# Intraspecific variation in *Mycocalicium subtile (Mycocaliciaceae)* elucidated by morphology and the sequences of the ITS1-5.8S-ITS2 region of rDNA

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The aim of the present paper is to investigate if *Mycocalicium subtile* as presently circumscribed is a morphologically highly variable species, or in fact represents two or more cryptic species, and further to investigate the status of *M. minutellum*. The morphological variation of 19 specimens of *Mycocalicium subtile* from five continents was investigated and the ITS1-5.8S-ITS2 region of their rDNA sequenced. Sequences from closely related taxa, 4 specimens of *M. albonigrum*, 2 of *Mycocalicium* sp., and *Chaenothecopsis nana* were also included. For comparison the corresponding sequence of *M. victoriae* and *C. pusilla* were also determined. We used the sequence of *Monascus purpureus* as the outgroup for the analysis. The sequences were used for phylogenetic inference using parsimony and distance methods. All the specimens assigned to *M. subtile* with the exception of two, form a well-supported monophyletic group. Those two specimens represent a morphologically cryptic, but genetically distinctive taxon. The infraspecific clades of *M. subtile* in the molecular phylogeny did not correspond to geographical origin. Only a weak correlation with geographical origin was found in the morphological analysis. The molecular analysis supports the suggestion that *Mycocalicium minutellum* is a taxonomic synonym of *M. subtile*.

## INTRODUCTION

*Mycocalicium subtile* is a non-lichenized calicioid species which is widely distributed on wood in the cool and temperate parts of both the Northern and Southern Hemisphere (Tibell 1994). It belongs to the *Mycocaliciaceae*, and its members have traditionally been referred to the calicioid lichens or allied fungi, which also include the phylogenetically not closely related *Caliciaceae* and *Sphaerophoraceae* in the *Lecanorales* (Wedin & Tibell 1997). In the vast majority of these widely distributed calicioid species a morphological differentiation between populations is not noticeable (Tibell 1987a). In *M. subtile*, however, considerable variability in the size of the apothecia, spore size, and structure and colour of the stalk and exciple has been noted (Tibell 1987a). This makes the identification of *M. subtile* difficult, and it has been suspected to possibly represent a species complex (Tibell 1987a). *M. minutellum* has been considered closely related, and to be characterized by very tiny ascomata (Poelt 1969).

There are also taxonomic problems in delimiting *Mycocalicium*. The genus was described by Vainio (1890) as differing from *Calicium* by not being lichenized. Schmidt (1970) emended generic concepts in the *Mycocaliciaceae*, and characterized *Mycocalicium* as having non-septate spores, short asci, periclinal hyphae in the ascoma stalk, and an evenly thickened ascus apex. When more species in the *Mycocaliciaeceae* were studied, however, these features were not consistent and a delimitation of *Mycocalicium* from *Phaeocalicium* and *Chaenothecopsis* as suggested by Schmidt proved difficult to maintain. Thus the circumscription of *Mycocalicium* and its phylogenetic relationships to other genera is unclear. Some ten species have been included in the genus, but some species now placed in other genera may be more closely related to *M. subtile* than some presently included in *Mycocalicium*. *M. albonigrum*, is morphologically very similar to *M. subtile*. The morphology of *M. albonigrum* and *M. subtile* was studied in detail by Tibell (1990), and in spite of the morphological similarity details of ascoma, ascospore and conidium ontogeny were shown to be different in light microscopical and ultrastructural investigations. This indicates that *M. albonigrum* is different from, but closely related to, *M. subtile*, and for this reason it was selected as an outgroup in our phylogenetic study. *Chaenothecopsis nana* was also selected due to its resemblance in anatomy and spore structure to *Mycocalicium subtile*. Its ascus apex, however, is of the *Chaenothecopsis*-type with a narrow canal in semi-mature stages (Tibell 1987a). *M. albonigrum*, however, like most *Chaenothecopsis* species has a short canal in the ascus apex.

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Two unidentified specimens of *Mycocalicium* were included due to their morphological resemblance to *M. subtile* and *M. albonigrum*. Two other representatives of *Mycocalicium* and *Chaenothecopsis* respectively, *M. victoriae* and *C. pusilla*, were selected as outgroups since they, judging from their morphology, have a more remote phylogenetic relationship to *M. subtile*. As a remote outgroup, *Monascus purpureus* was selected; that species belongs to the *Eurotiales*, a closely related order judging from analyses of the 18S gene sequence.

The objective of this work was to investigate the genetic variation within *Mycocalicium subtile* in order to find correlations with its morphological variation, and further to investigate the status of *M. minutellum*. Two kinds of data sets were used, sequences of the ITS1-5.8S-ITS2 region of rDNA, and morphological data. The patterns of variation obtained are compared with distribution data.

## MATERIAL AND METHODS

### *Fungal material*

Material of *Mycocalicium albonigrum*, *M. subtile*, *M.* sp., *M. victoriae*, *Chaenothecopsis nana*, and *C. pusilla* investigated is listed in Table 1. Voucher specimens are kept in UPS, and living cultures preserved in the Uppsala University Culture Collection of Fungi (UPSC). Both axenic cultures on agar plates or slant tubes, and fragments of apothecia, were used for DNA isolation.

