Phylogeny and taxonomy of the family
Arthrodermataceae (dermatophytes) using sequence
analysis of the ribosomal ITS region

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The internal transcribed spacer (ITS) region, covering the ITS1, ITS2 and 5·8S ribosomal DNA was used to evaluate phylogenetic relationships within the fungal family Arthrodermataceae. Sequences of variable length, ranging between 522 and 684 base pairs were aligned. An unrooted consensus tree based on parsimony analysis showed *Trichophyton* to be polyphyletic, and *Microsporum* to be paraphyletic. Non-monophyly of these two genera is in conflict with traditional classification. But this relation is not strongly supported by bootstrap analysis. Phylogenetic analysis showed that the two known members of the genus *Epidermophyton* grouped widely apart from each other. Within *Trichophyton*, our results suggest a separation of human pathogenic species and primarily geophilic species. Bootstrap support for these two groups is fairly high and both groups are recognized by current taxonomy. Three lineages were revealed within the *T. mentagrophytes* species complex. *Microsporum canis, M. audouinii* and *M. equinum* were found to be closely related. The topology of the tree was robust to various methods of analysis (parsimony and distance) and a different weighting scheme. Weighting of transversions over transitions did not improve the status of poorly supported branches of the tree.

Keywords dermatophytes, phylogeny, taxonomy

Introduction

Dermatophytes are fungi that, in humans, cause superficial infections such as ringworm, favus or athlete’s foot; other species occur on animals or in soil. They are usually seen in culture as conidial anamorphs, that is, as asexually reproducing forms. These anamorphs are classified in the genera *Epidermophyton, Keratinomyces, Microsporum* and *Trichophyton*. Related but non-pathogenic microconidial anamorphs are referred to the genus *Chrysosporium*. Teleomorphs (sexual stages), where known, are members of the family Arthrodermataceae of the ascomycetous order Onygenales. Until recently, teleomorphs of *Microsporum* were classified in *Namizzia* and those of *Chrysosporium, Keratinomyces* and *Trichophyton* in *Arthroderma*. The two genera were unified into *Arthroderma* by Weitzman et al. [1]. The dermatophytes are termed geophilic, zoophilic or anthropophilic depending upon whether their natural niche/reservoir is in soil, on animals or on humans. However, members of all three ecological groups are potentially able to cause human infections [2]. Criteria for distinction of dermatophyte species up to now mainly rest upon cultural, morphological and physiological characteristics. However, they may show considerable variation and pleomorphism within
established taxa. This makes the taxonomy of these fungi unsatisfactory. Several molecular approaches were used in an attempt to solve this problem, but hitherto provided insufficient taxonomic resolution. Nuclear DNA (nDNA) base composition was investigated but was not useful because of lack of differentiation between taxa [3]. To evaluate this group of fungi using nDNA hybridization a larger number of closely related taxa would have to be studied [4]. Mitochondrial DNA RFLP data failed to differentiate between some species that are considered to be distinct based on classical methods [5–7]. Partial ribosomal DNA sequence analysis of the nuclear small and large subunits provided insufficient variation to establish a hierarchy of species [8,9]. Given the shortcomings of these methods we used in the present study the more variable ITS region to evaluate phylogenetic relationships within the Arthrodermataceae.

**Material and methods**

**Strains and cultures**

Nucleotide sequences of the ribosomal ITS region were determined of 54 strains belonging to 41 currently recognized species and varieties of the genera *Chrysosporium*, *Epidermophyton*, *Keratinomyces*, *Microsporum* and *Trichophyton*, including their teleomorphs where known (Table 1). When available, type, paratype or authentic strains were used. All strains were grown at room temperature on Sabouraud glucose agar for 2–3 weeks. In an attempt to solve this problem, but hitherto provided insufficient taxonomic resolution. Nuclear DNA (nDNA) base composition was investigated but was not useful because of lack of differentiation between taxa [3]. To evaluate this group of fungi using nDNA hybridization a larger number of closely related taxa would have to be studied [4]. Mitochondrial DNA RFLP data failed to differentiate between some species that are considered to be distinct based on classical methods [5–7]. Partial ribosomal DNA sequence analysis of the nuclear small and large subunits provided insufficient variation to establish a hierarchy of species [8,9]. Given the shortcomings of these methods we used in the present study the more variable ITS region to evaluate phylogenetic relationships within the Arthrodermataceae.

