Phylogeny of the anamorphic genus *Chrysosporium* and related taxa based on rDNA internal transcribed spacer sequences

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Summary

Phylogenetic relationships of 57 species of *Chrysosporium* and related species belonging to Onygenales and Sordariales were studied by analysing the nucleotide sequences of the 5·8S rRNA gene and their flanking ITS1 and ITS2 regions. *Chrysosporium* is a polyphyletic taxon with affiliations to at least two orders of the Ascomycota. The phylogenetic trees obtained in this study divided the genus into nine highly supported monophyletic groups (clades I-IX). However, relationships among these groups were not resolved. These groups were consistent for both methods of comparison (neighbor-joining and parsimony) and clearly correlated with groupings based on classical morphological criteria, enzymatic capabilities and teleomorph morphology. The genus *Chrysosporium* should be restricted to anamorphs of Onygenales, while *Geomyces* should be restricted to anamorphs of Myxotrichaceae and *Myceliophthora* to anamorphs of Sordariales. This study also demonstrates that several pairs of species are very similar, which suggests that they are synonymous, e.g. *C. articulatum* and *A. reticulosporus*; *C. keratinophilum* and *A. keratinophilus* and *C. lucknowense* and *C. mephiticum*.

Key words

Fungi, Ascomycota, Onygenales, Molecular taxonomy

The genus *Chrysosporium* Corda comprises a large group of ubiquitous, mostly soil-borne and often keratinolytic anamorphic species which are responsible for occasional opportunistic infections in humans; a few morphologically related species, however, are frequent agents of severe infections [1]. *Chrysosporium* species are characterized by mostly white to yellowish colonies and fertile hyphae with few distinguishing characteristics. They have terminal and intercalar conidia, which release rhexolytically. Its teleomorphs are mostly in the Onygenales, an ascomycete order characterized by ascomata with very simple peridial structures (usually poorly differentiated interwoven hyphae), globose and evanescent asci with eight small, slightly coloured ascospores. Currah [2] examined the ascospore wall ornamentation, the enzymatic capabilities and the type of conidia dehiscence and split the order Onygenales into four families; Onygenaceae, Arthrodermataceae, Gymnoascaceae and Myxotrichaceae. Von Arx [3] did not accept the order Onygenales, and instead re-evaluated the order Eurotiales. Emphasizing the shape, size and symmetry of the ascospores, he divided it into the following four families: Eurotiaceae, Gymnoascaceae, Onygenaceae and Amauroascaceae. Delimitations were different from those of Currah. There is some consensus in the sense that Currah's approach provides compelling evidence of a relationship between at least three of the four families (Onygenaceae, Arthrodermataceae and Gymnoascaceae) [4]. The genus *Chrysosporium* shows perhaps the simplest asexual reproductive structures of the hyphomycetes. These are sessile, solitary, small and hyaline conidia that emerge laterally from vegetative hyphae. Primitive structures like these have also occasionally been associated with the genera of other orders of Ascomycota, such as *Thielavia*, *Zopfiella*, *Apiosordaria*, etc. *Chrysosporium* is therefore polyphyletic.

The first comprehensive review of *Chrysosporium* was made by Carmichael [5], who considered that some morphologically similar genera, such as *Geomyces* (Traaen), *Emmonsia* (Cif. & Montermartini), *Myceliophthora* (Cost.) and *Blastomyces* (Gilchrist & Stokes) were synonyms of *Chrysosporium*. Dominik [6] later included certain species of *Sepedonium* in *Chrysosporium* and Sigler and Carmichael [7] re-evaluated *Geomyces*. The most recent review of the genus was made by van Oorschot [8], who again separated *Chrysosporium* from the related genera mentioned above. Recently, numerous new species of *Chrysosporium* have been described from different geographical regions and substrates [9-28]. There are currently about 60 accepted species. This large number of species and the fact that

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they are not easy to distinguish makes it difficult for non-experts to identify them or to differentiate them from species of similar genera whose boundaries are not clearly established.

Although several approaches based on chemotaxonomy, e.g. those concerning the ubiquinone system [29,30] are useful for classifying fungi but they are not discriminative enough for *Chrysosporium* spp. There have been several attempts to obtain useful taxonomic markers based on DNA-typing techniques [31,32] but, although able to elucidate systematic ambiguities they have been restricted to only a few species and are not useful for routine identification. Some molecular studies have included some *Chrysosporium*. However, these studies concentrated mainly on resolving the relationship of pathogenic species rather than on taxonomic classification [33-39]. More recently, Sugiyama *et al.* [40] by analysing the SSU rDNA sequences, provided a reliable tool for better clarifying the taxonomy of Onygenales. This study, however, included few *Chrysosporium* species.

This study aims to establish phylogenetic relationships among *Chrysosporium* species and between this genus and morphologically related taxa. It also aims to assess the consistency of the most important morphological characters used for taxonomic purposes. We therefore analysed the sequences of ITS region of representative species of *Chrysosporium* and related species.

Isolates. We studied a total of 57 species. Isolates are shown in table 1. Some were provided by different Culture Collections.

DNA extraction. Fungi were grown in PYE broth on an orbital shaker at 250 rpm at room temperature (20-28°C). All cultures were tested for purity. DNA was extracted by the miniprep protocol of Lee and Taylor [41].

