

SHORT COMMUNICATION

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## Phylogenetic placement of *Diplocystis wrightii* in the Sclerodermatineae (Boletales) based on nuclear ribosomal large subunit DNA sequences

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**Abstract** *Diplocystis wrightii* is an enigmatic gasteroid basidiomycete from the Caribbean. It has taxonomic affiliations with Lycoperdaceae, Broomeiaceae, and Sclerodermataceae. This study sampled ITS and 28S ribosomal genes from three *D. wrightii* specimens to determine the phylogenetic placement and the closest relatives of this species. Results of database searches and phylogenetic analysis indicate this species to be a member of the Sclerodermatineae and most closely related to the genera *Astraeus* and *Tremellogaster*.

**Key words** *Astraeus* · Broomeiaceae · *Diplocystis* · Sclerodermatineae · *Tremellogaster*

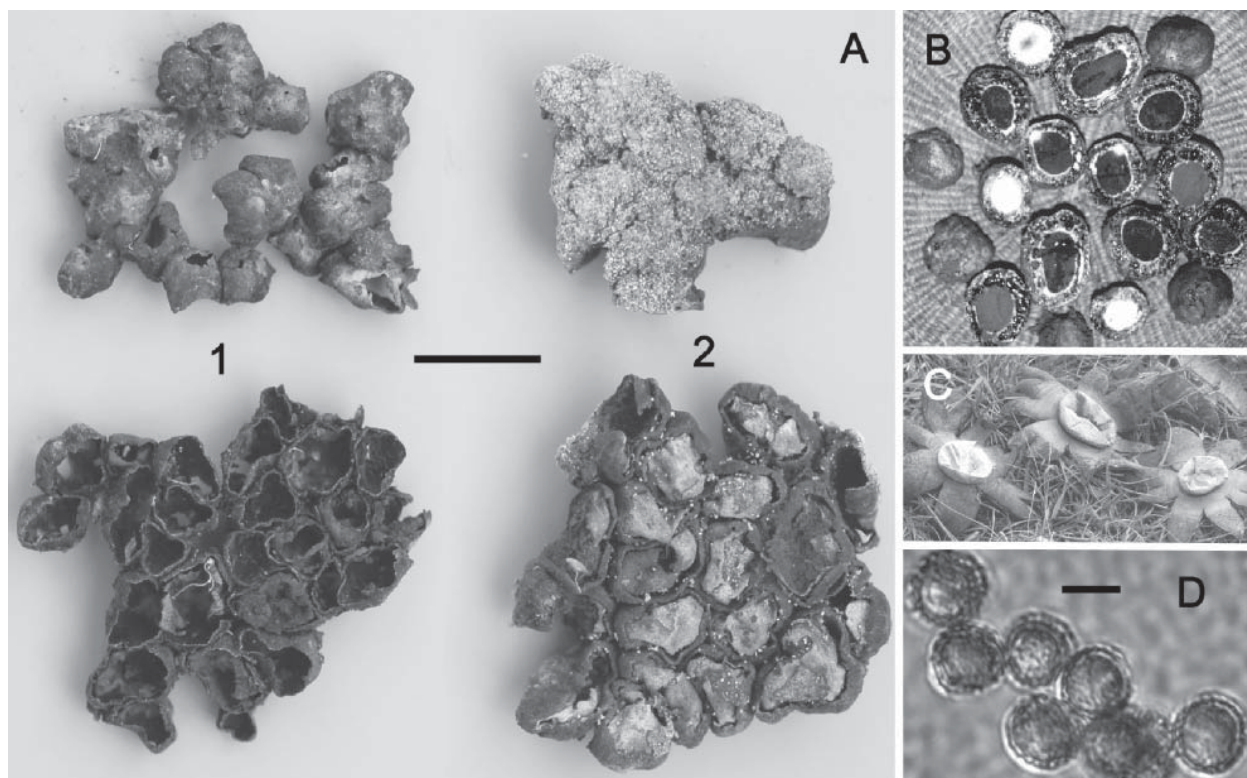
In view of the exceeding diversity of gasteroid basidiomycetes, despite the obvious limitations resulting from morphological constraints, the genus *Diplocystis* is perhaps the most enigmatic example among basidiomycetes. The genus *Diplocystis* includes only a single species, *D. wrightii* Berk. & M.A. Curtis, which forms aggregates of small gasteroid fruiting bodies borne on a sessile stroma (Fig. 1A) consisting of loosely woven hyphae. Each individual fruiting body forms a leathery, cup-shaped mesoperidium near the stroma that becomes fragile with age (Kreisel 1974). When young, the endoperidium is leathery, tough, and fits tightly within the mesoperidium. As it matures, the endoperidium becomes paperlike and ruptures in irregular cracks, allowing the exposure of glebal contents (Coker and Couch 1928). The fruiting bodies have a powdery black umber gleba, which Kreisel (1974) describes as being segregated internally by cream-white trama plates with chambers formed by the capillitium threads. The spores of *D. wrightii* are grayish-brown in color, and globose to sub-