### *Fungal isolates and culture conditions*

Cultures from ascospores were obtained by plating ascospores on MYE (malt yeast extract agar; Ahmadjian 1967) and PDA (potato dextrose agar; Oxoid) to germinate. Cultures were incubated in Petri dishes and culture tubes and kept under a variety of conditions. They grew well at 20 °C on a 12-h regimen of alternating near ultraviolet and cool daylight. After 2 wk the culture vessels were sealed with Parafilm.

### *DNA isolation and PCR reactions*

DNA was extracted from 100–200 mg of mycelium of colonies growing on agar. Generally the efficiency of extraction depended on the age of the culture. Young mycelia yielded a larger amount of DNA, probably because the cell walls were more easily ruptured during the extraction. Some of the samples did not yield detectable amounts of DNA on the assay gels, but were amplified in spite of this. When extracted from ascomata, 5–10 apothecia were used. The extraction method was based on the formation of CTAB-DNA complexes as described by Möller *et al.* (1992). Apothecia were first squashed, then subjected to three series of freezing-thawing and three pulses of (15–10–5 s) microwaves in order to improve the breakage of the cell walls of both ascomata and ascospores.

Isolation of DNA from herbarium material was attempted in numerous cases, but was only occasionally successful. This

**Table 1.** Origin of the material investigated. The code numbers are used as substitutions for specimen identification and origin. AP, fragments of dried apothecia; C, living cultures; DC, dried cultures. All voucher specimens are kept in UPS.

| Code | Species | Isolate/collection | Origin | Source | GenBank Accension number |
|---|---|---|---|---|---|
| Ma02087 | *Mycocalicium albonigrum* | UPSC 2087 | AA: New Zealand | C | AF223969 |
| Ma02088 | *M. albonigrum* | UPSC 2088 | AA: New Zealand | C | AF223968 |
| Ma02089 | *M. albonigrum* | UPSC 2089 | AA: New Zealand | C | AF223967 |
| Ma19038 | *M. albonigrum* | Tibell 19038 | AA: New Zealand | C | AF223966 |
| Ms00001 | *M. subtile* | Vinuesa 1 | NE: Sweden | C | AF225427 |
| Ms01161 | *M. subtile* | Goward 1161 | NAm: Canada | C | AF225428 |
| Ms01839 | *M. subtile* | UPSC 1839 | NE: Sweden | C | AF225429 |
| Ms01896 | *M. subtile* | UPSC 1896 | NE: Sweden | C | AF225430 |
| Ms01904 | *M. subtile* | UPSC 1904 | NE: Sweden | C | AF225431 |
| Ms02173 | *M. subtile* | UPSC 2173 | AA: New Zealand | C | AF225432 |
| Ms02504 | *M. subtile* | UPSC 2504 | NE: Sweden | C | AF225433 |
| Ms03832 | *M. subtile* | Hermansson 3832 | EE: Russia | AP | AF225434 |
| Ms03850 | *M. subtile* | Hermansson 3850 | EE: Russia | AP | AF225435 |
| Ms06747 | *M. subtile* | Selva 6747 | NAm: USA | C | AF225436 |
| Ms16207 | *M. subtile* | Tibell 16207 | NE: Sweden | DC | AF225437 |
| Ms16388 | *M. subtile* | Tibell 16388 | NE: Sweden | DC | AF225438 |
| Ms17361 | *M. subtile* | Tibell 17361 | NE: Sweden | AP | AF225439 |
| Ms17913 | *M. subtile* | Tibell 17913 | SAm: Argentina | C | AF225440 |
| Ms19319 | *M. subtile* | Tibell 19319 | EA: Russia | AP | AF225441 |
| Ms20093 | *M. subtile* | Tibell 20093 | NE: Sweden | C | AF225442 |
| Ms20539 | *M. subtile* | Streimann 20539 | AA: New Guinea | AP | AF225443 |
| Ms21003 | *M. subtile* | Tibell 21003 | NE: Sweden | C | AF225444 |
| Ms21020 | *M. subtile* | Tibell 21020 | NE: Sweden | C | AF225445 |
| Msp00975 | *M.* sp. | Goward 975 | NAm: Canada | C | AF243134 |
| Msp17604 | *M.* sp. | Tibell 17604 | SAm: Argentina | AP | AF243133 |
| Mv00021 | *M. victoriae* | Boom 21 | | C | AF213135 |
| Cn02083 | *Chaenothecopsis nana* | UPSC 2083 | | C | AF243131 |
| Cp02522 | *C. pusilla* | UPSC 2522 | | C | AF243132 |
| Mpu | *Monascus purpureus* | | | | U18356 |

**Table 2.** Morphological characteristics of nineteen specimens of *Mycocalicium subtile* and two specimens of an undescribed *Mycocalicium* species used in the PCA analysis. Quantitative characters (characters 1–8; based on the mean of 10 observations) and qualitative characters (9–14) are explained below.

