

## Molecular phylogeny of *Diploschistes* inferred from ITS sequence data

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**Abstract:** The phylogeny of the genus *Diploschistes* was investigated using nucleotide sequences of the nuclear ITS rDNA region (ITS1, ITS2 and 5.8S rDNA). Sequences of 22 *Diploschistes* species were aligned to those of six other species of *Thelotremataceae*, *Graphis scripta* and *Aspicilia cinerea*, with the last used as an outgroup. The alignment was analysed cladistically using maximum parsimony. In the most parsimonious trees, *Diploschistes* is monophyletic, with *D. ocellatus* being a sister-group to the remaining *Diploschistes* spp. (= *Diploschistes* s. str.). A previous cladistic analysis of morphological data suggested an evolutionary trend within the genus from perithecioid to urceolate ascomata. The present ITS data suggest the opposite: perithecioid ascomata are apparently an apomorphic character within the genus, with the *actinostomus* group forming a derived monophyletic clade. However, the topology within *Diploschistes* s. str. lacks strong bootstrap support.

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**Key words:** Ascomycota, *Diploschistes*, ITS, Ostrapales, phylogeny, *Thelotremataceae*.

### Introduction

The genus *Diploschistes* Norman belongs to the *Thelotremataceae* and includes crustose lichens with *Trebouxia* as photobiont, a blackish pigmented pseudoparenchymatous true exciple, and lateral paraphyses (Lumbsch 1989; Guderley *et al.* 1997). The genus is widely distributed in arid and semi-arid regions and occurs mainly on rocks and soil. It includes almost 30 species (Lumbsch & Guderley 1996). Recent monographic studies in this genus were begun in 1982 by one of us (HTL). The Holarctic taxa of *Diploschistes* have been monographed (Lumbsch 1989), and several additional papers on the genus have been published since that time (as cited in Guderley

& Lumbsch 1996), including revisions of species occurring on the Indian subcontinent (Pant & Upreti 1993), southern Africa (Guderley & Lumbsch 1996), and Australia (Lumbsch & Elix 2002). An exhaustive treatment of the morphology, chemistry, and history of taxonomic exploration, may be found in Lumbsch (1989).

A cladistic study using morphological, chemical, and ecological characters was published by Lumbsch & Tehler (1998). In this study, taxa with perithecioid ascomata (= *actinostomus* group) were found to be a basal, plesiomorphic group, while the taxa with urceolate or lecanoroid ascomata (= *scruposus* group) appeared as a derived monophyletic entity, which included *Diploschistes ocellatus*. This phylogenetic analysis indicated that the latter species is a highly derived member of the *scruposus* group. *Diploschistes ocellatus* differs from all other species in *Diploschistes* by having *Lecanora*-like ascomata, by the presence of depsidones belonging to the norstictic acid chemosyndrome, and by the absence of a

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TABLE 1. *Specimens used for phylogenetic analysis with GenBank accession number*