Amplification of DNA. The internal transcribed spacers (ITS1, ITS2) and the 5·8S rRNA gene, defined by primers ITS4 and ITS5 [42] were amplified using the polymerase chain reaction. Amplification was performed in 100 µL reaction volumes containing 0.2 - 0.3 µg of total genomic DNA as template, 1.5 µM of each primer (MedProbe, Norway), 200 µM of each deoxynucleotide triphosphate (Boehringer Mannheim) and 2 units of cloned *Taq* DNA polymerase (Promega), 10 mM Tris-HCl pH 9.5, 50 mM KCl, 0.1% Triton X-100 and 1.5 mM $MgCl_2$. Amplification was with an automated thermocycler (GeneAmp 2400, Perkin-Elmer) programmed for 30 cycles of 1 min at 95°C, 1 min at 50°C, 1 min at 72°C, a pre-denaturation step of 5 min at 95°C and a post-extension step of 10 min at 72°C. Negative controls were used in each set of reactions.

Sequencing. Doubled-stranded ITS4/ITS5 products of the strains listed in table 1 were sequenced using the Thermo-Sequenase kit (Amersham) according to manufacturer's instructions. Internal primers ITS1, ITS2, ITS3 (MedProbe) [42] labelled with ^{35}S-dNTP (5 µCi/reaction; Amersham) using the PCR products (2.5 µg) as templates, were employed at a concentration of 171 pM. Sequencing reactions were electrophoresed and autoradiographied according to Sambrook *et al.* [43]. Alternative sequencing reactions were performed on the ABI PRISM 377 DNA sequencer with the ABI PRISM™ dRhodamine Terminator Cycle Sequencing. Sequences of the following species were obtained from European Molecular Biology Laboratory (EMBL): *Ajellomyces* spp., *Corynascus* spp., *Emmonsia parva*, *Myxotrichum* spp., *Arthroderma benhamiae*, *Coccidioides immitis*, *Gymnostellatospora japonica*, *Paracoccidioides brasiliensis*, *Pseudogymnoascus roseus* and *Trichophyton rubrum*. Sequences of *Amauroascus aureus*, *Nannizziopsis albicans* and *Uncinocarpus reesii* were provided by Dr. Cano. EMBL accession numbers of the sequences obtained in this study are given in table 1.

Table 1. Strains used in this study.

Strain	Collection number	EMBL accession no
Ajellomyces capsulatus (*)		AF038354
Ajellomyces dermatitidis (*)		AF038355
Amauroascopsis perforatus	FMR 3882	AJ390377
Amauroascus aureus	CBS 593.71	-
Amauroascus volatilis-patellis	IMI 155647	AJ390378
Aphanoascus fulvescens	FMR 6154	AJ390379
Aphanoascus keratinophilus	IMI 319010	AJ390380
Aphanoascus reticulosporus	IMI 129854	AJ390381
Aphanoascus terreus	CBS 342.64	AJ005369
Arthroderma benhamiae (*)		AF038359
Arthroderma ciferrii	CBS 272.66	AJ007844
Arthroderma cuniculi	RV 20035	AJ390382
Arthroderma curreyi	IFO 31699	AJ005370
Arthroderma tuberculatum	IMI 86177	AJ390383
Candida albicans (*)		CA58RRNA
Chrysosporium articulatum	UAMH 4320	AJ007841
Chrysosporium carmichaelii	CBS 643.79	AJ007842
Chrysosporium europae	CBS 321.86	AJ007843
Chrysosporium evolceanui	RV 26475	AJ005368
Chrysosporium filiforme	CBS 187.82	AJ131680
Chrysosporium fluviale	IMI 378764	AJ005367
Chrysosporium keratinophilum	IFO 7584	AJ131681
Chrysosporium lobatum	CBS 666.78	AJ131688
Chrysosporium lucknowense	IMI 112798	AJ131682
Chrysosporium mephiticum	CBS 320.86	AJ131683
Chrysosporium merdarium	CBS 408.72	AJ390384
Chrysosporium minutisporosum	IMI 379912	AJ131689
Chrysosporium pilosum	FMR 4157	AJ390385
Chrysosporium pseudomerdarium	UAMH 4330	AJ390386
Chrysosporium siglerae	UAMH 6541	AJ131684
Chrysosporium submersum	FMR 6088	AJ131686
Chrysosporium sulfureum	CBS 634.79	AJ390387
Chrysosporium synchronum	IMI 282433	AJ390388
Chrysosporium tropicum	UAMH 691	AJ131685
Chrysosporium undulatum	IMI 375884	AJ007845
Chrysosporium vallenarense	ATCC 64421	AJ390389
Chrysosporium vespertilium	RV 27093	AJ007846
Coccidioides immitis (*)		CI18360
Corynascus sexualis (*)		CSE224202
Corynascus similis (*)		CSI224201
Corynascus verrucosus (*)		CVE224203
Emmonsia crescens (*)		AF038334
Emmonsia parva (*)		AF038331
Gymnostellatospora japonica (*)		AF062818
Geomyces asperulatus	IFO 31714	AJ390390
Myxotrichum chartarum (*)		AF062813
Myxotrichum stipitatus (*)		AF062816
Nannizziopsis albicans	IMI 155645	-
Nannizziopsis vriesii	CBS 407.71	AJ131687
Paracoccidioides brasiliensis (*)		AF038360
Pectinotrichum llanense	RV 22834	AJ390391
Pseudogymnoascus roseus (*)		AF062819
Renispora flavissima	IMI 241796	AJ390392
Trichophyton rubrum (*)		TR18352
Uncinocarpus orissi	CBS 340.89	AJ390393
Uncinocarpus queenslandicum	IMI 121675	AJ390394
Uncinocarpus reesii	CBS 121.77	-

(*) sequences obtained from EMBL data bank
ATCC: American Type Culture Collection, Maryland, USA.
CBS: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.
FMR: Facultat de Medicina de Reus, Spain.
IFO: Institute for Fermentation, Osaka, Japan.
IMI: International Mycological Institute, Egham, UK.
RV: Institut de Médecine Tropicale "Prince Léopold", Antwerpen, Belgium.
UAMH: University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada.