globose with warty or spiny ornamentation (Fig. 1D). Fruiting bodies of *D. wrightii* have been collected in calcareous, sandy, and alkaline habitats, growing in dry areas at low elevations (1–100m) without having obvious associations with ectomycorrhizal woody plants (Kreisel 1974). Collection sites are mostly in the Caribbean, including the islands of the Bahamas and Cuba (Kreisel 1974), Guadeloupe in the Lesser Antilles (Coker and Couch 1928; Herrera 1972), Puerto Rico (this study), and in Mexico (Herrera 1972).

Berkeley and Curtis (1868) described *Diplocystis wrightii* from Cuba and suggested that it is related to *Lycoperdon*, based on the claim that the capillitium formed chambers in the fruiting bodies during early development. Coker and Couch (1928) arrived at a similar conclusion and independently supported the placement of *Diplocystis* in the Lycoperdaceae. Kreisel (1974) questioned this classification and argued that *D. wrightii* was more closely related to the Sclerodermatales, specifically *Astraeus* and *Calostoma*, because of its multiple peridial layers, which are not present in *Lycoperdon*. *Diplocystis* has been compared with *Broomeia* (Coker and Couch 1928; Miller and Miller 1988) because each consists of aggregated sporocarps united by a basal stroma. However, *Broomeia* has sporocarps with peristomes, an elevated stroma, noncartilaginous mesoperidium, and a universal volva, and none of these are seen in *Diplocystis* (Coker and Couch 1928; Kreisel 1974). The goal of this study is to identify the phylogenetic placement of *D. wrightii* using sequences of the nuclear ribosomal large subunit rRNA gene (LSU) and the internal transcribed spacers (ITS) 1 and 2 and the 5.8S rRNA gene.

Sequence data (Table 1) were generated from herbarium materials, including three specimens of *D. wrightii* from Puerto Rico (DSH 2004 and PR4718) and Anguilla (Ang1). DNA was extracted using the E.Z.N.A. Fungal DNA Miniprep Kit (Omega Bio-tek, Doraville, GA, USA). Polymerase chain reaction (PCR) and cycle sequencing were performed on an MJ Research PTC 200 DNA Engine thermal cycler (Bio-Rad, Waltham, MA, USA) using standard protocols. The LSU region was amplified with primers LROR and LR5, and the ITS region was amplified with