Species	Collection	GenBank
<i>Aspicilia cinerea</i>	France, Brittany, <i>Lumbsch</i> 8113 (hb. Lumbsch)	AJ458278
<i>Diploschistes actinostomus</i>	Spain, Catalonia (BCC-Lich 13394)	AF229194
<i>D. aeneus</i> 1	Mexico, Sonora (ESS-16971)	AJ458279
<i>D. aeneus</i> 2	Mexico, Chihuahua (ESS-16968)	AJ458280
<i>D. badius</i>	USA, Massachusetts, <i>May</i> 5334 (FH)	AJ508685
<i>D. candidissimus</i>	Australia, South Australia (ESS-20699)	AJ458281
<i>D. cinereocaesius</i>	Venezuela, Merida (ESS-9364)	AJ458282
<i>D. diacapsis</i> 1	Spain, Catalonia (BCC-Lich 13392)	AF228317
<i>D. diacapsis</i> 2	Spain, Catalonia (BCC-Lich 13393)	AF228318
<i>D. diploschistoides</i>	Australia, Queensland, <i>Lumbsch</i> 11115 <i>n</i> (hb. Lumbsch)	AJ458283
<i>D. gypsaceus</i>	Switzerland, Graubünden (ESS-9047)	AJ458284
<i>D. gyrophoricus</i>	Spain, Catalonia (BCC-Lich 11883)	AJ458285
<i>D. hensseniae</i>	Australia, Western Australia (ESS-16604)	AJ458291
<i>D. muscorum</i> 1	Spain, Catalonia (BCC-Lich 13390)	AF228319
<i>D. muscorum</i> 2	Spain, Catalonia (BCC-Lich 13391)	AF229193
<i>D. ocellatus</i> 1	Spain (BCC-Lich 13208)	AF098410
<i>D. ocellatus</i> 2	Spain (BCC-Lich 13207)	AF098411
<i>D. ocellatus</i> 3	Australia, South Australia, <i>Archer</i> 1024 (hb. Lumbsch)	AF227960
<i>D. ocellatus</i> 4	Australia, South Australia, <i>Lumbsch</i> 10734 (hb. Lumbsch)	AF228316
<i>D. rampoddensis</i>	Papua New Guinea, <i>Aproot</i> 39679 (hb. Lumbsch)	AJ458286
<i>D. scruposus</i>	Germany, Rheinland-Pfalz (ESS-21508)	AJ458287
<i>D. thunbergianus</i> 1	Australia, New South Wales, <i>Eldridge</i> 3800 (hb. Lumbsch)	AJ458289
<i>D. thunbergianus</i> 2	Australia, South Australia, <i>Lumbsch</i> 10728 <i>d</i> (hb. Lumbsch)	AJ458290
<i>Graphis scripta</i>	Sweden (UME 41530)	AF229195
<i>Myriotrema urceolare</i>	Costa Rica, Prov. San José, <i>Sipman</i> 44328 (B)	AJ508680
<i>Ocellularia interponenda</i>	Singapore, Nee Soon, <i>Sipman</i> 46129 (B)	AJ508681
<i>O. perforata</i>	Costa Rica, Prov. San José, <i>Sipman</i> 44335 (B)	AJ508682
<i>O. croceopora</i>	Malaysia, Johor, <i>Sipman</i> 46421 (B)	AJ508683
<i>Thelotrema lepadinum</i>	UK, Hampshire, <i>Wedin</i> 6231 (hb. Wedin)	AF546077
<i>T. suecicum</i>	Turkey, Kackar, <i>Guttova, Halda &amp; Palice</i> (ESS 21521)	AJ508684

pseudoparenchymatous true exciple with lateral paraphyses. Hence, this species was distinguished as the only member of a third group (= *ocellatus* group) within *Diploschistes* by [Lumbsch \(1989\)](#).

As part of our continuing studies on the systematics and phylogeny of ostropalean fungi, the phylogeny of *Diploschistes* has been studied using molecular methods. Internal transcribed spacer (i.e. ITS1, ITS2 and 5.8S rDNA) sequence data have been examined to determine whether the genus is monophyletic and whether the evolution of selected characters within the group, as suggested by [Lumbsch & Tehler 1998](#), is supported by molecular data. The phylogenetic position of *D. ocellatus* is especially interesting in this regard, since initial molecular

studies failed to support the monophyly of *Diploschistes* and also revealed remarkable sequence variation within that species ([Martín et al. 2000](#); [Winka et al. 1998](#)).