Phylogenetic analysis. Sequences were aligned using the CLUSTAL W program [44] with a GOP of 10·0 and a GEP of 5·0. *Candida albicans* was used as an outgroup. Phylogenetic analysis were performed using both additive distances and parsimony analysis methods. The neighbor-joining (NJ) method was applied to DNA distance matrices calculated according to Kimura two-parameter model [45] using TREECON 1.3b [46]. The maximum parsimony (MP) was performed using PAUP 3·1·1 [47].

Alignment gaps were treated as missing data and characters were equally weighted. First, 1000 heuristic searches were performed with random taxon addition sequences and with the following settings: no topological constraints enforced, no steepest descent option, collapsing zero length branches and keeping all minimal trees (MULPARS). Second, all the shortest trees from the first part of the analysis were used as starting trees for complete tree-bisection-reconnection (TBR) branch swapping algorithm. Clade stability was assessed by 100 bootstrap replications [48] and by decay index (DI) [49,50]. These were calculated from a tree file containing 15283 trees up to five steps longer than the most parsimonious trees (MPTs).

We have analysed the ITS1-5·8S-ITS2 region of the rDNA of 38 species of *Chrysosporium* and 18 species of related genera (Table 1). ITS1 and ITS2 regions were aligned along with the intervening 5·8S rDNA (177-182 base pairs, bp) and flanking partial sequences of the ribosomal small (SSU rDNA) and large (LSU rDNA) subunits (112 bp in total). The average size of this region for *Chrysosporium* spp. was 647 base pairs (bp), of which the smallest was that of *Chrysosporium filiforme* (578 bp) and the largest that of *Renispora flavissima* (716 bp). ITS1 region ranged from 160 to 263 bp and ITS2 from 125 to 171 bp. The ITS1-5·8S-ITS2 region of the members of Myxotrichaceae and *Corynascus* spp. never exceeds 595 bp while that of Onygenales, except for *C. filiforme* and *C. pseudomerdarium*, always exceeds 595 bp. On average, the Arthrodermataceae displayed sequences of 697 bp. Sequences from 57 OTUs were used to obtain a data matrix of 819 characters, 532 of which were variable sites and 432 were parsimony informative.

NJ analysis is shown in figure 1. Cladistic analysis yielded 8 equally parsimonious trees (MPT) of 3125 steps. Consistency (CI), retention (IR) and rescaled consistency (RC) indices for each of these trees was 0·365, 0·635 and 0·229, respectively. The low consistency index of the parsimonious trees indicates that data from a more conserved region would probably be more suitable for our purpose. The eight equally parsimonious trees did not differ in the overall topology. The 50% majority rule consensus of the MPTs along bootstrap values and DI of the internal branches is shown in figure 2. In general, there was a high degree of congruence between the resulting phylogenies of the NJ and cladistic analysis. However, bootstrap support for most branches was lower in MP.

Chrysosporium was revealed as a polyphyletic taxon that has species associated with at least two currently recognized ascomycete orders, Onygenales and Sordariales. Our analyses resulted in nine main monophyletic lineages (clades I-IX) supported by bootstrap values ranging from 69 to 100%. Five of them grouped species belonging to the Onygenaceae *sensu* Currah [2] (clades I-V) and one to each of the following families, Arthrodermataceae (clade VII), Chaetomiaceae (clade VIII) and Myxotrichaceae (clade IX). The species included in clade VI have never been associated with meiotic states. Taxonomic relationships among these clades were not well resolved because support for internal branches forward the root received lower measures of clade stability. Therefore, the exact order of branching of the nine clades remains uncertain. The majority of these evolutionary clusters can be recognized mainly by teleomorphic rather than anamorphic features, and peridium type, ornamentation and shape of the ascospores are the most distinctive characters. These clusters were consistent irrespective of the method for inferring phylogeny. However, six *Chrysosporium* species changed their position according to the topology obtained. These were: *C. europae*, *C. merdarium*, *C. lobatum*, *C. pilosum*, *Amauroascopsis perforatus* and *Nannizziopsis vriesii* (Figures 1 & 2). In general, our results agree with those of Leclerc *et al.* [35] and Sugiyama *et al.* [40], although these authors concentrated mainly on resolving higher taxonomic relationships among the onygenalean fungi rather than discussing the phylogenetic relationships among species of *Chrysosporium*.

