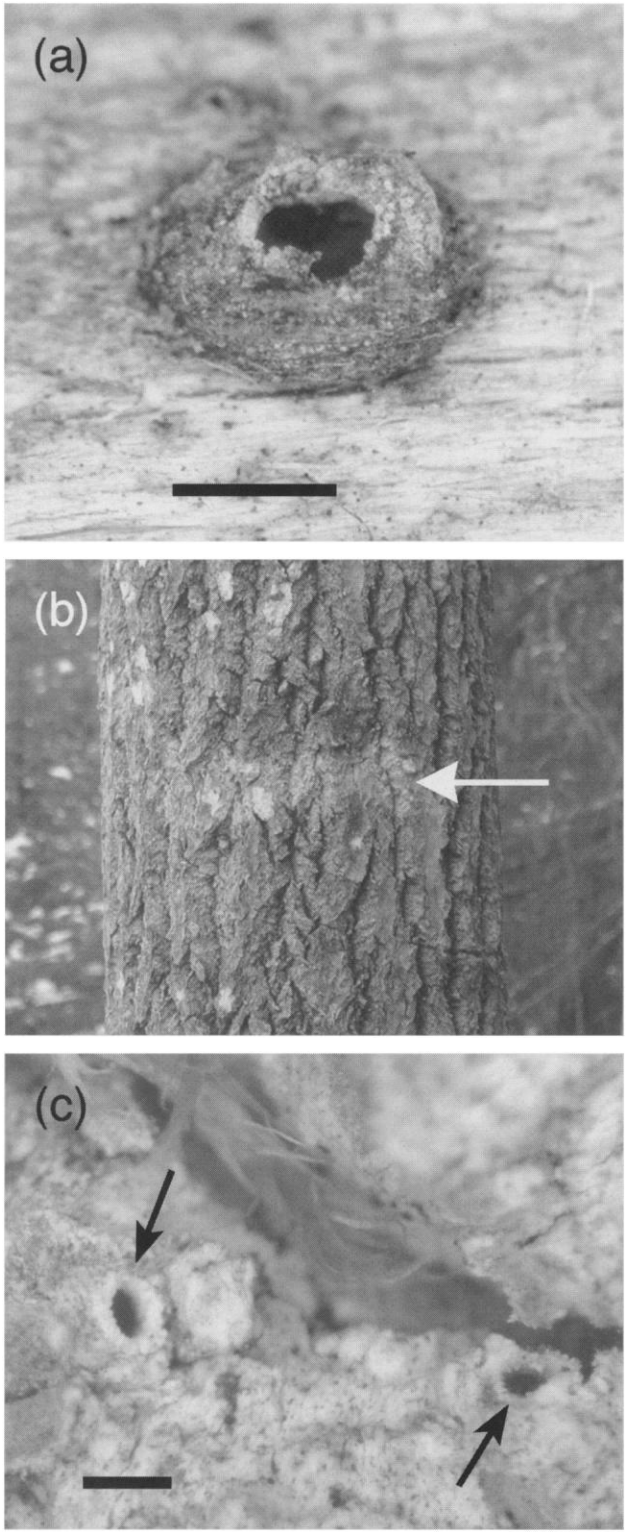

Fig. 1 Optional lichenization in *Stictis* species 2. (a) A fruitbody of a saprotrophic colony (bar, 1 mm) growing on a decorticated twig of *Populus tremula*. (b) The corresponding '*Conotrema*' lichen, forming white patches (arrow) around cracks in the thick bark of an older *Populus* trunk. The two genera *Stictis* and *Conotrema* were traditionally distinguished only by the presence of a lichen thallus containing algae in the latter. (c) Fruitbodies (arrows) of the lichenized colony (bar, 1 mm). The whitish area in which the fruitbodies develop is the lichen thallus, which contains the algal cells.

based on all variable sites. Parsimony settings: steepest descent off, collapse branches if minimum length is 0, character-state optimization ACCTRAN. Heuristic settings: 1000 random addition sequence replicates with start from random trees, TBR branch swap, multiple trees saved. The trees were rooted with specimens of the distantly related *Stictis radiata* as outgroup. The settings for parsimony jack-knifing in PAUP* were as in the parsimony analyses, with the following exceptions: 1000 jack-knife replicates with 'JAC' emulation, nominal deletion of characters 36.79% (emulating the procedure used in Farris *et al.*, 1997), full heuristic search with 10 random addition replicates per jack-knife replicate.