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ms00001 | 0.34 | 0.13 | 0.04 | 9 | 40.3 | 3.5 | 6.8 | 3.6 | 1 | 0 | 1 | 1 | 1 | 1 |
| Ms01161 | 0.70 | 0.23 | 0.06 | 5 | 36.9 | 3.8 | 7.1 | 4.0 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms01839 | 0.64 | 0.14 | 0.05 | 4 | 44.8 | 4.1 | 7.8 | 3.5 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms01896 | 0.56 | 0.26 | 0.06 | 5 | 41.9 | 3.5 | 7.6 | 3.6 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms01904 | 0.44 | 0.17 | 0.05 | 4 | 44.4 | 3.6 | 7.1 | 4.0 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms02173 | 0.94 | 0.15 | 0.05 | 8 | 38.3 | 3.1 | 6.8 | 3.3 | 1 | 1 | 0 | 1 | 0 | 0 |
| Ms03832 | 0.65 | 0.16 | 0.06 | 7 | 40.4 | 4.1 | 7.2 | 3.3 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms03850 | 0.50 | 0.17 | 0.07 | 5 | 43.0 | 3.4 | 7.2 | 3.3 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms06747 | 0.73 | 0.20 | 0.07 | 6 | 38.0 | 3.6 | 6.9 | 4.3 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms16207 | 0.38 | 0.07 | 0.04 | 5 | 39.5 | 3.9 | 6.8 | 3.4 | 1 | 0 | 1 | 1 | 1 | 1 |
| Ms16388 | 0.46 | 0.14 | 0.05 | 5 | 38.4 | 3.3 | 8.0 | 3.7 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms17361 | 0.56 | 0.10 | 0.04 | 6 | 43.5 | 3.2 | 7.4 | 3.9 | 1 | 0 | 1 | 1 | 1 | 1 |
| Ms17913 | 0.70 | 0.18 | 0.07 | 7 | 49.7 | 3.8 | 8.4 | 3.9 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms19319 | 0.66 | 0.23 | 0.07 | 5 | 42.3 | 3.7 | 7.0 | 3.6 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms20093 | 0.76 | 0.24 | 0.08 | 6 | 43.0 | 3.7 | 6.9 | 3.6 | 1 | 0 | 1 | 1 | 1 | 1 |
| Ms20539 | 0.74 | 0.16 | 0.05 | 3 | 35.3 | 3.3 | 5.9 | 3.3 | 1 | 0 | 0 | 0 | 1 | 1 |
| Ms21003 | 0.67 | 0.16 | 0.06 | 7 | 42.9 | 3.3 | 7.1 | 4.0 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms21020 | 0.74 | 0.17 | 0.07 | 6 | 39.8 | 3.4 | 7.6 | 4.0 | 1 | 0 | 1 | 0 | 1 | 1 |
| Ms02504 | 0.47 | 0.18 | 0.06 | 7 | 41.3 | 3.6 | 7.8 | 3.3 | 1 | 0 | 1 | 1 | 0 | 1 |
| Msp00975 | 0.83 | 0.17 | 0.07 | 2 | 41.6 | 3.1 | 7.3 | 3.1 | 0 | 0 | 1 | 0 | 0 | 0 |
| Msp17604 | 0.33 | 0.11 | 0.05 | 4 | 40.0 | 3.9 | 6.4 | 3.0 | 0 | 0 | 1 | 0 | 0 | 0 |

A, Apothecium height including the capitulum (mm). B, Capitulum diam (mm). C, Stalk diam (mm). D, Number of layers of hyphae in the excipulum. E, Ascus length (μm). F, Ascus width (μm). G, Spore length (μm). H, Spore width (μm). I, Exciple dark brown to blackish (1; reddish: 0). J, Hypothecium brown (1; hypothecium hyaline; 0). K, Hypothecium aeruginose (1; hypothecium hyaline: 0). L, Stalk in section aeruginose (1; stalk in section hyaline: 0). M, Stalk in section brown (stalk in section hyaline; 0). N, Ascus apex strongly and evenly thickened, without canal (1; ascus apex unevenly thickness, with distinct canal: 0).

resulted in a low representation of material from areas from which cultured material was not easily available. 10–150 ng of extracted DNA was used to amplify the ITS1-5.8S-ITS2 region of ribosomal DNA (rDNA). The primers used for amplification were ITS5 and ITS4 according to White *et al*. (1990). PCR reactions were carried out in a Perkin Elmer Gene Amp PCR System 2400 according to White *et al*. (1990).

### *DNA sequencing*

For sequencing, 100 ng of PCR products were cleaned using exonuclease I and alkaline phosphatase supplied in the reagent pack for PCR product pre-treatment (Amersham). In order to completely cover the region of interest with overlapping sequences, three internal oligonucleotides ITS1, ITS2 and ITS3 as recommended by White *et al*. (1990), were used as primers in separate sequencing reactions. PCR products were sequenced by the cyclic reaction method using the Thermosequenase cycle sequencing kit (Amersham) according to the protocol described by the manufacturer. Sequencing reactions were run on 6% polyacrylamide gels.