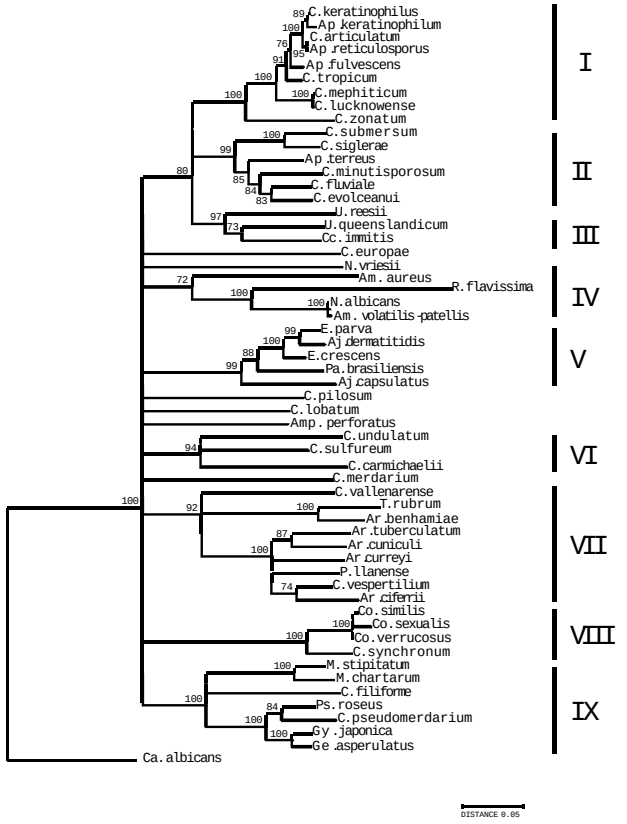

Figure 1. Phylogenetic relationships of *Chrysosporium* and related species by the ITS1-5·8S-ITS2 rDNA sequences analysing of the 57 strains listed in table 1. These distance tree was generated with the neighbor-joining method by TREECON. Bootstrap values > 50 % from 100 replicates are indicated above the respective internodes. Horizontal branch lengths are drawn to scale and represent the expected number of substitutions. *Ca. albicans* was used as outgroup. Bracketed groups I-IX are discussed in the text. (Aj.: *Ajellomyces*; Am.: *Amauroascus*; Amp.: *Amauroascopsis*; Ap.: *Aphanoascus*; Ar.: *Arthroderma*; C.: *Chrysosporium*; Ca.: *Candida*; Cc.: *Coccidioides*; Co. *Corynascus*; E.: *Emmonsia*; Ge.: *Geomyces*; Gy.: *Gymnostellatospora*; M.: *Myxotrichum*; N.: *Nannizziopsis*; P.: *Pectinotrichum*; Pa.: *Paracoccidioides*; Ps.: *Pseudogymnoascus*; R.: *Renispora*; T.: *Trichophyton*; U.: *Uncinocarpus*).

Clades I-II were strongly supported (each receiving ≥ 99% bootstrap support in NJ and 87-97% and ≥ 5 values of bootstrap and DI, respectively in MP), they included five species with an *Aphanoascus* teleomorph and some *Chrysosporium* species with an unknown teleomorph, e.g. *C. evolceanui*, *C. fluviale*, *C. lucknowense*, *C. mephiticum*, *C. minutisporosum*, *C. siglerae*, *C. submersum* and *C. zonatum*. *C. zonatum* was recently connected with *Uncinocarpus orissi* [51]. All these species are strongly keratinolytic.

Clade I includes thermotolerant species with spreading colonies and smooth-walled conidia arising from non-swollen conidiogenous cells, e.g. *Aphanoascus keratinophilum*, *A. reticulosporus*, *A. fulvescens*, *C. tropicum*, *C. lucknowense* and *C. mephiticum*. *Chrysosporium zonatum* operates as the common ancestor of this set with

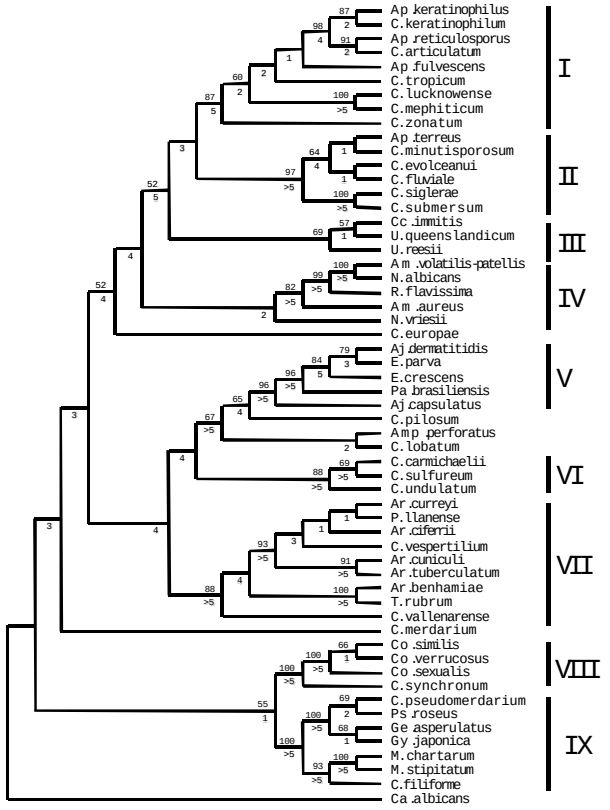

Figure 2. Phylogenetic relationships of *Chrysosporium* and related species by the ITS1-5·8S-ITS2 rDNA sequences analysing of the 57 strains listed in table 1. The 50 % majority-rule consensus cladogram based on the 8 MPTs (3125 steps) was inferred from heuristic search by PAUP. Bootstrap values and decay indices are indicated above and bellow branches, respectively. *C. albicans* was used as outgroup. Bracketed groups I-IX are discussed in the text. (Aj.: *Ajellomyces*; Am.: *Amauroascus*; Amp.: *Amauroascopsis*; Ap.: *Aphanoascus*; Ar.: *Arthroderma*; C.: *Chrysosporium*; Ca.: *Candida*; Cc.: *Coccidioides*; Co. *Corynascus*; E.: *Emmonsia*; Ge.: *Geomyces*; Gy.: *Gymnostellatospora*; M.: *Myxotrichum*; N.: *Nannizziopsis*; P.: *Pectinotrichum*; Pa.: *Paracoccidioides*; Ps.: *Pseudogymnoascus*; R.: *Renispora*; T.: *Trichophyton*; U.: *Uncinocarpus*).

a bootstrap support value of 100% in NJ and 87% in MP and a DI of 5 in MP. Clade II included species with verrucose-walled conidia which frequently emerge from swollen conidiogenous cells, i.e. *C. evolceanui*, *C. fluviale*, *C. indicum*, *C. minutisporosum*, *C. siglerae* and *C. submersum*.