Results

For two data sets (nITS-LSU rDNA and mtSSU rDNA) we obtained sequences of 27 specimens representing 15 geographically separate localities, whereas for the two other data sets (GPD and EF-1a) it was not possible to obtain sequences for all specimens, due to amplification failure or sequencing problems. All data matrices are available online as Table S1 (see Supplementary Material section for details).

The phylogenetic trees obtained from the nITS-LSU rDNA and mtSSU rDNA data sets are shown in Fig. 2. The nITS-LSU rDNA data set comprises 1161 nucleotide positions with a total of 179 variable sites (160 parsimony-informative) and resulted in a single most-parsimonious tree of 205 steps (consistency index CI = 0.95, retention index RI = 0.99). The mtSSU rDNA data set comprises 786 nucleotide positions. A total of 116 variable sites (113 parsimony-informative) were included in the analyses, after 34 positions (a major insertion only occurring in the outgroup) were excluded. A single most-parsimonious tree of 130 steps (CI = 0.99, RI = 1.0) was obtained. Our samples form three identical, distinct clades in both analyses, with very little within-group sequence variation. In these three groups, the *Stictis*- and *Conotrema*-specimens are intermixed. In the remainder of this article, these clades (which are supported by all analyses and individual datasets and thus are interpreted as phylogenetic species) will be referred to as species 1, 2 and 3 (the latter including *Conotrema populorum*). In the parsimony jack-knife analyses all three clades gain 100% support in the separate nITS-LSU rDNA analysis. In the separate mtSSU rDNA analysis, species 1 and the group composed by species 2 and 3 gain 100% support, and species 2 and 3 get 66 and 69% jack-knife support, respectively.

The GPD data set (20 specimens) comprises 934 nucleotide positions, including two spliceosomal introns. An intron with a length of 45 or 49 nucleotides is present in all samples. This intron site is homologous to the third intron

Table 1 List of investigated specimens with collecting sites and GenBank accession numbers for the four genetic markers analysed

Species and life-style	Specimen	Origin	GenBank nrDNA	GenBank mtSSU	GenBank GPD	GenBank EF-1a
SPECIES 1						
lichenized *Conotrema* sp. 1	Gilenstam 2351	Sweden, Lycksele lappmark, Lycksele, Furuvik	AY527312	AY527341	AY527375	AY527296
lichenized *Conotrema* sp. 1	Gilenstam 2370	Sweden, Lycksele lappmark, Lycksele, Furuvik	AY527310	AY527339	AY527376	AY649845
lichenized *Conotrema* sp. 1	Wedin 6739	Sweden, Lycksele lappmark, Lycksele, Norrås	AY527311	AY527340	–	–
lichenized *Conotrema* sp. 1	Wedin 7193	Norway, Troms, Skibotn, Fieldstation	AY527314	AY527343	AY527379	AY649848
lichenized *Conotrema* sp. 1	Wedin 7200	Norway, Troms, Skibotn, Campingground	AY527313	AY527342	AY527378	AY649846