### *Alignment and phylogenetic analysis*

Sequences were introduced and edited in the computer package DNASTAR (Lasergene system for sequence analysis) obtaining the complete sequence of the fragment including primers ITS1 and ITS4.

A preliminary alignment of the sequences was performed with the program CLUSTAL W (Thompson *et al*. 1994). The alignment was reviewed manually and corrected moving the position with gaps in order to improve the number of aligned sites, using the GENEDOC multiple sequence alignment editor (Nicholas *et al*. 1997). Regions of ambiguous alignment were removed and corrected alignments (available upon request) were used as input for parsimony and distance (PHYLIP package) analyses.

Parsimony analyses were carried out by changing the input order of the sequence 10 times. Distances were calculated following the maximum likelihood algorithm for distance generation, using a transition/tranversion ratio = 2, and frequencies of nucleotides calculated from the alignment. Parsimony analyses considering gaps as a fifth character and as unknown nucleotides were developed and compared. Kishino & Hasegawa (1989) tests were performed using the resulting trees from parsimony and distances as user trees in DNAML. Jack-knifing for identification of well supported monophyletic groups (Farris *et al*. 1996) was performed using Farris Parsimony Jack-knifing program version 4.22 (Farris 1995), with the cut-off level set to 50%, and the number of replicates to 10000.

Trees were rooted using TREEVIEW program version 1.6 (Page 1996) by selecting the *Monascus purpureus* sequence as outgroup.

### *Principal component analysis*

For the elucidation of morphological variation in *Mycocalicium subtile*, both quantitative and qualitative characters were utilized; the 14 characters used are listed in Table 2. Measurements are mean values of ten observations for each character in each collection. Character states for qualitative characters are indicated in Table 2. A principal component analysis was carried out using the SAS package according to standard procedures.

## RESULTS

Phylogenetic relationships were investigated by sequencing the ITS1-5.8S-ITS2 region of rDNA of nineteen samples identified as *M. subtile* and seven samples of closely related taxa, four specimens of *M. albonigrum*, two specimens of *Mycocalicium* sp., and one of *Chaenothecopsis nana*. *M. victoriae* and *C. pusilla*, supposedly more remotely related to *M. subtile*, were also sequenced. The homologous rDNA region of *Monascus purpureus* was obtained from GenBank, and used as outgroup for the analysis. The size of the sequenced region in *Mycocalicium subtile* showed little variation (576 to 580 bp). The size of the homologous region in *M. albonigrum* is larger, ranging from 589 to 591. The size of the sequenced region of the two representatives of *Mycocalicium* sp. is 589 and 596 nt respectively.

**Table 3.** Analysis of the variable sites observed in the final alignment in which ambiguous alignment regions have been removed.

| | Complete sequence | ITS1 | ITS2 |
|---|---|---|---|
| Total number of sites | 539 | 146 | 150 |
| Variable sites | 234 | 99 | 110 |
| Significant sites | 125 | 67 | 53 |
| Transitions | 53 | 21 | 21 |
| Transversions | 49 | 14 | 28 |
| Gap sites | 40 | 19 | 15 |
| Hypervariable sites | 92 | 44 | 47 |

### *DNA sequence alignments*

For the analysis of intraspecific variation within *M. subtile*, we generated an alignment of the DNA sequences of the ITS1-5.8S-ITS2 region of rDNA. The analysis of the final alignment is shown in Table 3.

