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Morphological variation and ITS phylogeny of *Chaenotheca trichialis* and *C. xyloxena* (Coniocybaceae, lichenized ascomycetes)

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Chaenotheca trichialis and *C. xyloxena* have been characterized by differences in their morphology, anatomy and ecology. They are, however, often difficult to distinguish. In a molecular phylogeny based on ITS1-5.8S-ITS2 rDNA sequences the specimens of *C. xyloxena* but not those of *C. trichialis* formed a monophyletic group. To some extent the analysis also revealed regional groupings.

Key words: Ascomycotina, classification, Coniocybaceae, *Chaenotheca*, ITS, phylogeny, rDNA

Introduction

Several *Chaenotheca* species have wide distributions, occur in a variety of niches, and are morphologically very plastic. Since the ecophenotypic variation is considerable and not easily understood, it is often difficult to identify specimens, and species delimitation is often problematic. In some habitats, the species are very easily recognised, but in others different species seem to intergrade morphologically.

Chaenotheca trichialis (Ach.) Th. Fr., which occurs on bark and sometimes on wood, and *C. xyloxena* Nádv., which exclusively occurs on wood, have during the second half of the twentieth century been accepted as probably closely related but morphologically distinct species. *Chaenotheca trichialis* was described by Acharius already in 1808, and *C. xyloxena* was described by Nádvorník in 1934. They were accepted as distinct species by, for example, Tobolewski (1966), Tibell (1980, 1987, 1999), Puntillo (1994), Goward (1999), and Selva and Tibell (1999). They have been reported to occur over large areas in the temperate parts of both the Southern and Northern Hemisphere.

Chaenotheca trichialis (Fig. 1a–d) is characterized by usually having a well-developed,