saprophytic *Stictis* sp. 1	Gilenstam 2425	Sweden, Lycksele lappmark, Lycksele, Furuvik	AY527319	AY527348	AY527373	–
saprophytic *Stictis* sp. 1	Gilenstam 2440c	Sweden, Lule lappmark, Jokkmokk par., Randijaur	AY527317	AY527346	AY527377	AY527294
saprophytic *Stictis* sp. 1	Gilenstam 2441c	Sweden, Lule lappmark, Jokkmokk par., Randijaur	AY527315	AY527344	AY527371	–
saprophytic *Stictis* sp. 1	Gilenstam 2445a	Sweden, Lycksele lappmark, Lycksele, Furuvik	AY527318	AY527347	AY527374	AY527295
saprophytic *Stictis* sp. 1	Gilenstam 2458b	Sweden, Lycksele lappmark, Lycksele, Furuvik	AY527316	AY527345	AY527372	AY527293
saprophytic *Stictis* sp. 1	Wedin 7209	Norway, Troms, Skibotn, Campingground	AY527320	AY527349	AY527380	AY649847
SPECIES 2						
lichenized *Conotrema* sp. 2	Nordin 3222	Sweden, Dalarna, Los, Mt.Lillkullen	AY527336	AY527365	–	AY527299
lichenized *Conotrema* sp. 2	Nordin 3233	Sweden, Dalarna, Hamra, Mt.Harjamägg	AY527335	AY527364	–	AY527301
lichenized *Conotrema* sp. 2	Gilenstam 2353	Sweden, Lycksele lappmark, Lycksele, Furuvik	AY527326	AY300882	AY649855	AY527300
lichenized *Conotrema* sp. 2	Gilenstam 2357	Sweden, Lycksele lappmark, Lycksele, Tannberget	AY527325	AY527354	–	–
lichenized *Conotrema* sp. 2	Wedin 7033	Sweden, Lycksele lappmark, Tärna, Stor-Björkvattnet	AY527330	AY527359	–	–
lichenized *Conotrema* sp. 2	Wedin 7051	Sweden, Lycksele lappmark, Tärna, Övre Boksjön	AY527328	AY527357	–	AY649850
lichenized *Conotrema* sp. 2	Wedin 7059	Sweden, Lycksele lappmark, Stensele, Storskog	AY527329	AY527358	AY527366	AY649851
saprophytic *Stictis* sp. 2	Gilenstam 2413	Sweden, Lycksele lappmark, Lycksele, Norrås	AY527322	AY527351	AY649853	AY527297
saprophytic *Stictis* sp. 2	Gilenstam 2542a	Sweden, Lycksele lappmark, Stensele, Kyrkberget	AY527323	AY527352	AY649854	AY527298
saprophytic *Stictis* sp. 2	Gilenstam 2609a	Sweden, Lycksele lappmark, Lycksele, Furuvik	AY527324	AY527353	AY527367	AY649849
SPECIES 3						
lichenized *Conotrema populorum*	Gilenstam 2610a	Sweden, Lule lappmark, Jokkmokk par., Parkijaur	AY527327	AY527356	AY527369	AY527306
lichenized *Conotrema populorum*	Gilenstam 2618	Sweden, Lule lappmark, Jokkmokk par., Parkijaur	AY527331	AY527360	–	AY527307
lichenized *Conotrema populorum*	Wedin 7301	Sweden, Västerbotten, Umeå	AY527334	AY527363	AY649856	AY527302
saprophytic *Stictis* sp. 3	Gilenstam 2440b	Sweden, Lule lappmark, Jokkmokk par., Randijaur	AY527321	AY527350	–	AY527303
saprophytic *Stictis* sp. 3	Gilenstam 2619	Sweden, Lule lappmark, Jokkmokk par., Parkijaur	AY527333	AY527362	AY527370	AY527305
saprophytic *Stictis* sp. 3	Gilenstam 2620b	Sweden, Lule lappmark, Jokkmokk par., Nautijaurälven	AY527332	AY527361	AY527368	AY527304
Outgroup						
Stictis radiata	Gilenstam 2449a	Sweden, Lule lappmark, Jokkmokk par., Kvikkjokk	AY527308	AY340532	–	–
Stictis radiata	Wedin 6493	Sweden, Öland, Algotsrum, Färjestaden	AY527309	AY527338	AY649852	–