## Materials and Methods

### Material

Ascomata and thallus material for ITS rDNA sequence analyses were used in this investigation from the 22 specimens of *Diploschistes*, six additional *Thelotremataceae*, *Graphis scripta* and *Aspicilia cinerea* listed in [Table 1](#). Although we tried to include as many species of *Diploschistes* as possible in this study, no material was available of some species, such as *Diploschistes awasthii* Pant & Upreti or *D. nepalensis* Pant & Upreti. Other species, such as *D. megalosporus* Lumbsch & Mayrhofer and *D. thelenelloides* Lumbsch & Aproot, are known only from the type or from very

scanty additional specimens (e.g. *D. isabellinus*, *D. prominens*) and no attempt was made to isolate DNA from this material. Furthermore, the probable presence of lichenicolous fungi frequently hampered the investigation, since repeat sequences of unrelated fungi were often obtained. Despite these difficulties, however, sequences were obtained from a sufficient number of species to ensure that several representatives of each of the groups proposed by Lumbsch (1989) were included. We also obtained ITS sequences from six additional *Thelotrema* taxa. Among these is an ITS sequence from *Thelotrema lepadinum* that was kindly provided by Mats Wedin (Umeå).

#### DNA extraction, PCR amplification and sequencing

Total DNA was extracted from fresh and herbarium material using modified CTAB methods following Cubero *et al.* (1999) and Martín & Winka (2000).

Dilutions ( $10^{-1}$  or  $10^{-10}$ ) of the total DNA were used for PCR amplification of the nuclear rDNA ITS and 5-8S genes. Primers (primer nomenclature follows Gargas & DePriest 1996) for amplification were: nu-SSU-1752-5' (=ITS-1F; Gardes & Bruns 1993) and nu-LSU-0041-3' (=ITS-4; White *et al.* 1990). Conditions for PCR amplification and cycle sequencing have been described previously (Martín *et al.* 2000). Sequence fragments were assembled with SeqMan 4.03 (DNASTar) or Sequencher 3.0 (Gene Codes Cooperation) and manually adjusted.

#### Sequence alignment

ITS rDNA sequences of the 22 *Diploschistes* specimens (Table 1) were aligned with sequences of *Aspicilia cinerea*, *Graphis scripta* and six other *Thelotrema* taxa. The ITS data set contains portions of the sequence that are highly variable and cannot be aligned unambiguously. Since standard multiple alignment programs become less reliable when sequences are highly divergent, we used an alignment procedure employing a linear Hidden Markov Model (HMM) as implemented in the software SAM (Hughey & Krogh 1996; <http://www.cse.ucsc.edu/research/compbio/sam.html>). All regions that were not aligned with statistical confidence were excluded from the subsequent phylogenetic analysis.

#### Phylogenetic analysis

The alignment was analysed using the PAUP\* 4.0 software package (Swofford 2002), using *Aspicilia cinerea* as the outgroup. Maximum parsimony (MP) trees were inferred using the heuristic search option with 200 random sequence additions. Gaps were treated as missing data. Branch lengths equal to zero were collapsed to polytomies. Nonparametric bootstrap support (Felsenstein 1985) for each clade was tested based on 2000 replications, using the heuristic bootstrap option of PAUP\* 4.0. Phylogenetic trees were drawn using Treeview (Page 1996). The consistency index (CI;

Kluge & Farris 1969), retention index (RI; Farris 1989), and rescaled consistency index (RC; Farris 1989) were obtained from PAUP\*.

## Results

The sequences obtained for the ITS rDNA region varied in length from 493 bp in *Diploschistes badius* to 572 bp in *Myriotrema urceolare*. More than 95% of the sequence lengths were sequenced in both directions.

Sequences of the 30 taxa were aligned to produce a matrix of 578 unambiguously aligned nucleotide-position characters, which included 266 parsimony-informative sites. The alignment is available in TreeBASE (<http://herbaria.harvard.edu/treebase/>).