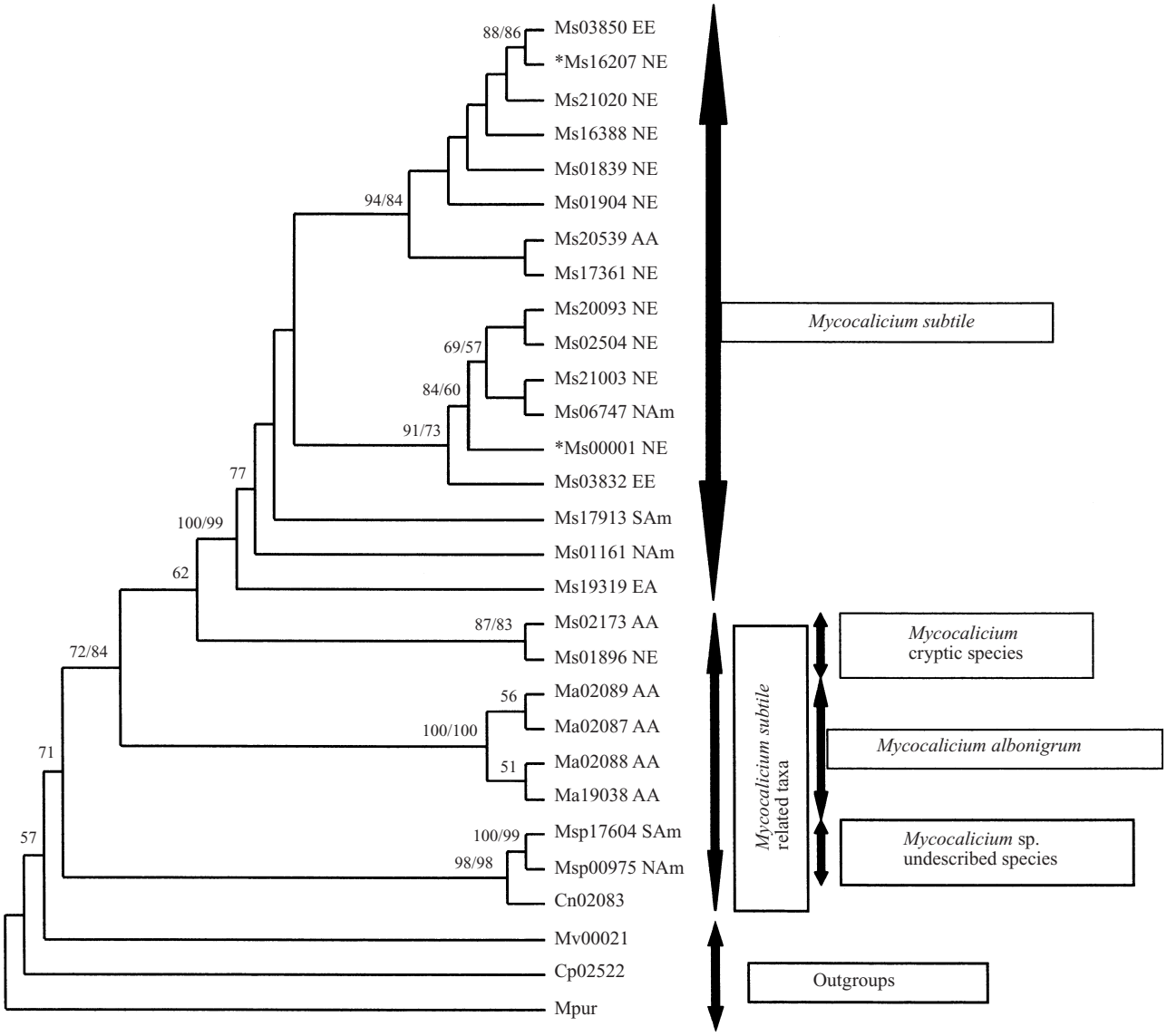


**Fig. 1.** Phylogenetic trees showing relationships among 26 specimens of *Mycocalicium*. The trees were constructed by a parsimony-based method, using the DNAPARS options from the PHYLIP software package with 10 randomization of the sequence input order. The tree was obtained from an alignment where gap positions were treated as a fifth character. Bootstrap values are shown as a percentage of 100 replications and Jack-knife values after the slash (/). AA, Australasia; EA, eastern Asia; EE, east Europe; NAm, North America; NE, northern Europe; SAm, South America. *, '*M. minutellum*' specimens.

Among specimens of a species, most of the variation is due to transitions. Sequences of *M. subtile* Ms02173 and Ms01896 are especially interesting, because they include transversions among specimens of this species. The sequences of Msp00975 and Msp17604 are quite similar. Transversions and insertion/deletion(indel) events otherwise mainly occur when different species are compared. Most of the indels are produced due to the alignment of the more divergent sequences, *M. victoriae*, *C. pusilla* and *Monascus purpureus*.

### *Phylogenetic analyses*

For phylogenetic inference we used an alignment where positions with gaps were maintained and then treated as a fifth character or as unknown nucleotides. An analysis of the intraspecific relationships of sequences of 26 collections of *Mycocalicium* was undertaken. Phylogenetic analysis based on parsimony methods were carried, out, and the result is shown in Fig. 1. The topology of the one resulting tree is identical when gaps are treated as a fifth character rather than as unknown nucleotides, the difference appears in the support values being higher when gaps are treated as a fifth character. In this analysis the majority of the collections of *M. subtile* formed a well supported (100%) monophyletic group. Two specimens, from Sweden (Ms01896) and New Zealand (Ms02173) respectively, formed a separate clade. This appears as a sister clade to the majority of *M. subtile* with 62% support value. When distance methods are applied to generate phylogenetic inference this clade appears as more closely related to *M. albonigrum* than to *M. subtile*, but again showing a low support value (65%; data not shown). Within the main clade of *M. subtile*, three specimens, from South America (Ms17913), North America (Ms01161) and East Asia (Ms19319) occupied isolated positions. The rest of the specimens are distributed in two main clades with eight and six members respectively, both well supported (94%, 91%). The largest clade contains specimens from northern and eastern Europe, and Australasia. The second largest clade contains specimens from northern and eastern Europe, and North America. The two specimens characterized by their small ascoma and considered to be *M. minutellum* (*Ms00001, *Ms16207) do not cluster together in the analysis.

*M. albonigrum* forms a well supported monophyletic group (100%) and appears as a sister group to *M. subtile* (72% support). Two specimens (Msp00975 and Msp17604) formed a separate clade together with the sequence of *Chaenothecopsis nana*.