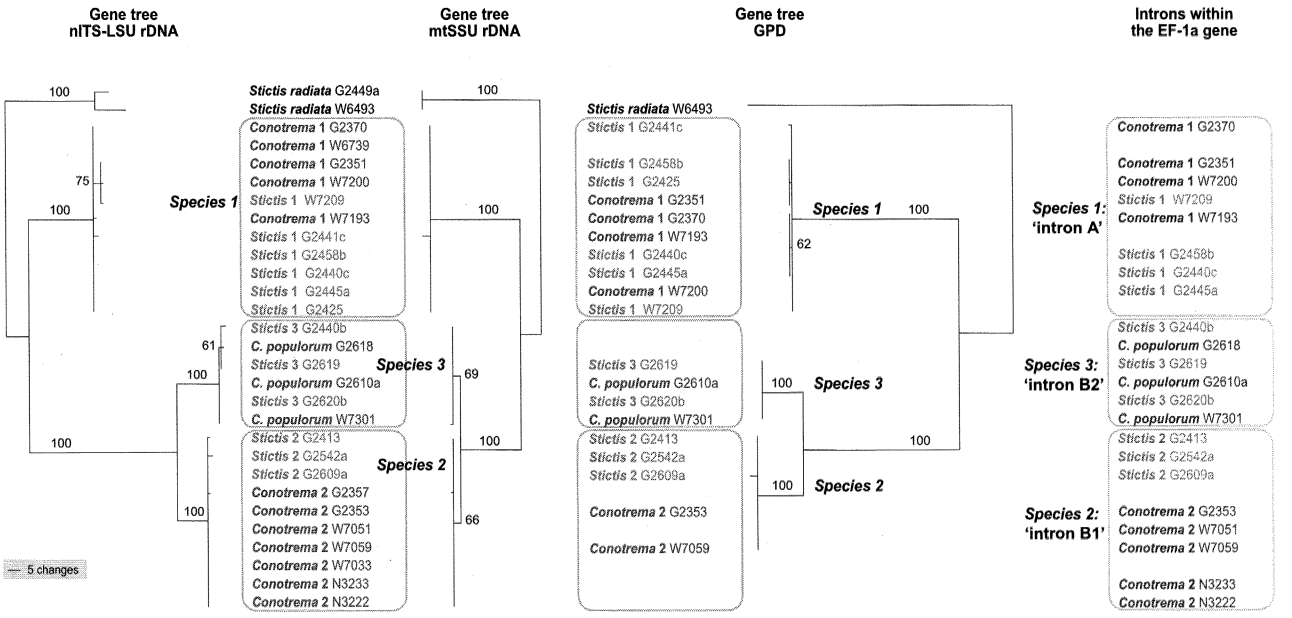

Fig. 2 Phylogenetic analyses and congruent groupings show three cases where lichens of the genus *Conotrema* and saprotrophs of the genus *Stictis* are only different expressions of the same fungal species. The single most-parsimonious gene trees from maximum parsimony and parsimony jack-knifing analyses of nITS-LSU rDNA and mtSSU rDNA sequences, illustrates the hypothesis of the evolutionary relationships between the specimens. Three groups are formed, all containing lichenized (*Conotrema*) and saprotrophic (*Stictis*) specimens. These groups are here interpreted as distinct phylogenetic species, due to the lack of genetic recombination between them. The GPD gene tree shows the same three phylogenetic species, but within-species recombination may be indicated in species 1 by minor incongruence with the nITS-LSU rDNA gene tree. The groupings of introns in the EF-1a gene ('A1', 'B1', 'B2') also support the three phylogenetic species. In all trees, branch-lengths correspond to nucleotide changes along each branch. Jack-knife values are given above the branches.

present in the GPD gene of *Cochliobolus heterostrophus* (GenBank X63516). This intron is four nucleotides shorter in species 1 compared to species 2, 3, and the outgroup. The second intron is only present in the specimen of *Stictis radiata*, and has a length of 45 nucleotide positions. This intron site is homologous to the fourth intron present in the GPD gene of *C. heterostrophus* (GenBank X63516). As this intron occurs only in the outgroup, it was excluded from further phylogenetic analysis. There are no insertions/deletions in the coding region of the GPD gene analysed here. The GPD data set contains a total of 238 variable sites (156 parsimony-informative) and resulted in two most-parsimonious trees of 294 steps (CI = 0.98, RI = 0.99), which are congruent with the nITS-LSU rDNA and the mtSSU rDNA trees (Fig. 2). All three species gain 100% jack-knife support in the separate GPD analysis. The two most-parsimonious trees differ only within species 1.

The amplified region of the EF-1a gene spans an intron site, and the data set comprises 386 nucleotide positions (22 specimens). The first 373 nucleotide positions are within this spliceosomal intron, which is located at a site homologous to the second intron present in the EF-1a gene of *Neurospora crassa* (D45873; positions 728–970 of that GenBank entry). The introns in species 1 ('intron A') are too different from the introns in the other two species to align unambiguously (data matrix presented in the online supplementary material) and no phylogenetic analysis was therefore performed from this data set. The introns in species 2 ('intron B1', 351 bp) and species 3 ('intron B2', 336 bp) show several nucleotide changes, but are comparatively similar and can be aligned. The length differences are mainly due to two distinct insertion/deletion events distinguishing intron B1 from B2. The sequence variation of the introns in the EF-1a gene, however, is congruent with the topology of the trees resulting from the other data sets (Fig. 2). There is furthermore almost no within-species variation in these introns; within intron A there are three variable nucleotide positions, one bp-change in B1, and none in B2.

Discussion

The gaps in our knowledge on the taxonomy and ecology of microfungi are clearly huge. Here, we show that molecular phylogenetics, in combination with intensive field studies, provide good tools to investigate these issues.

The separate data set-analyses of the nITS-LSU rDNA and mtSSU rDNA resulted in fully congruent phylogenetic trees where three main clades, here interpreted as phylogenetic species, are present. The sequence variation of introns in the EF-1a gene sequences also supports the same three groups. The GPD analysis also resulted in trees supporting the same three groups, but the topology within one of these (species 1) was slightly different. The samples G2351, W7200 and W7209 form a distinct clade in the nrDNA trees (j = 75%; Fig. 2), but these samples are found in different subgroups within species 1, in the GPD trees. A combined analysis of the nuclear

and mitochondrial rDNA data sets resulted in a fully congruent single most-parsimonious tree (not shown here) with a length of 335 steps (CI = 0.97, RI = 0.99). The same three groups are formed here as in the separate analyses, without extra steps added to the tree length; demonstrating that the homoplasy is not increased in the reconstruction of a tree from the combined data sets. An increase in homoplasy could have indicated genetic recombination (Burt *et al.*, 1996) and could have suggested that the three species are not completely genetically separated. This, however, is not the case here. The slight incongruence between the GPD gene trees and the nrDNA gene tree may imply genetic recombination within species 1, but the amount of information in our datasets is too small to investigate within-species variation.