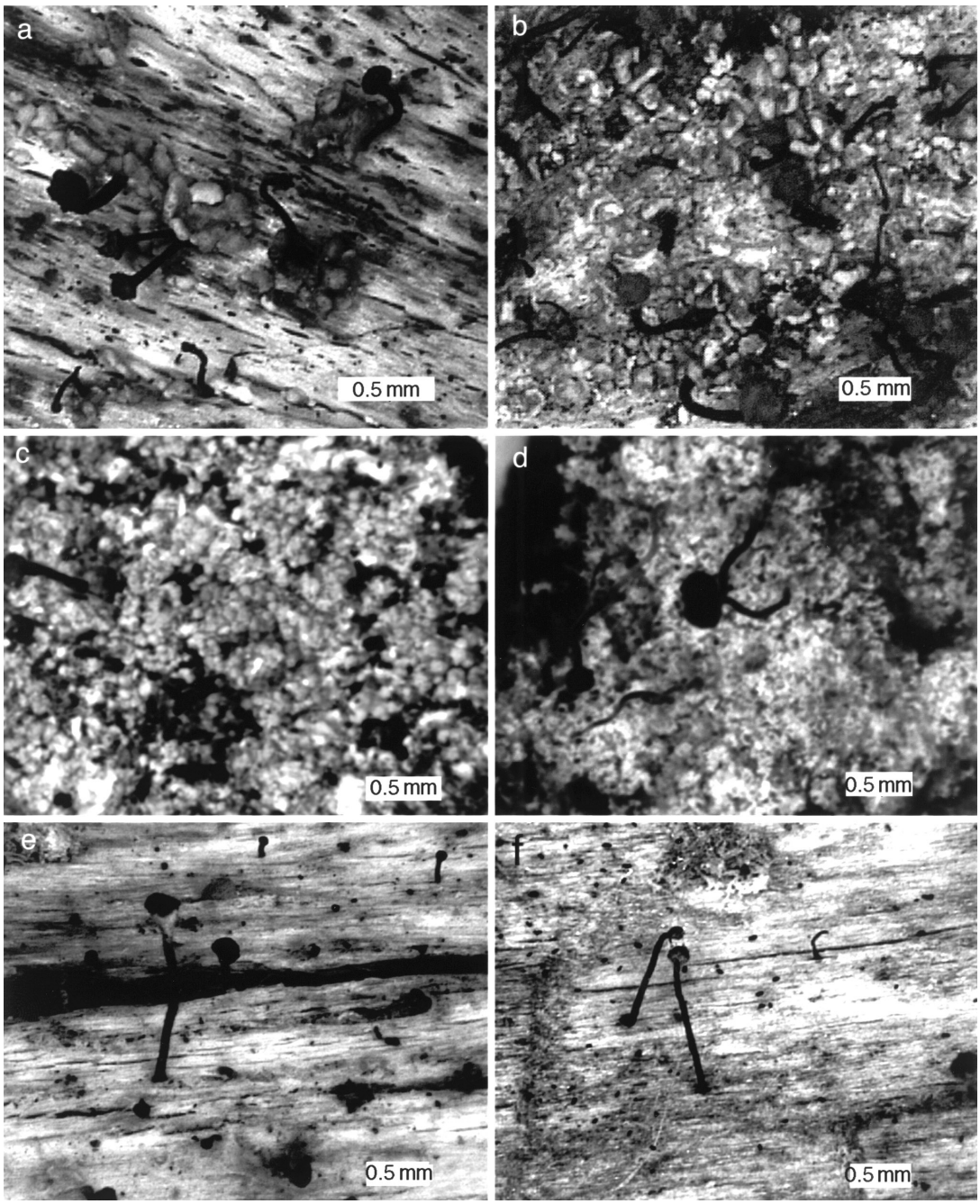

Fig. 1. Thallus structure in *Chaenotheca trichialis* (**a**–**d**) and *C. xyloxena*. (**e** and **f**). — **a**: Coarsely squamulose thallus (Sweden, Dalarna, Lima par., *Tibell 16178*, UPS). — **b**: Minutely squamulose thallus (Sweden, Uppland, Dannemora par., *Tibell 16878* = T038, UPS); — **c**: Verrucose to coarsely granular thallus (Sweden, Jämtland, Undersåker par., *Tibell 22355* = T130, UPS). — **d**: Minutely granular thallus (Argentina, Tierra del Fuego, *Tibell 17478* = T012, UPS). — **e**: Largely immersed thallus with some minutely granular parts visible among the wood fibres (Sweden, Jämtland, Åre par., *Tibell 22171* = T103, UPS). — **f**: Completely immersed thallus (Sweden, Uppland, Bondkyrko par., *Tibell 22361* = T131, UPS).