A Kishino & Hasegawa (1989) test was carried out taking the parsimony and the distance trees as user trees, in order to select the best topology for the MS1896-Ms02173 group using maximum likelihood criteria; the results are shown in Table 4. The position of Ms01896-Ms02173 as sister clade to the rest of *M. subtile* specimens is selected as the best topology, but distance trees are not significantly worse.

A cladistic support analysis of the same alignments was undertaken by the Parsimony Jack-knifing program. This analysis yields trees that allow multifurcations in the case of not clearly monophyletic groups. It resulted in a more restrictive topology, showing a trifurcation that contains *M. albonigrum*, a group formed by Ms01896 and Ms02173, and a third group composed by the rest of the *M. subtile* collections. All these three groups are supported by high jack-knife values (100, 83, and 99 respectively); jack-knife support values are indicated in Fig. 1.

**Table 4.** Kishino & Hasegawa tests for the same alignment but with different gap treatments. The tests compare the Ln likelihood of two user trees obtained each using a different phylogenetic analysis. The arrows indicate the best trees.

| Gap treatment | Phylogenetic analysis | Ln L | Diff Ln L | Significantly worse? |
|---|---|---|---|---|
| Fifth character | Parsimony | −272.788.885 | ←— best | |
| | Distances ML | −272.877.365 | −0.88479 | No |
| Unknown nucleotide | Parsimony | −272.788.901 | ←— best | |
| | Distances ML | −272.877.365 | −0.88464 | No |

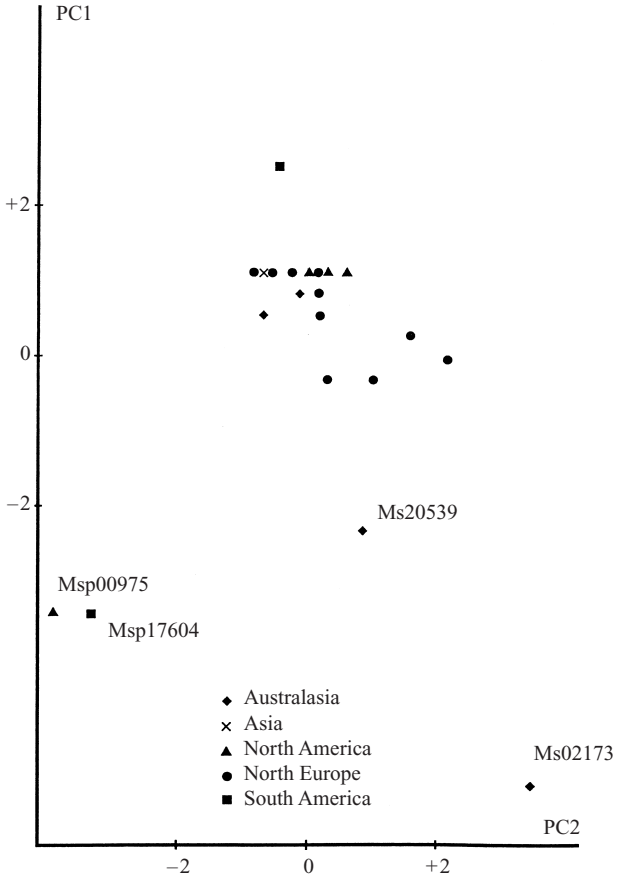


**Fig. 2.** Principal component analysis of nineteen specimens of *Mycocalicium subtile* and two of an undescribed *Mycocalicium* species (Msp00975 and Msp17604) based on fourteen morphological characters (Table 2) identifying the geographical origin of the specimens. PCA1 *vs* PCA2.

### *Morphological data*

The morphological variation of *Mycocalicium subtile* was studied to investigate the interrelationships of the collections in comparison to their geographical origin. The morphological

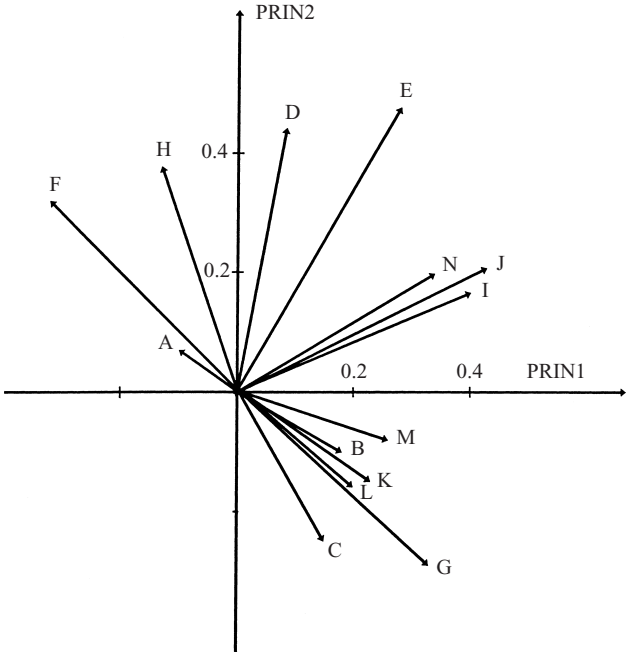


**Fig. 3.** Contributions of the original characters to the first two principal components of the PCA in Fig. 2. Letters denoting characters explained in Table 2.