Twenty-one most parsimonious trees were obtained with a tree length of 974 steps, CI=0.57, RI=0.68 and RC=0.40. In the strict consensus tree (Fig. 1), *Diploschistes* is monophyletic with a bootstrap support of 84%. *Diploschistes ocellatus* is sister to all the other *Diploschistes* taxa examined. The individual samples of *D. ocellatus* form a monophyletic group with 100% bootstrap support. The other *Diploschistes* species form a separate monophyletic group (= *Diploschistes* s. str.), which is also supported by 100% bootstrap support. Within *Diploschistes* s. str., the taxa belonging to the *D. actinostomus* group are a derived monophyletic group (with 73% bootstrap support) nested within the basal, paraphyletic *D. scruposus* group. Within the *D. actinostomus* group, *D. aeneus* and *D. badius* form a sister-group, as does *D. gyrophoricus* together with these two species, but the latter relationship lacks strong bootstrap support. Within the paraphyletic *D. scruposus* group, *D. diacapsis*, *D. gypsaceus* and *D. scruposus* form a group.

The six additional *Thelotrema* taxa and *Graphis scripta* form two groups: *Myriotrema urceolare* clusters with the three *Ocellularia* spp., and *G. scripta* with the two *Thelotrema* spp. The *Thelotrema* taxa (including *G. scripta*) form a monophyletic group with strong support (100%).

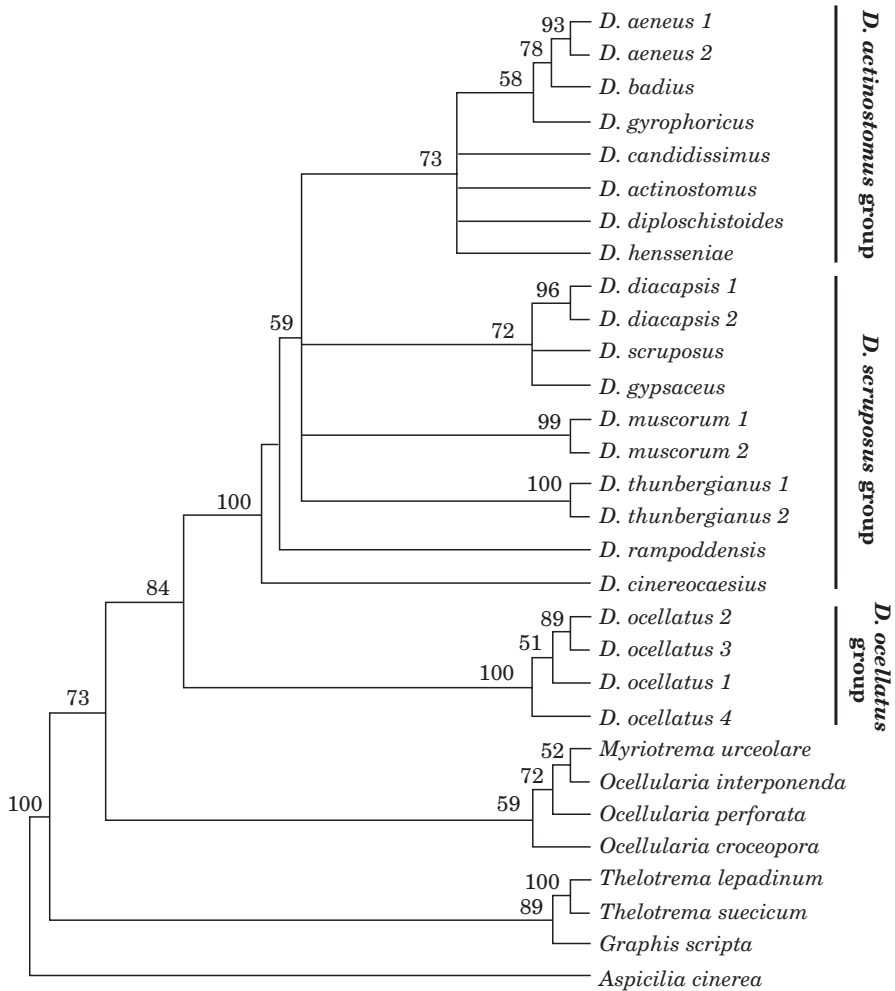


FIG. 1. Phylogeny of *Diploschistes* inferred from ITS region sequences. Strict consensus of 21 most parsimonious trees obtained from a heuristic search using PAUP\*. Numbers at nodes are bootstrap frequencies above 50%. Group placement according to Lumbsch (1989) indicated on the right.