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**DNA isolation**

DNA isolation was according to Gruber [10], with some modifications. Briefly, one fungal colony (0.5–1.0 cm in diameter) was cut from the agar plate with a scalpel, transferred to a mortar and ground in liquid nitrogen. The mycelial powder was transferred to an Eppendorf tube and resuspended in 1 ml of extraction buffer (100 mM Tris-HCl [pH 8.0], 1.4 M NaCl, 20 mM EDTA, 2% cetyltrimethylammonium bromide, 2% β-mercaptoethanol). The cells were subjected to three rounds of freezing and thawing. Then proteinase K (AGS, Heidelberg, Germany) was added to give a final concentration of 1 mg ml⁻¹ and the mixture was incubated for 1.5 h at 60 °C. After centrifugation for 5 min at 13000 g one volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the supernatant and the tube was vortexed and centrifuged again at 13000 g for 15 min. The upper phase was removed. This procedure was repeated four to five times; for the last treatment only one volume of chloroform was added. The DNA was precipitated with 1/10 volumes of sodium acetate (4.2 M, pH 5.2) and 1 ml of cold isopropanol. After incubation for 16 h at −20 °C, the DNA solution was centrifuged for 10 min, the pellet was washed with 70% ethanol and air dried. DNA was dissolved in 1 x TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and treated with RNase at a final concentration of 100 μg ml⁻¹ (Sigma, Deisenhofen, Germany) for 15 min at 37 °C, after which it was stored at 4 °C. DNA concentration was estimated by measuring the optical density at 260 nm.

**Sequence determination**

The ribosomal ITS region was amplified using universal primers LR1 (5’GGTTGTTTTTCCTTTTCTCT) and SR6R (5’AAGTTAAAGGTCAAAAGGG), corresponding to positions 73–57 of the 25S and positions 1744–1763 of the 18S nuclear rDNA gene of *Saccharomyces cerevisiae*, respectively. For sequencing both strands, one of the two primers was biotinylated in two reciprocal PCR reactions. Single stranded DNA was obtained for direct sequencing by using streptavidin-coated magnetic beads (Dynabeads® M 280, Dynal, Oldendorf, Germany). Each of the strands were sequenced using the same IR (infrared)-labelled primers in the sequencing reaction (Se- quiTherm Exel™ Long Read Cycle Sequencing Kit, Biozym Technologies, Oldendorf, Germany), combined with a LI-COR automatic DNA sequencer.

**Sequence alignment and phylogenetic analysis**

The amplified products ranged in length from 640 to 800 nucleotides. The amplicons included the ITS1 and ITS2 regions, the 5.8S rDNA gene (156 bp) as well as parts of the 18S and 25S rDNA genes (total 124 bp). Four separate alignments were performed with groups of strains having mutually similar nucleotide sequences, using CLUSTAL V as the alignment program. A complete alignment of all dermatophyte species was subsequently obtained with the help of PROFILALIGN (DKFZ, Heidelberg). Sequences could be aligned unambiguously since (i) variable domains were flanked by conserved regions (e.g. 5.8S DNA with only seven substitutions and parts of the 18S and 25S rDNA), and (ii) regions with length mutations were recoded as a fifth character state to yield informative characters for phylogenetic analysis [11]. Parsimony analysis was conducted excluding uninformative characters and using stepwise addition of sequences of the heuristic search option of PAUP 3.1.1. [12]. For distance trees Kimura’s two parameter model matrix and the neighbor-joining method were applied [13] using TREECON 1.1. [14]. Distance trees were calculated excluding the recoded characters. Subtrees were generated with
Table 1 Strains analysed in the study—anamorph

<table>
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<th>Species</th>
<th>CBS No.</th>
<th>Teleomorph</th>
<th>CBS No.</th>
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<td>417·65 T</td>
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<td></td>
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<td><em>T. tonsurans</em> †</td>
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<td><em>A. quadrididum</em></td>
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<td><em>A. gertleri</em></td>
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<tr>
<td>*M. canis var. <em>canis</em> †</td>
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<td><em>A. otae</em></td>
<td>496·86 MT</td>
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<td><em>A. fulsum</em></td>
<td>167·64 MT +; T</td>
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<td><em>A. incurvatum</em></td>
<td>173·64 MT +</td>
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<tr>
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<td><em>A. persicolor</em></td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<tr>
<td><em>E. floccosum</em> †</td>
<td>358·93</td>
<td>Unknown</td>
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</table>

Species which are: * geophilic; † zoophilic; †† anthropophilic. MT, mating type (sexually compatible); T, type strain; PT, paratype; AUT, authentic strain; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

both analysis methods using the separate alignments mentioned above, taking all variable characters into account (gaps were not longer than five nucleotide positions). The robustness of branches for the parsimony trees was assessed by bootstrap analysis with 100 replicates. No bootstrap analysis was performed for the
neighbor-joining tree. A different weighting scheme was applied where transversions were weighted two times that of transitions.