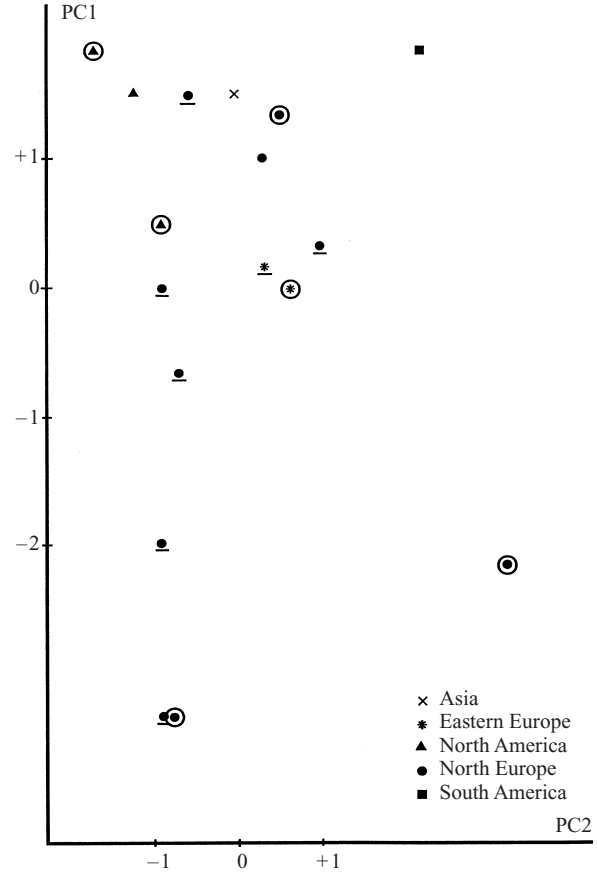


**Fig. 4.** PCA ordination based on ten morphological characters of seventeen specimens of *Mycocalicium subtile* identifying the geographical origin of the specimens. Underlined symbols belong to the clade Ms20539-Ms03850 in Fig. 1, the encircled specimens to the clade comprising Ms17913-Ms02504.

analysis was based on the investigation of 14 characters, six of them qualitative and eight quantitative (Table 2). A Principal Component Analysis (PCA) was chosen since it can accommodate both quantitative and qualitative data in the same analysis. Results from the PCA (Fig. 2) show that the first three components explain 63% of the variation. Some of the characters were strongly correlated (brown stalk in section/evenly and strongly thickened ascus apex: 0.84; stalk width/evenly and strongly thickened ascus apex: 0.79; capitulum diam *vs* stalk diam: 0.73), while most characters were moderately or only weakly correlated. Ascus apex structure, ascus and spore width, stalk width and stalk colour are characters most strongly contributing to the first principal component (Fig. 3), while exciple colour, the number of layers in the exciple, stalk colour, and hypothecium colour contribute most strongly to the second principal component. Apothecium height, capitulum diameter, and stalk diameter, contributed strongly to the third component. Msp00975 and Msp17604 in the low left-hand corner of Fig. 2 were originally identified as *M. subtile*. In the morphological analysis, however, they were far removed from other specimens. In the molecular analysis Ms02173 and Ms01896 formed a distinct clade, but in Plots PC1 *vs* PC2 (Fig. 2), PC2 *vs* PC3 (not shown), and PC2 *vs* PC4 (not shown), of the morphological analysis they were far apart. Except for the collections Ms02173 (New Zealand) shown as an outlier in the plot of PC1 *vs* PC2 (Fig. 2), in PC1 *vs* PC3, and PC1 *vs* PC4, and Ms20539 (Papua New Guinea), also rather far removed from the main cluster, the other specimens keep together.

The morphology of all the specimens was reinvestigated and they were all (apart from Msp00975 and Msp17604) considered to belong to *M. subtile*, including the outlying specimen Ms02173 from New Zealand. The peripheral Ms20539 originates from New Guinea. The fairly coherent central cluster harbours specimens of diverse geographical origins, with European, eastern Asian, and North American specimens being close, whereas the South American and Australasian collections occur at the fringe of the cluster. This pattern is consistent also in projections PC1 *vs* PC3, PC1 *vs* PC4, PC2 *vs* PC3, and PC2 *vs* PC4. In a further analysis, outlying specimens (Msp00975, Msp17604, Ms20539 and Ms02173) were removed in order to obtain a more detailed resolution of the remaining specimens (Fig. 4). Here only 10 characters were informative. The first three components explained 60% of the variation. In this analysis the collections from North and South America were found in the upper part of the ordination, and the two collections from eastern Europe kept together but were integrated in a wide area occupied by North European material. There are thus tendencies for material of similar origin to associate in the morphological analysis. There were no tendencies for the two major clades identified in the molecular analysis to show congruence in morphology. This was also the case in the other projections of the first three components.