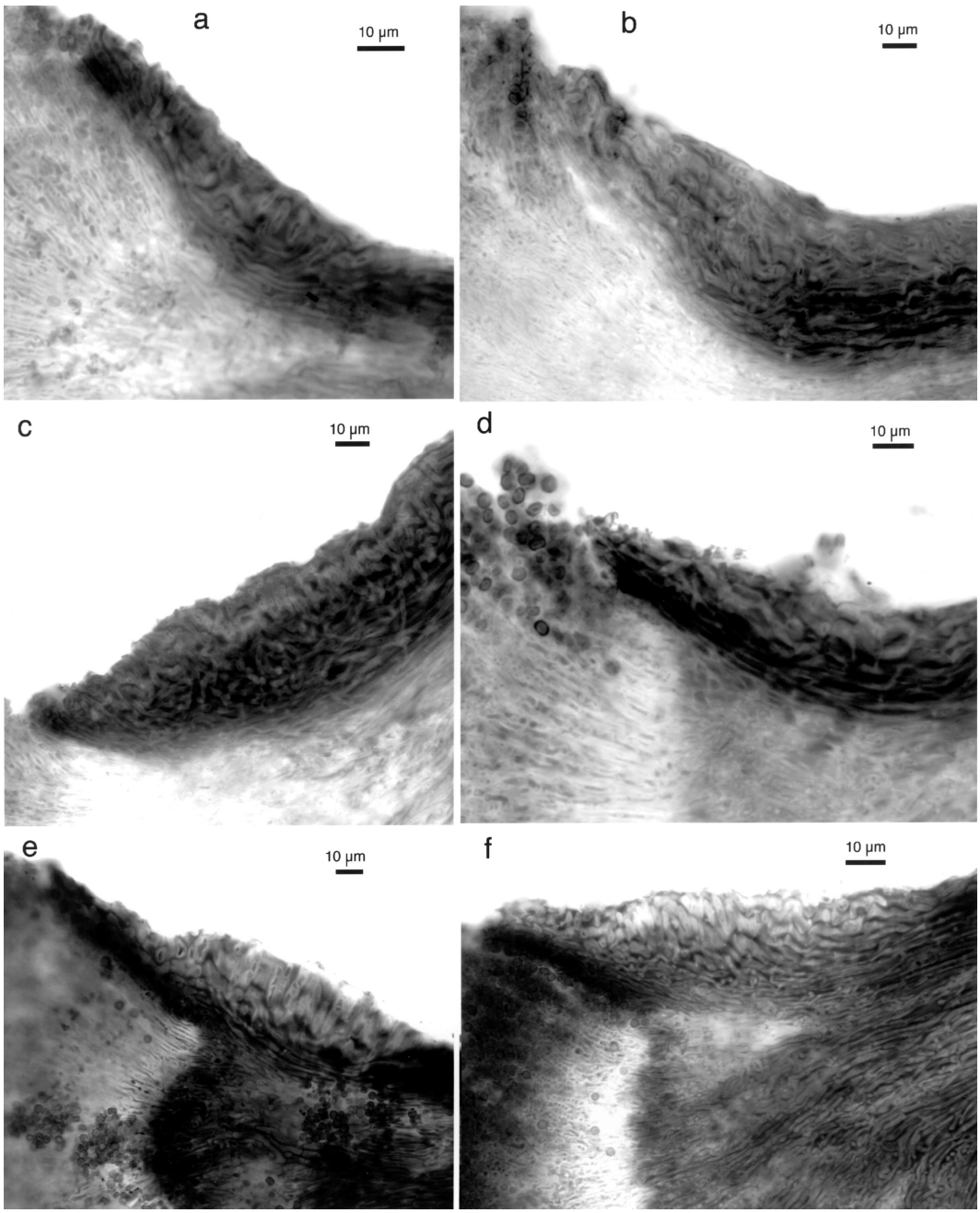

Fig. 2. Excipulum structure in *Chaenotheca trichialis* (**a**–**c**) and *C. xyloxena* (**d**–**f**). — **a**: Moderately thick excipulum with outer hyphae irregularly to anticlinally arranged (Sweden, Västmanland, *Tibell 3834*, UPS). — **b**: Thick excipulum with largely periclinally arranged hyphae (Sweden, Uppland, Björklinge par., *Tibell 3767*, UPS). — **c**: Thick excipulum with outer hyphae irregularly to anticlinally arranged (Sweden, Dalarna, *Tibell 3904*, UPS). — **d**: Rather thin excipulum consisting of periclinally arranged, only weakly intertwined hyphae (Argentina, Rio Negro, *Tibell 18431* = T011, UPS). — **e**: Excipulum differentiated into an inner part consisting of a few layers of periclinally arranged hyphae and an outer, thicker part with anticlinally arranged hyphae (Sweden, Västmanland, *Tibell 3828*, UPS). — **f**: Thick excipulum with less clear differentiation between the inner, periclinally arranged hyphae and an outer, irregularly to anticlinally arranged hyphae (Sweden, Uppland, *Tibell 3731*, UPS).

minutely squamulose to granular thallus, being associated with *Stichococcus*, by having small, spherical spores and asci produced in chains, and by having obconical to lenticular capitula usually provided with a white pruina on the lower side of the capitulum. The excipulum (Fig. 2a–c) is formed by largely periclinally arranged hyphae.

Chaenotheca xyloxena (Fig. 1e and f) differs from *C. trichialis* in having an immersed thallus, and often very slender apothecia with a narrowly conical capitulum covered by a thick white pruina on the lower side. In contrast to *C. trichialis*, the excipulum of *C. xyloxena* in the outer part consists of a more or less distinctive layer of anticlinally arranged hyphae forming a small cushion (Fig. 2d–f). Very robust apothecia occur in some specimens of *C. trichialis*, while the apothecia of *C. xyloxena* always are slender. Most specimens of *C. trichialis* and *C. xyloxena* can easily be referred to one taxon or the other, but sometimes the decision is very difficult.

Specimens of *Chaenotheca trichialis* often have a very well-developed, squamulose to minutely squamulose thallus (Fig. 1a and b), but sometimes the thallus is verrucose to granular (Fig. 1c) or very thin, minutely granular (Fig. 1d) or even almost completely immersed. The excipulum is often thin and formed of an inner layer of periclinally arranged hyphae and outer layer of moderately intertwined, more or less periclinally arranged hyphae (Fig. 2a). Sometimes it is thicker with mainly periclinally arranged (Fig. 2b) or more irregularly arranged and intertwined hyphae (Fig. 2c). The outline of the lower part of the capitulum is concave and the capitulum is more or less clearly lenticular. Apothecium size is also very variable, as is the robustness of the apothecia.