## Discussion

The phylogenetic analysis of ITS rDNA sequence data suggests that the genus *Diploschistes*, as currently circumscribed, is monophyletic. *Diploschistes ocellatus* is basal to all other *Diploschistes* spp. (= *Diploschistes* s. str.) examined. Since the placement of *D. ocellatus* in the genus had become uncertain based on preliminary molecular studies, the genetic distances between *Diploschistes* spp. were calculated to determine whether the

genetic distances between *Diploschistes* s. str. and *D. ocellatus* fall within the range of different genera in euascomycetes. Following Lumbsch (2002), pairwise differences were calculated using Tree-Puzzle (Strimmer & von Haeseler 1997). The model of evolution used in the calculations was the HKY model (Hasegawa *et al.* 1985) with among-site variation assuming a discrete gamma distribution with six rate categories. Pairwise differences were divided into distances within *Diploschistes* s. str. and

*D. ocellatus* and distances between these two groups. The differences within *Diploschistes* s. str. varied between 0.004 and 0.120, while the differences within *D. ocellatus* varied from 0.01 to 0.07. The differences between *Diploschistes* s.str. and *D. ocellatus* were between 0.35 and 0.42, thus exceeding the modal class of intergeneric distances in euascomycetes (Lumbsch 2002). These sizeable genetic distances suggest that *D. ocellatus* is a distant basal member of *Diploschistes*, or may even represent a distinct lineage within the *Thelotremataceae*.

The exact affinities of *D. ocellatus* must await a future, comprehensive phylogenetic analysis of the *Thelotremataceae* as a whole. This family includes more than 500 species worldwide (Hawksworth et al. 1995). The majority of species in the family are tropical, largely corticolous, and have *Trentepohlia* as their photobiont. Our current knowledge about the phylogeny of these tropical lichens is extremely poor. Currently, c. 13 genera are accepted in the family (Eriksson 1999), but their circumscription, especially of the tropical genera, is controversial. In the traditional classification proposed by Müller Argoviensis (1887), the ascospore septation and coloration were used schematically to distinguish genera. Since the work of Salisbury (1972), the genera in the family have been delimited mainly by the structure and coloration of the true exciple, as well as the presence of lateral paraphyses (e.g. Hale 1980). This situation is further complicated by the fact that the distinction between the *Thelotremataceae* and the *Graphidaceae*, another largely tropical family, remains unclear. The clustering observed in this study of *Graphis scripta* with the two *Thelotrema* spp. with 89% bootstrap support may be regarded as evidence of synonymy of the families. However, the placement of *G. scripta* may be a result of long branch attraction, since no additional taxa of this family were included in our study.

Within *Diploschistes* s. str., the species of the *D. actinostomus* and *D. scruposus* groups form a monophyletic group with 100% bootstrap support. These taxa share the same exciple type, and they all produce orcinol

depsides related to lecanoric acid (e.g. diploschistesic and gyrophoric acids). Although very similar in their anatomy and chemistry, these two groups are readily distinguished by their ascoma openings, being perithecioid in the *D. actinostomus* group and urceolate in the *D. scruposus* group. The molecular analysis suggests that the monophyletic *D. actinostomus* group (with 73% bootstrap support) is nested within the paraphyletic *D. scruposus* group. In contrast, the morphological analysis of Lumbsch & Tehler (1998) and a preliminary molecular analysis which included only one species of the *D. actinostomus* group (Martín et al. 2000) suggested the opposite. Unfortunately, none of these analyses provides strong bootstrap support for the backbone of the topology within *Diploschistes* s.str. Sequence data from additional genes are necessary to resolve the phylogeny within this group; consequently, the evolution of the ascoma openings within *Diploschistes* remains an enigma.

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