The PCA analyses based on morphological features (Figs 2–4) showed *Mycocalicium subtile* to have quite a continuous morphological variation, particularly with respect to Northern Hemisphere material.

## DISCUSSION

### *Morphology, molecular data, and taxonomy*

The variation in the ITS1-5.8S-ITS2 region among specimens initially referred to *Mycocalicium subtile* is substantial (0–16%), and is far greater than that of *M. albonigrum* (0–1%). This may to some extend depend on *M. albonigrum* sequences being obtained from a limited area. Four collections (Ms01896, Ms02173, Msp00975, and Msp17604) have sequences that differ considerably from those of the majority of *M. subtile*. If they are disregarded, the range of variation for *M. subtile* is more limited (0–3%).

In other fungi a complicated pattern of intraspecific variation in the ITS1-5.8S-ITS2 regions was found in *Fusarium sambucinum* (O'Donnell 1992), for which an intraspecific variation as high as 15% was noted. Yan, Rogers & Wang (1995) found interspecific variation in the internal spacers in the range of 1–10% in two species of *Phialophora*. On the other hand, a low variability in these regions has also been reported. Hallenberg *et al*. (1996) found it to be less than 2% in the ITS2 region of the basidiomycete *Peniophora*, whereas ITS1 was found to be more variable. In *Sclerotiniaceae* intraspecific variation in the ITS1 region was reported to be minimal by Carbone & Kohn (1993). A very low variation in the same region was also reported by Sreenivasaprasad, Brown & Mills (1993) for *Colletrotrichum kahawae* and *C. gloeosporoides*.

The molecular data show that the majority of the specimens, i.e. *M. subtile*, form a well supported clade. Thus the genomic variation in the ITS1-5.8S-ITS2 region of the samples studied supports the recognition of *M. subtile* as a species different from *M. albonigrum* (Fig. 1). This is also supported by morphological data. *M. subtile* has been noted to have a large intraspecific morphological variability, and it has been suggested that *M. subtile* may contain populations that could be recognized as distinct taxa (Tibell 1987a). In this context in particular the variability of the exciple structure was pointed out, and it was suggested that populations with different types of exciple may inhabit different geographical areas or niches. Exciple thickness (character D; Table 2), however, showed a continuous variation and there was no correlation between exciple thickness and the molecular phylogeny obtained.

Two specimens belonging to *M. minutellum*, in accordance with the concept of Poelt (1969), were included in our study (Ms00001 and Ms16207). In the molecular analysis (Fig. 1) they proved to be well nested in *M. subtile*, but rather far apart. In the morphological analysis they associated, but were still part of the main *M. subtile* cluster. In our opinion, specimens identified as *M. minutellum* only differ from *M. subtile* in characteristics associated with ascoma size, and they do not merit recognition at species level. The suggestion that *M. minutellum* is a taxonomic synonym of *M. subtile* (Tibell 1987b) is thus supported.

Four specimens included in the study and originally thought to belong to *M. subtile* do not belong to this taxon. Msp00975 (Canada) and Msp17604 (Argentina) are similar to each other and quite distinct from *M. subtile* in the morphological analysis (Fig. 2). Their ITS sequences are also quite different from those of *M. subtile* (Fig. 1). They have, for example, a different type of ascus apex and most probably represent an undescribed species, but more material is needed for an adequate morphological characterization.

Ms01896 (Sweden) and Ms02173 (New Zealand) form a sister-clade to *M. subtile* in the molecular phylogeny; the percentage similarity between their ITS1-5.8S-ITS2 sequences is 92%. The sequences of both collections were re-analyzed starting from newly culture material in order to eliminate the possibility of contamination, but the same sequences were obtained. As a clade they have a rather high support, but are only rather weakly supported to form a clade with *M. subtile* or *M. albonigrum*. These specimens, however, were far apart in the morphological analysis (Fig. 2). Trusting the molecular evidence, they may, however, represent a morphologically cryptic, undescribed taxon.

In conclusion, the molecular phylogeny showed *M. subtile* to form a well-supported clade distinct from *M. albonigrum*. It also revealed the existence of a morphologically distinctive, undescribed species (Msp00975 and Msp17604) with quite a different ITS-region. Further, it identified a morphologically cryptic taxon more closely related to *M. subtile* as shown by parsimony analysis.

### *Biogeography*

In the *M. subtile s. str*. clade, the geographical origin of the material was not correlated with the genomic structure (Fig. 1).

In the morphological analysis (Fig. 2) there were some weak tendencies for a geographical grouping of the material. Two collections from eastern Europe are close, and the North American collections tend to aggregate in the upper left corner of the ordination. What is astonishing is that the north European collections are very scattered. This may, at least in part, reflect that this area is strongly over-represented in the material. It may also reflect that north European populations may have been established from widely different sources after the latest glaciations.

The distinct clade of Ms1896 (Sweden) and Ms02173 (New Zealand) in the molecular phylogeny is puzzling. It may be seen as support for the possibility of long distance dispersal in the genus (*cfr* Tibell 1994). The possibility that this clade represents an old, separate, and slow-evolving, possibly widely distributed taxon cannot, however, be ruled-out.

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