In *Chaenotheca xyloxena* the thallus is usually completely immersed (Fig. 1f), but sometimes a thin thallus is visible among disrupted fibres of the wood surface (Fig. 1e). The excipulum is sometimes very thin, with mainly periclinally arranged hyphae (Fig. 2d), but often differentiated into an inner part consisting of a few layers of periclinally arranged hyphae and an outer, thicker part with anticlinally arranged hyphae forming a distinctive cushion (Fig. 2e). Sometimes the excipulum is quite thick and the layer of anticlinally arranged hyphae is not so distinct (Fig. 2f). The outline of the lower part of the capitulum is usually straight and the capitulum is more or less conical.

No secondary substances have been identified in these two species, and their thalli are C–, K– and PD–.

Does this variation in thallus and apothecium morphology correlate with other phylogenetic markers? Recently, in lichenology, sequence comparisons have been used to elucidate phylogenetic relationships of the mycobiont in polymorphic species and critical species complexes, for example in *Chaenotheca* (Tibell & Beck 2002), *Dendrographa* (Lohtander *et al.* 1998b), *Diploschistes* (Martin *et al.* 2000), *Lasallia* (Niu & Wei 1993), *Peltigera* (Goffinet & Miadlikowska 1999), *Nephroma* (Goffinet & Goward 1998), *Phaeophyscia* (Lohtander *et al.* 2000), *Platismatia* (Thell *et al.* 1998), *Ramalina* (Groner & LaGreca 1997, LaGreca 1999), *Roccella* (Myllys *et al.* 1999), *Roccellina* (Lohtander *et al.* 1998a), *Tuckermannopsis* (Thell 1998), and *Xanthoria* (Franc & Kärnefelt 1998). All these studies were based on the ITS region. In a few studies other sequence information has been used for the same purpose, Group I intron sequences in cetrariod lichens (Thell 1999, Thell & Miao 1999), and β-tubulin in *Physcia* (Myllys *et al.* 2000).

DNA sequence comparisons offer an independent data set for comparison, and in this investigation sixteen collections of *Chaenotheca trichialis* and *C. xyloxena* are compared to test whether *C. trichialis* and *C. xyloxena* as identified by morphological features are supported by a phylogeny inferred from the ITS1-5.8S-ITS2 rDNA sequences.

Material and methods

Total DNA was extracted from 13 samples with the Qiagen DNeasy Plant Mini Kit. Either 10–30 apothecia or about 0.5 cm^2 of an axenic culture of the mycobiont was extracted. Collections identified by UPSC numbers were isolated from cultivated material. Lichen sample vouchers are kept in UMFK and UPS, and cultures in UPSC as presented in Table 1.

PCR amplification was conducted by using the primers ITS1-F (Gardes & Bruns 1993), and ITS 4 (White *et al*. 1990) to specifically amplify the fungal ITS1-5.8S-ITS2. The PCR ran for 35 cycles (1 min at 94 °C, 1 min at 54 °C, 45 sec at 72 °C with a 4 sec/cycle extension at 72 °C) using ABI or Promega Taq. Before sequencing, the PCR product was purified using the Qiaquick Spin kit and protocol by Qiagen.

Sequencing reactions were carried out with the following primers: ITS1-F, ITS2, ITS3, ITS4, and ITS5 (White *et al*. 1990). The sequencing reaction ran for 26 cycles (30 sec at 96 °C, 15 sec at 50 °C, 4 min at 60 °C) for sequencing by the BigDye labelling method (Perkin-Elmer) and for 30 cycles (20 sec at 95 °C, 15 sec at 50 °C, 3 min at 60 °C) for sequencing by the MegaBACE labelling method (Pharmacia-Amersham). Contigs were assembled manually.

In addition to the 13 new sequences presented here, five additional, previously published sequences were aligned by using Clustal W (gap opening penalty = 5, gap extension penalty = 5) of the DAMBE software packet by X. Xia*. The alignment can be obtained from the author upon request.

The data matrix was analysed by the computer software PAUP* 4.0b4a (Swofford 1998). In a previous analysis (Tibell 2001), *Chaenotheca cinerea* was with very high support shown to be the sister species of *C. trichialis* and *C. xyloxena* (*C. xyloxena* AF298138 in this paper erroneously having been identified as *C. trichialis*). The polarity of the characters was assessed by using two sequences of *C. cinerea* as outgroup. A branch and bound search (MaxTrees =100, collapse branches if maximum branch length = 0, MulTrees = yes, gaps treated as missing data, characters given equal weight) was undertaken. The branchlength to the two *C. cinerea* sequences corresponds to what is found within *C. trichialis*/*xyloxena*.