Leclerc *et al.* [35] used the LSU rDNA sequences comparison of several species of Onygenales to point out that *C. keratinophilum* and *A. fulvescens* should be separated at a varietal rather than a species level. However, our results agree with those of Cano and Guarro [15] who reviewed the genus and stated that *C. keratinophilum* was more closely related to *A. keratinophilus* than to *A. fulvescens*. The connection between *C. keratinophilum* and *A. keratinophilus* is moderately well supported by bootstrap values of 89% in NJ and 87% in MP. Interspecific sequence divergence of both species is low (0·6%), with only four nucleotide substitutions while sequences of *C. keratinophilus* and *C. fulvescens* are different in 20 bp (3·2%). Similarly, *C. articulatum* and *A. reticulosporus* appeared in our study in the same branch with a bootstrap support of over 91%. Their sequences were identical. The morphological features of both species are also practically identical (data not shown).

Our study also revealed the presence in the *Aphanoascus* clade of several terminal clusters with high bootstrap support and low interspecific sequence divergence, i.e. those formed by the following pairs of species: *C. lucknowense* / *C. mephiticum*; C. *siglerae* / *C. submersum* and *C. evolceanui* / *C. fluviale*. The two species of the first pair, with 100% bootstrap support and a DI higher than 5, were closely related (two base differences out of 644 bp). Both species are practically identical morphologically and their synonymyzation seems the most appropriate. In contrast, members of the other two terminal clusters were morphologically and molecularly different. The relationship between *C. siglerae* and *C. submersum* received a bootstrap support of 100% in both NJ and MP and a DI higher than 5. However, their ITS sequences showed 63 nucleotide substitutions (10·8% sequence divergence). Both taxa were also differentiated easily by their colony growth rates and the shape of their conidia. The cluster formed by *C. evolceanui* and *C. fluviale* was moderately well supported in NJ analysis (83%) but unsupported in MP. Their ITS1-5·8S-ITS2 sequences differed in 44 bp (7·5% of sequence divergence). These species are also similar morphologically, but they can be clearly distinguished from each other by the width of their conidia [28].

The most surprising aspect of the *Aphanoascus* clade I was the inclusion of *C. zonatum*. Morphological characteristics prompted Sigler *et al.* to associate *C. zonatum* with *U. orissi* [51]. Our analysis was demonstrated a greater affinity of this species to the *Aphanoascus* clade than to the *Uncinocarpus* species (clade III). This is not surprising since *Aphanoascus* species as well as *U. orissi* lack peridial appendages, while the ascomata of *Uncinocarpus* have typical uncinate appendages. These results suggest that *Uncinocarpus sensu* Sigler [51] is clearly polyphyletic and that this name should probably be restricted to those species with uncinate or loosely or irregularly spiraled appendages. Nevertheless, the taxonomic affinities of *U. orissi* remain unclear because *Aphanoascus* spp. have ascomata with a membranous peridium which is lacking in the teleomorph of *C. zonatum*. However, one explanation for this could be that *Gymnoascus arxii* [52], which was originally described as having a rudimentary membranous peridium formed by a unique layer of hyaline to pale, flattened cells, was considered cospecific with *U. orissi* by Sigler *et al.* [51]. This could be the ancestral structure of the well-developed peridium of *Aphanoascus*, which would justify the position of this species in the phylogram.

The *Uncinocarpus* group (clade III) included *Uncinocarpus reesii*, *C. queenslandicum* and *Coccidioides immitis*. The taxonomic affinity of the dimorphic fungus *C. immitis* to *Uncinocarpus* has been discussed at length by many authors [7,34,37,40,51,53-57]. Our findings provide further evidence of this relationship. Sigler *et al.* [51] transferred *Apinisia queenslandica*, the teleomorph of *C. queenslandicum*, to the genus *Uncinocarpus* mainly because of morphological similarities between their anamorphs [51,57]. Our molecular results are in agreement with this proposal.

Clades I, II and III were sister groups, which together received a moderate (80%) and low (52%) bootstrap support in NJ and MP, respectively, and a high DI value (5) in MP. They share the presence of dorsoventrally flattened ascospores (oblate, discoid or lenticular). However, the peridial structures of the members of group III were clearly different from the other groups. Leclerc *et al.* [35] from the analysis of the D1 and D2 domains of LSU rRNA also found a close relationship between *Uncinocarpus reesii* and *Aphanoascus* spp.