Results and discussion
The topologies of the trees were robust to various methods of analysis (parsimony and distance) and to a different weighting scheme. Weighting of transversions over transitions did not improve the status of poorly supported branches of the trees. For this reason only parsimony trees are shown.

General phylogenetic tree
Fig. 1 shows an unrooted consensus tree out of the 56 shortest parsimony trees. *Trichophyton* appears to be polyphyletic, while *Microsporum* is paraphyletic. Nomenonophyly of these two genera conflicts with traditional classification, but it should be stated that only weak bootstrap support (BS) was obtained for this result. Our data suggest that *Microsporum* as well as the zoophilic and anthropophilic *Trichophyton* species have evolved from a geophilic member of the latter genus. Bootstrap value (99%) supports separation of a mainly geophilic group from the remaining members of the Arthrodermataceae, which were referred to as the ‘true dermatophytes’ by Leclerc et al. [9]. The geophilic group seems to be rather heterogeneous, containing *Arthroderma* species with microconidia only (*Chrysosporium* anamorphs), *Trichophyton terrestre* with thin-walled micro- and macroconidia, *Epidermophyton stockdaleae* with macroconidia only, as well as *Keratinomyces ajelloi* with thick- and smooth-walled macroconidia and sparse microconidia. The group contains a single purported zoophilic species, viz. *Trichophyton equinum*, CBS 292-81. This strain was isolated from hair of a non-infected horse, which was probably contaminated accidentally; the possibility of a misidentification cannot be excluded. In that case the entire group would consist of fungi that have their primary niche in soil. Leclerc et al. [9] obtained similar results in their 25S rRNA analysis of *Trichophyton*, where *K. ajelloi* and *T. terrestre* were also separated from the ‘true dermatophytes’. Restriction analysis of mtDNA [15] also showed a more distant grouping of the teleomorphs of *T. terrestre*, *Arthroderma quadrifidum* and *A. insinulare* to the dermatophytic species of the family Arthrodermataceae. Even Davison and Mackenzie [4] found in DNA homology studies that DNA from *T. terrestre* had little sequence relatedness when compared with DNA from other dermatophytes. Thus, a subdivision of *Trichophyton* into a group including human and animal pathogenic species and a group containing the primarily geophilic species seems warranted. This would favour the maintenance of the name *Keratinomyces* for the geophilic fungi rather than *Trichophyton*, as was advocated by de Hoog and Guarro [16]. The group seems to be defined by ecological rather than by morphological features. The separate positions of *Arthroderma quadrifidum* and *A. insinulare* within this group confirm the subdivision of *Trichophyton terrestre* in two entities. The third teleomorph of *T. terrestre*, *A. lenticularum*, has not been investigated in this study.

Within the genus *Epidermophyton* two species have traditionally been recognized, both of which are characterized morphologically by the presence of macroconidia and absence of microconidia. Phylogenetic analysis showed that the two *Epidermophyton* species were located widely apart with fairly high bootstrap support. These results suggest that absence of microconidia may not necessarily be relevant for classification at the generic level. The separate position of the *Epidermophyton* species is ecologically consistent since the geophilic species *E. stockdaleae* grouped within the geophilic *Tricho phyton/Keratinomyces* group, while the anthropophilic species *E. floccosum* is more closely related to the anthropophilic *Trichophyton* species.