Apparently, the lichenized and non-lichenized specimens do not form separate groups, identifying the *Conotrema*-specimens as lichenized representatives of the corresponding saprotrophic *Stictis*-species (Fig. 1). We interpret our results as showing that we have three distinct phylogenetic species, including both lichenized and saprophytic specimens. This leads to the conclusion that the lichenized *Conotrema*-specimens cannot be regarded as separate species, distinct from the corresponding *Stictis*. Microscopical studies of the dead wood that the non-lichenized specimens grew on, failed to show associated algae, confirming that these colonies are not interacting with algae hidden in the wood. This suggests that these three fungal species all develop into a saprotrophic colony if the spores germinate on bark-free wood, but form a lichen symbiosis on bark if compatible photobionts are present.

In addition to the three species investigated here, microscopic observations suggest at least two further cases in the Stictidaceae where optional lichenization is likely to occur, and we suggest that this might be a common strategy in this family of ascomycetes, where closely related species are known to have different nutritional modes (Sherwood, 1977). As a consequence of these fungi being congeneric, all three species should eventually be treated within *Stictis*, an older name than *Conotrema*. Valid formal descriptions of these taxa and the necessary generic transfers will be presented elsewhere.

These *Stictis*-species are the first known cases where individuals of the same fungal species have the option to develop either as lichens or saprotrophs, depending on the substrate (bark or wood), and in this way increase their ecological amplitude. All our cases have been found connected to *Populus tremula*, as a substrate. *P. tremula* is a rapidly growing tree forming stands in post-fire forest successions in the Scandinavian coniferous forests (Esseen *et al.*, 1997). Here, *Populus* forms a very important habitat, essential for epiphytic lichen biodiversity in the boreal forests (Kuusinen, 1994).

Optional lichenization may be an advantageous strategy for these three *Stictis*-species. They play somewhat different ecological roles in the forest; in species 1, the lichenized samples occur on young and thin, smooth-barked *Populus*-stems. Here, the lichen symbiosis enables colonization to take place in very young *Populus*-stands, already before dead branches suitable for the saprotrophic lifestyle have been produced. This can explain why species 1 seems to be more common and widespread than the other two, also in forests much affected by commercial logging activities. In the other two species, the lichenized samples grow in and around deep cracks in the bark of thick and comparatively old *Populus*-trunks. Optional lichenization may allow them to establish as saprotrophs, utilizing decorticated *Populus*-branches in somewhat younger stands, before the trunks become thick enough to offer a suitable substrate for the lichenized lifestyle.

The option to utilize different nutritional modes within a fungal population is an amazing example of environmental plasticity, even if the genetic or physiological background remains unknown. It allows the fungi both to exploit different niches on a tree, as well as *Populus*-stands of various successional stages during forest succession. In fact, this plasticity represents a strategy, which we predict to also occur among other fungi adapted to successional habitats that are unpredictable in space and time, and we suggest that it should be actively searched for among such fungi.

Optional lichenization indicates that the evolutionary step between fungal lifestyles is much smaller than earlier anticipated, which may open up a new perspective for the discussion of the role of lichen symbiosis in fungal evolution (Gargas *et al.*, 1995; Lutzoni *et al.*, 2001), and in the colonization of land (Taylor & Osborn, 1996; Heckman *et al.*, 2001). The finding of this degree of plasticity not only corroborates the integration of the classification systems of lichenized and other fungi (Santesson, 1952; Eriksson *et al.*, 2004), but also questions the relevance of estimating numbers of lichenization and de-lichenization events in fungal phylogeny (Aptroot, 1998; Lutzoni *et al.*, 2001) if these are merely different options in life in some fungi.

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Supplementary Material

The following material is available as Supplementary material at http://www.blackwellpublishing.com/products/journals/suppmat/NPH/NPH1198/NPH1198sm.htm

Table S1 Analysed matrices of nITS-LSU rDNA, mt SSU rDNA, GPD, and EF-1a sequence data.

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