Jackknife values (Fig. 3) were obtained from an heuristic search (5 random additional replicates, TBR branch swapping algorithm, steepest descent off, MulTrees off, collapse zero length

Table 1. Material used in the study and GenBank accession numbers. Jmt = Jämtland, NS = Nova Scotia, Upl = Uppland.

GenBank accession number	Species	Code number	Origin	Voucher
AF298122	*Chaenotheca cinerea*	T089	Sweden	*Jonsson & Nordin* (UPS)
AF 421201	*Chaenotheca cinerea*	T163	Estonia	*Tibell 22374*
AF298138	*Chaenotheca trichialis*	T012	Argentina	*Tibell 17478* (UPS); culture
AF298139	*Chaenotheca trichialis*	T038	Sweden: Upl	*Tibell 16878* (UPS); UPSC2297
AF421202	*Chaenotheca trichialis*	T040	Sweden: Upl	*Tibell 21818* (UPS)
AF421203	*Chaenotheca trichialis*	T129	Sweden: Jmt	*Tibell 22300* (UPS)
AF421204	*Chaenotheca trichialis*	T130	Sweden: Jmt	*Tibell 22355* (UPS)
AF421205	*Chaenotheca trichialis*	T151	India	*Tibell 22090* (UPS)
AF421206	*Chaenotheca trichialis*	T187	USA: Maine	*Selva 8274a* (UMFK)
AF421207	*Chaenotheca trichialis*	T196	Sweden: Upl	*Tibell 22384* (UPS)
AF421208	*Chaenotheca xyloxena*	T006	New Zealand	*Tibell 16673* (UPS); UPSC2079
AF298138	*Chaenotheca xyloxena*	T011	Argentina	*Tibell 18431* (UPS)
AF421209	*Chaenotheca xyloxena*	T028	New Zealand	*Tibell 16605* (UPS); UPSC2072
AF298140	*Chaenotheca xyloxena*	T066	Sweden: Jmt	*Tibell 22188* (UPS)
AF421210	*Chaenotheca xyloxena*	T103	Sweden: Jmt	*Tibell 22171* (UPS)
AF421211	*Chaenotheca xyloxena*	T131	Sweden: Upl	*Tibell 22361* (UPS)
AF421212	*Chaenotheca xyloxena*	T181	Sweden: Jmt	*Tibell 22329* (UPS)
AF421213	*Chaenotheca xyloxena*	T188	Canada: NS	*Selva 7753* (UMFK)

* Xia, X.: *http://web.hku.hk/~xxia/software/software.htm*

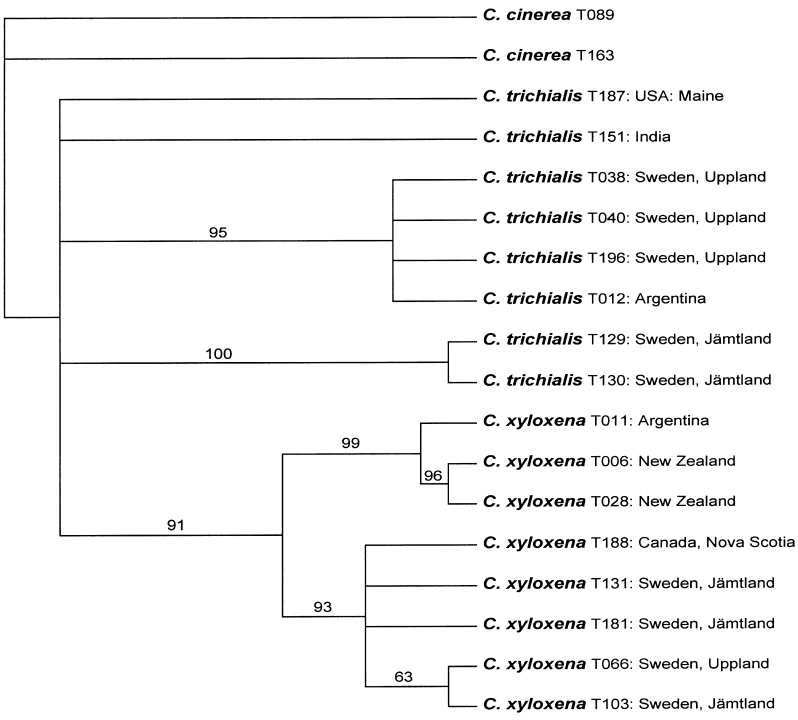

Fig. 3. Strict consensus tree of 39 most parsimonious trees of an alignment of 16 ITS1-5.8S-ITS2 rDNA sequences of *Chaenotheca trichialis* and *C. xyloxena* using two sequences of *C. cinerea* as outgroup. Numbers associated with branches are jackknife values.

branches when maximum length is zero, gaps treated as missing data) using 1000 replicates, resample = normal, with 36.9% of the characters deleted in each replicate.