Fig. 1 Parsimony consensus tree of 56 trees obtained for ITS sequences of all dermatophyte species listed in Table 1. The tree was generated by using stepwise addition of sequences of the heuristic search option of PAUP (version 3.1.1). Forty-four of the 855 selected sites were informative and the tree length is 105 steps. All trees were midpoint rooted and only bootstrap values above 70% are shown. Number in brackets refer to CBS numbers listed in Table 1. Teleomorphs, where known, are given with anamorphs in brackets otherwise anamorphs are listed. * Geophilic, + zoophilic and # anthropophilic species. EMRL accession numbers for the sequences are: *Trichophyton rubrum*, Z97993; *T. megnini*, Z97994; *T. mentagrophytes* var. *mentagrophytes*, Z97995; *T. mentagrophytes* var. *erinacei*, Z97996-7; *T. mentagrophytes* var. *goetii*, Z97998; *T. mentagrophytes* var. *nodulare*, Z97999; *T. mentagrophytes* var. *quincunx*; Z98000; *T. mentagrophytes* var. *interdigitale*, Z98001; *T. verrucosum* var. *verrucosum*, Z98002-3; *T. verrucosum* var. *ochraceum*, Z98004; *T. tonsurans*, Z98005-8; *T. equinum* var. *equinum*, Z98009; *T. schoenleini*, Z98010-11; *T. concentricum*, Z98012; *T. canbreseghemii*, Z98013; *Microsporum canis* var. *canis*, AJ000617-18; *M. canis* var. *distortum*, AJ000619; *M. gallinae*, AJ000620; *M. gyipseum* var. *gypseum*, AJ000621; *M. audouini* var. *audouini*, AJ000622-3; *M. audouini* var. *langeronii*, AJ000624; *M. audouini* var. *rivulare*, AJ000625; *M. equinum*, AJ000626; *M. fulvum*, AJ000627; *Epidermophyton stockdaleae*, AJ000628; *E. floccosum*, AJ000629; *Arthroderma canbreseghemii*, Z98014; *A. benhamiae*, Z98015-16; *A. simii*, Z98017-18, AJ000605; *A. quadrifidum*, AJ000632; *A. insinulare*, AJ000606; *A. uncinatum*, AJ000607-8; *A. cuniculi*, AJ000609; *A. curryi*, AJ000610; *A. otae*, AJ000611; *A. incurvatum*, AJ000630-1; *A. grubyi*, AJ000612-13; *A. persicolor*, AJ000614-15; *A. fulvum*, AJ000616.
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Fig. 1.

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Within *Microsporum*, the general tree shows little resolution among *M. canis, M. canis* var. *distortum, M. equinum, M. audouinii, M. audouinii* var. *langeronii* and *M. audouinii* var. *ritleri* (BS 80%). Our data confirm the close relatedness of the putative asexual species *M. gallinae* with *A. grubyi* (BS 100%) as previously inferred from restriction analysis of mtDNA [17]. Our results support Georg’s et al. [18] suggestion that *Arthroderma grubyi* comprises the two anamorphs *Microsporum vanbreuseghemii* and *M. gallinae*. *Microsporum gallinae* did not produce ascomata when crossed with tester strains of *A. grubyi* [19], but a negative mating test does not provide conclusive evidence for distinctness of species.

The phylogenetic data suggest that teleomorphs were lost several times during the evolution of dermatophytes. Teleomorphic species are present within every lineage (Fig. 1). They are relatively frequent in the geophilic group and less abundant in the anthropophilic *Trichophyton* species. For example, the very common species *Trichophyton rubrum* and *T. tonsurans* are hitherto exclusively known as anamorphic species. Such taxa seem to have switched to clonal reproduction.

**Phylogenetic subtrees**

Within *Trichophyton mentagrophytes* molecular data support lineages that correspond with several of the currently recognized varieties (Fig. 2). However, these lineages are found at large distances from each other, which might justify a species status for the oldest varieties *T. mentagrophytes* var. *interdigitale* and *T. mentagrophytes* var. *erinacei*. Nishio’s et al. [7] restriction analysis of mtDNA also revealed heterogeneity within *T. mentagrophytes*, but they were unable to resolve members of complexes 1 and 2. The phylogeny corresponds well with ecology, since anthropophilic (complex 1) and zoophilic (complexes 2 and 3) strains group with taxa having similar preference. The zoophilic *T. mentagrophytes* var. *erinacei* and var. *granulosum* (complex 3), are closely related to *T. verrucosum* (including its var. *ochraceum*), forming a single group at BS 100%. The var. *granulosum* is a mating partner of *A. benhamiae* [20]. *Trichophyton verrucosum* is a zoophilic species showing no sporulation. In addition this group contains an anthropophilic species, *T. concentricum*, which is difficult to identify in culture morphologically since sporulation is absent. Its clinical picture (tinea imbricata) is quite characteristic, but nevertheless a second strain identified as *T. concentricum* clustered within the *M. canis/M. audouinii* complex (data not shown). Strains assigned morphologically to *T. concentricum* thus may be degenerate forms of divergent taxa. The varieties *T. mentagrophytes* var. *interdigitale*, var. *goetzi* and var. *nodulare* (complex 1) on the one hand, and *T. mentagrophytes* var. *mentagrophytes* and var. *quinckeaeum* (complex 2) on the other were recognized as two individual lineages (Fig. 2). The var. *goetzi* was described for strains with dark olivaceous colonies, which were already known as var. *batonrougei* [21]. *Trichophyton mentagrophytes* complex 1 (BS 85%) was closely related to *T. tonsurans*, whereas complex 2 showed a close relatedness to *T. schoenleinii* (BS 96%; Fig. 2). In the mtDNA RFLP data published by Nishio et al. [7], *T. tonsurans* also formed a group with members of complex 1, while *T. mentagrophytes* var. *quinckeaeum* and *T. schoenleinii* were indistinguishable. This latter association is quite surprising, since *T. schoenleinii* can be distinguished by the formation of favic chandeliers and by its characteristic clinical picture with scutellae. The strains originated from human scalp hair are without confirmed report of favus and might be a misidentification. Thus, this item needs further study to verify their classification.