The two *Amauroascus* species included in this study, *A. aureus* and *A. volatilis-patellis* together with *Nannizziopsis albicans* and *Renispora flavissima*, were in the same group (clade IV) with a bootstrap support value of 72% in NJ and 82% in MP and a DI higher than 5. Moreover, in all the MPTs, *Nannizziopsis vriesii* was also included in this clade, with a DI of 2, as the common ancestor of the preceding taxa. All these species have *Chrysosporium* anamorphs and their peridia are formed by loosely interwoven undifferentiated hyphae without distinct appendages. No other genus included in the Onygenaceae *sensu* Currah [2] has been described as having this type of peridium. *Amauroascus* and *Nannizziopsis* are morphologically similar genera. *Nannizziopsis* was proposed by Currah [2], based on *N. vriesii*, as a monotypic genus characterized by asperulate peridial hyphae with septal constrictions. Guarro *et al.* [58] later considered hyaline ascospores to be a key feature of *Nannizziopsis* and transfered *Amauroascus albicans* to this genus, although this species has no asperulate peridial hyphae with constrictions. In this study *Nannizziopsis albicans* and *Amauroascus volatilis-patellis* were on the same branch, with a bootstrap value of 100%, a DI higher than 5 and a very low interspecific sequence divergence (7 nucleotide substitutions and 1·1% dissimilarity). Both species have undifferentiated white peridial hyphae, similar ascospores and a *Chrysosporium* anamorph. When Guarro *et al.* [58] re-evaluated the genus *Nannizziopsis*, they did not include *A. volatilis-patellis* because they considered its ascospores to be pale, not hyaline. However, the close molecular relationship between these two species indicates that they could belong to the same genus. In all the phylogenetic trees, *R. flavissima* was also closely related to these species and to *A. aureus*, a sister species of the other three. The ascospores of all these taxa are globose, except those of *R. flavissima* which are typically reniform to bacilliform. This indicates that the reniform shape of the ascospores may be a more recent evolved character. The *Chrysosporium* anamorph of *R. flavissima* ressembles the asexual state of *Ajellomyces capsulatus*. Both species have tuberculate aleurioconidia which are similar in shape and size, but both taxa were widely separated in our study. This demonstrated that conidial morphology is not always a good marker of phylogenetic relationship. Previous phylogenetic studies associated *R. flavissima* with *Malbranchea* spp. [34,37,40]. Our study included some *Malbranchea* spp., i.e. *Malbranchea* anamorph of *Uncinocarpus reesii* and the close morphological species, *Coccidioides immitis*, but these taxa clustered markedly away from *R. flavissima*. Generic concepts of *Nannizziopsis*, *Renispora* and *Amauroascus* must be re-examined.

The distinction between the genera *Chrysosporium* and *Malbranchea* is unclear. Species of both genera are frequently found as anamorphs of the same onygenalean genus e.g. *Aphanoascus*, *Uncinocarpus* and *Amauroascus*. They have been separated mainly by the predominance of aleurioconidia in the first and arthroconidia in the second, and by comparing the width of their conidia with that of the fertile hyphae [7,11,59]. The existence of some species with intermediate features, like *C. articulatum*, *C. europae*, *M. chrysosporoidea* and *C. queenslandicum*, demonstrates the weakness of this character. No other molecular, physiological or ecological criteria can separate these genera, so we think that they should be considered synonyms and limited to the anamorphs of Onygenales. Other authors have made similar arguments, which indicates that the distinction between aleurioconidia and arthroconidia may be more semantic than biological [7,37,60].

The dimorphic pathogenic fungi, i.e. the species of *Ajellomyces* and *Paracoccidioides immitis* were in the same group (clade V), as other authors have indicated [35,38-40]. Carmichael [5] placed nearly all the anamorphs of these species in *Chrysosporium* but later van Oorschot [8] and von Arx [61] accommodated some of them in different genera, like *Emmonsia*, *Zymonema* or *Blastomyces*. Our molecular studies placed all these genera in the same cluster, interspersed with other *Chrysosporium* species. In fact, there are not enough morphological reasons to distinguish all of them from *Chrysosporium*. However, for practical reasons and because these generic names are commonly used by most medical mycologist, it seems reasonable to keep them. Although with weak support our phylogenetic analysis, especially the parsimony method, established that the supposedly non pathogenic species, *C. pilosum*, *Amauroascopsis perforatus* and *C. lobatum*, are the ancestors of the other members of the *Ajellomyces* clade. These three species share several distinctive morphological features, like the violaceous to brown colonies and the pyriform and verrucose-walled conidia. However, their relationships with *Ajellomyces* are unclear since there are striking morphological differences between the two groups. These differences may be due to the latter's adaptation to parasitism.

Group VI encompasses apparently non keratinophilic and non cellulolytic *Chrysosporium* spp. that are unable to grow above 30°C and without teleomorph. It is made up of *C. carmichaelii*, C. *sulfureum* and *C. undulatum*. The monophyletic origin of this clade was supported by a DI higher than 5 and bootstrap values of 94% and 88% in NJ and MP, respectively. With the latter method, this clade looks like the ancestor of the *Ajellomyces* clade with a DI of 4.