Results

Thirteen ITS1-5.8S-ITS2 sequences are reported here for the first time. The alignment contained 551 sites. Of these 484 did not vary, 26 variable sites were parsimony-uninformative, while 41 were parsimony-informative. The branch-and-bound search of 18 samples of the alignment containing 551 sites resulted in 39 most parsimonious trees of 102 steps. The strict consensus tree is shown in Fig. 3. Values at branches indicate jackknife values. In this alignment all specimens of *Chaenotheca xyloxena* form a well supported clade (jackknife = 91). One clade of specimens of *C. trichialis* from Sweden and Argentina obtains a high support (jackknife = 95). The other parts of the polytomy with five branches are a strongly supported clade of *C. trichialis* from Jämtland, Sweden (jackknife = 100) along with one specimen from India and one from the U.S.A.

Discussion

Chaenotheca xyloxena forms a well supported clade in the analysis, whereas *C. trichialis* is paraphyletic with regard to *C. xyloxena*.

There is a considerable intraspecific sequence difference between different regions in both *Chaenotheca trichialis* and *C. xyloxena*. The number of nucleotide differences in pairwise comparisons in *C. trichialis* ranges between 0 (Sweden, Uppland/Sweden, Uppland) and 24 (India/USA), and in *C. xyloxena* between 0 (Sweden, Uppland/Sweden, Jämtland) to 19 (New Zealand/Canada). Generally sequence differences increased with the distance between collections, and cladistically material from adjacent localities tend to group together. The grouping together of material from the same area can be seen on Swedish material in both *C. trichialis* and *C. xyloxena*. On a larger scale a

clear grouping in *C. xyloxena* of the material from the Southern Hemisphere as distinct from that from the Northern Hemisphere is evident (Fig. 3). There are, however, also interesting exceptions. The Swedish material of *C. trichialis* is divided into two clades, one from Uppland (southern) and one from Jämtland (northern). One possibility is that this may reflect different immigration histories after the latest glaciation. Two or more alleles may, however, occur in the ITS sequences of one species (Zoller *et al.* 1999).

It is possible that the part of the genome studied here does not provide information enough for resolving the phylogenetic relationships between *Chaenotheca trichialis* and *C. xyloxena*; *C. trichialis* may, after all, be monophyletic. It is, however, also possible that *C. trichialis* as conceived from morphological evidence contains several morphologically cryptic species that are not resolved in this study because of limited sampling. Thus Swedish material of *C. trichialis* occurs in two separate, well-supported groups.

It is, however, also possible that the gene investigated is subject to ancestral polymorphism in a monophyletic species. This could possibly be revealed by studying more genes.

Chaenotheca trichialis and *C. xyloxena* are often associated with commensalistic *Chaenothecopsis* species. *Chaenothecopsis epithallina* exclusively occurs on *Chaenotheca trichialis*. *Chaenothecopsis pusiola* is often associated with *Chaenotheca xyloxena,* although it also may occur with other *Chaenotheca* species. Whether this difference has a physiological/phylogenetic basis or also depends on the ecology of the host is not clear.

Alternatively, *Chaenotheca trichialis* and *C. xyloxena* could be considered conspecific with a considerable, ecologically induced morphological plasticity. We would then, according to the present analysis, deal with a monophyletic taxon. Since *C. trichialis* is the older name *C. xyloxena* should then be considered a synonym of *C. trichialis*. In this view *C. trichialis* and *C. xyloxena* as traditionally used merely represent different ecophenotypes, which, however, often are quite distinctive. It may, however, be prudent to refrain from such a conclusion until more data are available (Grube & Kroken 2000).

A discrepancy between phylogenies based on morphological and molecular data like the one presented in this paper is awkward for species recognition. Morphologically indistinguishable species may certainly be a phylogenetic reality, and will no doubt turn up when molecular data are playing an increasingly larger role for recognising taxa and reconstructing phylogenies. A discussion of cases like the one dealt with in this paper and investigations of other parts of the genome for corroboration of these and other results are certainly needed.

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