Mochizuki et al. [22] showed with mtDNA restriction analysis that *T. mentagrophytes* var. *interdigitale* is a genetically homogeneous taxon derived from *Arthroderma vanbreuseghemii* as postulated earlier on the basis of morphology by Takashio et al. [23]. *Arthroderma simii* and *A. benhamiae* are clearly different, despite similarities in their anamorphs. Our data are compatible with this finding, confirming the close relationship of *T. mentagrophytes* var. *interdigitale* and *A. vanbreuseghemii* (Fig. 2). *Trichophyton rubrum* and *T. megnini* were found to be closely related, composing a monophyletic group supported by a bootstrap value of 100% (Fig. 2). Both species differ physiologically in that *T. megnini* requires histidine. Nowadays both species mostly cause tinea corporis [16]. Frequently, *T. rubrum* also is a agent of tinea manuum and tinea pedis, with the last-named infection usually progressing to distal-subungual onychomycosis as the patient ages. *Trichophyton megnini* was historically mostly an agent of tinea barbae, but home shaving has virtually ended that.

The unrooted consensus tree in Fig. 3 shows a better resolution of the relationships within the *M. canis/M. audouinii/M. equinum* complex than the general tree. Based on a subalignment using the variable sites among the strains of this complex which had been excluded and

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**Fig. 2** Parsimony consensus tree for 25 strains, including the common human pathogenic dermatophytes. The tree was generated by using stepwise addition of sequences of the heuristic search option of PAUP (version 3.1.1). One hundred and twenty-two characters of 749 nucleotides were parsimony informative. Uninformative characters were excluded and the length of the three shortest trees was 176 steps.
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Fig. 2.

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Fig. 3
recoded as a single character state in the general alignment, *M. canis* was separated from *M. audouinii* with a bootstrap support of 100%. Microsporum equinum always grouped with *M. canis* var. *distortum* (BS 75%). This finding is in agreement with identical mtDNA restriction patterns yielded for both species and with the view that *M. equinum* probably is an anamorph of *A. otae*, although crossing experiments thus far have failed [17]. In general, the ITS region showed up to 2.6% intraspecies variation, i.e. between proven mating partners. This value is within the range of intraspecies variation reported for other fungi (data not shown). This excludes the extremely high values among the three *T. mentagrophytes* complexes (up to 15%). The ranges of variability within each complex were similar to those of single species, viz. 0.45–0.9%. Within the complex *M. equinum* and *M. canis* var. *distortum* the interspecies variation was 0.7% and within the group *M. audouinii* var. *audouinii*, *M. audouinii* var. *langeronii* and *M. audouinii* var. *rivalieri* this was 0.4%. Between *M. canis* var. *canis* and *M. audouinii* var. *audouinii* the ITS region showed 2.2% variation. This value is relatively high and the separation of the two taxa is supported by a BS of 100%. On the other hand, is their close relationship supported by similar morphology and physiology. Most features appear to be rather quantitative than qualitative. This also holds true for clinical symptoms, which in *M. audouinii* are less inflammatory and more chronic than with *M. canis* as etiological agent. Thus, it remains uncertain whether or not these taxa should be maintained separately. More strains of this complex were similar to those of single species, viz. *Trichophyton mentagrophytes* and *Epidermophyton stockdaleae*. From our data the following main taxonomic conclusions can be drawn, (i) retaining *Trichophyton* and *Microsporum* as separate genera does not reflect the natural phylogeny; (ii) the two *Epidermophyton* species are unrelated and *E. stockdaleae* should be reclassified; (iii) *T. mentagrophytes* consists of three species; (iv) the number of varieties of *T. mentagrophytes* should be reduced; (v) the genus *Keratinomyces* should be restored; (vi) the following taxa are so closely related that they are probably conspecific: *T. rubrum* and *T. meginitii, T. gallinae* and *A. grubyi*, and *M. audouinii* var. *rivalieri, M. audouinii* var. *langeronii* and *M. audouinii, M. canis and M. equinum.*

**Acknowledgements**

We thank Jaqueline Rudolph and Helga Melle for excellent technical assistance. Funding was provided by the Deutsche Forschungsgesellschaft, GR 1147/1-1, to H.-J. Tietz and Y. Gräser.

**References**