Clade VII received a bootstrap support of 88-92% and a DI higher than 5. It comprises apart from several soil-borne *Chrysosporium* spp., the species of *Arthroderma* and the dermatophytes included in this study. Currah [2] included most of these species in the Arthrodermataceae. *Arthroderma* spp. are characterised by smooth ascospores and anamorphs with rhexolytically dehiscent conidia that belong to the anamorphic genera *Chrysosporium*, *Microsporum* and *Trichophyton*. Two highly supported subclades, with bootstrap values of 93-100% in NJ and MP and DI higher than 5, consistent with current ecological and morphological concepts, were distinguished, i.e. the pathogenic group with *Trichophyton* anamorphs and the non pathogenic group with *Chrysosporium* anamorphs. The pathogenic subclade contained zoophilic and anthropophilic species (*T. rubrum* and *A. benhamiae*), and the non pathogenic subclade was made up exclusively of geophilic species (*A. cuniculi*, *A. tuberculatum*, *A. curreyi*, *A. ciferrii*, *Pectinotrichum llanense*, *C. vallenarense* and *C. vespertilium*). These last two have not been associated with any teleomorph. The two heterothallic species of *Arthroderma*, *A. tuberculatum* and *A. cuniculi* clustered together with bootstrap values of 87% and 91% in NJ and MP, respectively, and DI higher than 5. These two subclades were also included in Arthrodermataceae group by previous authors [35,62,63].

There are morphological reasons to suggest that *C. vespertilium* and *Pectinotrichum llanense* are accommodated in the non pathogenic subclade. Like most members of this clade, *C. vespertilium* produces multicellular conidia which usually arise from orthotropically branched fertile hyphae, characteristically wavy or loosely coiled appendages and hyphae constricted at the septa similar to those of the *Arthroderma* peridium. *Pectinotrichum* is a

monotypic genus currently included in Onygenaceae *sensu* Currah [2], although more recently it was transferred to *Auxarthron* [64]. Molecular analysis based on SSU rDNA sequences [40] demonstrated that *P. llanense* is more related to Onygenaceae than to Arthrodermataceae. However, like the *Arthroderma* spp., this species has peridial hyphae with pectinate appendages and knuckle joints and smooth ascospores under light microscopy. This agrees with the molecular findings and supports the inclusion of this species in the Arthrodermataceae clade. Previously, Varsavsky and Orr [65] have suggested that *Pectinotrichum* may be an evolutionary forerunner of *Arthroderma* and *Ctenomyces*.

Chrysosporium vallenarense appears in the phylogram as the ancestor of the other species of clade VII and several morphological features support its inclusion in this group. Like other members of the clade, such as *A. tuberculatum*, *A. curreyi* and *C. vespertilium*, it has yellow colonies and pale yellow and tuberculate conidia. However, the conidia of *C. vallenarense* are obovoid while those of the other species are clavate. The conidia of *C. vallenarense* resemble those of the *Chrysosporium* anamorph of *Renispora flavissima* [10], although no close phylogenetic relationship was found between the two species.

Chrysosporium synchronum clustered with species of *Corynascus* (Sordariales) to form clade VIII. It was strongly supported by bootstrap (100% in NJ and MP) and DI values (> 5). Although the teleomorph of *C. synchronum* remains unknown, its morphology indicates that it has a certain affinity to *Myceliophthora*, whose species are anamorphs of *Corynascus*. *Myceliophthora* was considered a synonym of *Chrysosporium* by Carmichael [5] but it was later re-evaluated by von Arx [66] and van Oorschot [67]. Van Oorschot [8] broadened the genus concept and included in it some anamorphs of *Arthroderma*, together with *Chrysosporium asperatum* and the anamorph of *Ctenomyces serratus*. However, this was not accepted by McGinnis *et al.* [53], Sigler [59] and Sigler *et al.* [68]. *Myceliophthora* spp. are differentiated from *Chrysosporium* spp. by narrower conidiopherous denticles, their thermotolerance and non keratinolytic capabilities, and the fact that most species of *Myceliophthora* are cellulolytic. Van Oorschot [8] considered that the formation of conidia on ampulliform swellings provided a basis for separating *Myceliophthora* from *Chrysosporium*. However, this is not a good discriminatory feature because the *Chrysosporium* species of clade II also form conidia on swollen stalks. *Chrysosporium synchronum* has not been re-examined in this study but, very narrow conidiophorous denticles (0·5-1 µm), were originally reported [8] and it was described as being thermotolerant but not keratinolytic. It seems appropriate, therefore, to transfer this species to *Myceliophthora*.

The species belonging to the Myxotrichaceae (*Pseudogymnoascus roseus*, *Myxotrichum stipitatum*, *M. chartarum* and *Gymnostellatospora japonica*) form another monophyletic group (clade IX). It was strongly supported by bootstrap (100% in NJ and MP) and DI values (>5). This family includes non keratinolytic and markedly cellulolytic species belonging to the anamorphic genera *Geomyces*, *Malbranchea* and *Oidiodendron*. They have fusiform, hyaline ascospores and rhexolytically dehiscent conidia. Its taxonomic placement is still unclear. Currah [2] placed it in the Onygenales, although he later pointed out that it merits placement in its own order [64]. Myxotrichaceae have recently been excluded from Onygenales and considered *incertae sedis* [4]. Molecular analysis of the SSU rDNA sequences confirmed this and excluded the Myxotrichaceae from the Onygenales [40]. This study recognized two lineages in the Myxotrichaceae based on the ascospores ornamentation. One was a cluster with longitudinally striate ascospore and the other was a cluster without such a feature. We also found two subclusters within the Myxotrichaceae. However, *Gymnostellatospora japonica*, which has ascospores with longitudinal crests, was in the cluster with non-striate ascospores.

Our study revealed that two *Chrysosporium* species, *C. pseudomerdarium* and *C. filiforme*, are closely related to the Myxotrichaceae. Traditionally, the anamorphs of Myxotrichaceae have been included in *Geomyces*, *Malbranchea* and *Oidiodendron* [69]. Species of *Geomyces* are the anamorphs of *Pseudogymnoascus* and species of *Malbranchea* and *Oidiodendron* are anamorphs of *Myxotrichum*. The morphological distinction between *Chrysosporium* and *Geomyces* is unclear. Carmichael [5] and Domsch *et al.* [70] considered these genera to be synonyms. However, according to Sigler and Carmichael [7] and van Oorschot [8] the erect and acutely branched conidiophores in *Geomyces* are enough to separate it from *Chrysosporium*. We have not examined *C. pseudomerdarium*, but it is originally described as having a tree-like branching pattern similar to that of *Geomyces*. It is not surprising, therefore, to find *C. pseudomerdarium* in the same clade as *Geomyces* spp. and it should probably be transferred to this genus. On the other hand, the presence of *C. filiforme* in this group is more difficult to explain because it is morphologically closer to *Chrysosporium* than to *Geomyces*. This species is weakly cellulolytic and not keratinolytic, and it was isolated from chitinous substrates [9]. This controversial aspect requires further study.

This study has not resolved the phylogenetic relationships of *Chrysosporium merdarium*, the type species of the *Chrysosporium* genus. This species is mesophilic, not keratinolytic, and is characterized by highly variable colonies (yellow, pink or green), sparsely echinulate and subglobose conidia and frequent intercalar conidia. Van Oorschot associated this species with *Gymnoascus uncinatus* [8]. Currah later transferred *G. uncinatus* to *Uncinocarpus* and rejected its association with *C. merdarium* because *U. uncinatus* degrades keratin and forms conidia which look quite different from those of *C. merdarium* [2]. Its phylogenetic position could be resolved if additional onygenalean species were included in the analysis.

Some of the species included in this study are known pathogens or are occasionally reported as opportunistic pathogens. These species are: *Ajellomyces capsulatus*, *Ajellomyces crescens*, *Ajellomyces dermatitidis*, *Aphanoascus fulvescens*, *Aphanoascus keratinophilus*, *Arthroderma benhamiae*, *Chrysosporium evolceanui*, *Chrysosporium tropicum*, *Coccidioides immitis*, *Emmonsia parva*, *Myxotrichum chartarum*, *Nannizziopsis vriesii*, *Paracoccidioides brasiliensis*, *Trichophyton rubrum*, *Uncinocarpus queenslandicum* and *Uncinocarpus orissi*. These species were not grouped in the same. Clades I, II, III, IV, V, VII and IX contained at least one pathogenic or opportunistic species. Neither were the species most adapted to parasitism, such as the dermatophytes and systemic pathogens (except *Paracoccidioides brasiliensis*) placed in the same phylogenetic lineage. From this framework, we concluded that pathogenicity would have evolved independently many times. These findings are largely consistent with those of other studies more specifically designed to test the origin of fungal pathogenicity [34,36,37,71].

Enzymatic ability has proved to be a highly reliable criterion for classifying the members of this fungal group, at least at high taxonomic ranks. Taxa which are mostly keratinolytic and non cellulolytic were included in the Onygenales groups (clades I-VII) and those which are cellulolytic were excluded from these clades and placed in Sordariales (clade VIII) and Myxotrichaceae (clade IX). In the Onygenales, the surface of the ascospores was a distinguishing feature. All taxa with smooth ascospores were placed in the same clade (group VII), the Arthrodermataceae *sensu* Currah, while those with ornamented ascospores were included in the Onygenaceae (clades I, II, III, IV, V) *sensu* Currah. Leclerc *et al.* [35] who analysed the LSU rDNA, had already established that these were the best criteria for separating families within the Onygenales. The type of ascomata peridium was a useful criterion at genus level for the Onygenaceae. These were arranged in five monophyletic clades, the *Aphanoascus* clade (groups I and II), the *Uncinocarpus* clade (group III), the *Amauroascus-Nannizziopsis-Renispora* clade (group IV) and the *Ajellomyces* clade (group V). Other morphological characters, such as the colour of the colony, the growth rate of temperature, pathogenicity, anamorph (conidiogenesis and conidia), and certain characteristics of ascospores like colour and size, are subject to homoplasy (convergence and reversal) and cannot therefore resolve supra-specific taxonomic levels very well.

In conclusion, molecular data generally agree with morphological aspects. They indicate that *Chrysosporium* should be limited to those mitosporic species that are generally mesophilic, that have keratinolytic but not cellulolytic capabilities, and whose teleomorphs are in Onygenales. On the other hand, *Geomyces* should be restricted to those species that are generally psychrophilic, that have cellulolytic but not keratinolytic capabilities and whose teleomorphs are in Myxotrichaceae. *Chrysosporium* and *Geomyces* share the formation of conidia with wide basal scars (more than 1 µm). *Myceliophthora* should be restricted to those mitosporic fungi that are usually thermophilic, that have cellulolytic but not keratinolytic capabilities, that have teleomorphs in Sordariales and are characterized by the formation of conidia with narrow basal scars (up to 1·0 µm). There are, therefore, morphological, physiological and molecular reasons for transferring *C. synchronum* to the genus *Myceliophthora* and *C. filiforme* and *C. pseudomerdarium* to the genus *Geomyces*. The delimitation of the *Uncinocarpus* should be re-evaluated because *Uncinocarpus orissi*, the only species of the genus without uncinated appendages, clustered within the *Aphanoascus* clade. To resolve the phylogenetic relationships of the *Amauroascus*, *Nannizziopsis* and *Renispora* genera, more of their species should be studied.

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