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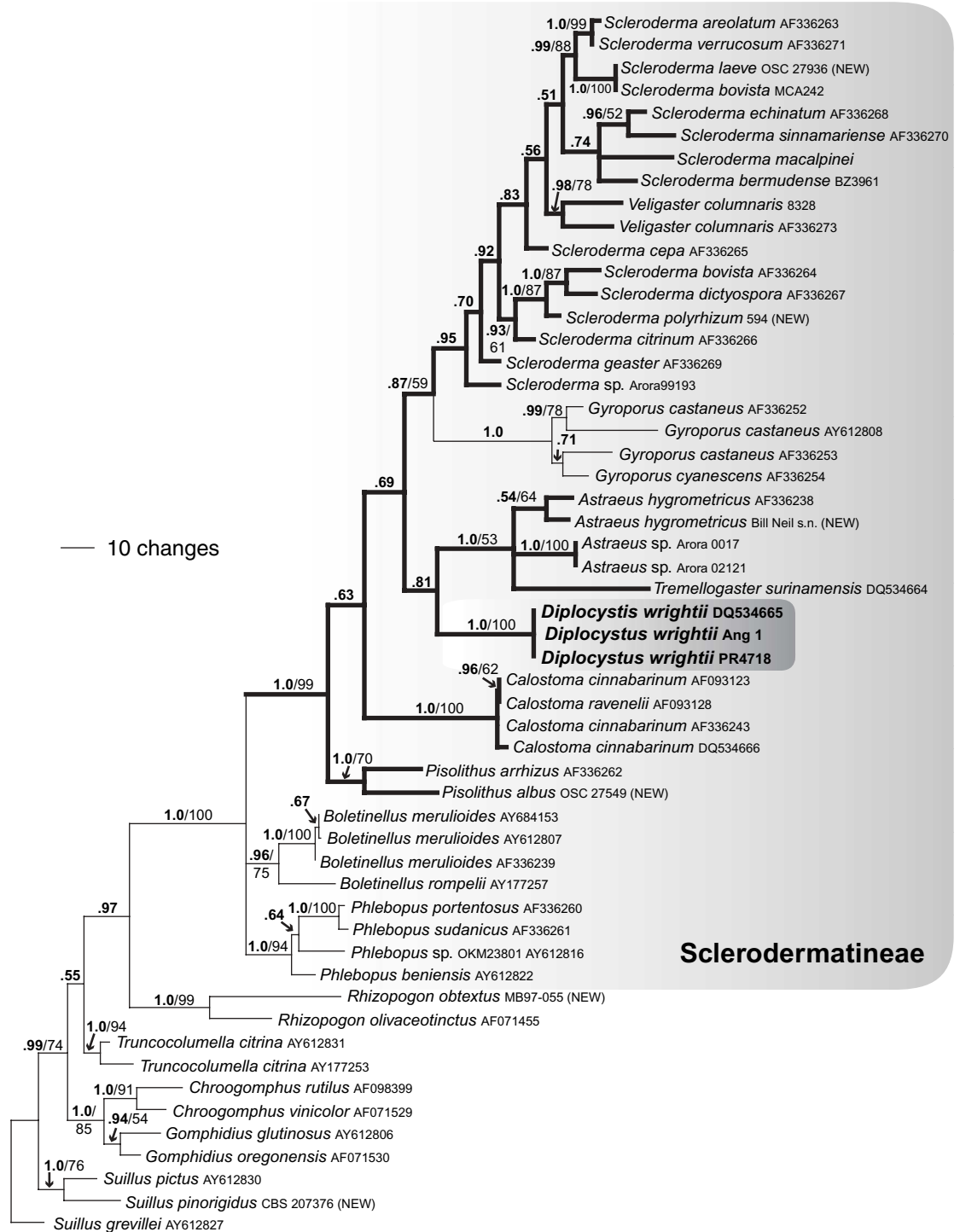
**Fig. 1.** *Diplocystis*, *Tremellogaster*, and *Astraeus*. **A** Profiles of the bottom and top of *Diplocystis wrightii* fruiting bodies from St. John, Virgin Islands (USA) (1) and Anguilla (2). **B** *Tremellogaster surinamensis*: different stages of development, section and surface view. **C** *Astraeus hygrometricus*. **D** Spores of *Diplocystis wrightii* are globose, thick walled with warty ornamentation ( $\times 100$ ). Bars **A** 1 cm; **D** 5  $\mu$ m. (Photo credits: **A**, **D**, A.W. Wilson; **B**, M.C. Aime; **C**, P.B. Matheny)

**Table 1.** Specimen identification, collection information and Genbank accession numbers for 28S sequences generated in this study

Specimen ID	Species	Date	Origin	Genbank number
MB 05-029	<i>Astraeus hygrometricus</i>	7/10/05	U.S.A., Massachusetts, Cape Cod	DQ682996
Arora 02-121	<i>Astraeus</i> sp.	8/18/02	Thailand, Udorn Thani Province	DQ644133
Arora 00-17	<i>Astraeus</i> sp.	June 2000	Cambodia, Near Ankor Wat	DQ517425
Ang1	<i>Diplocystis wrightii</i>	7/10/93	United Kingdom, Lesser Antilles, Anguilla	DQ644134
PR4718	<i>Diplocystis wrightii</i>	12/23/97	Puerto Rico, Rio Grande, near Loiza	DQ644135
OSC 27549	<i>Pisolithus albus</i>	2002	Australia	DQ682997
MB 97-055	<i>Rhizopogon obtextus</i>	8/23/97	Germany, Bavaria, Regensburg	DQ682998
BZ3961	<i>Scleroderma bermudense</i>	11/22/04	Belize, Inner Cayes	DQ644137
MCA242	<i>Scleroderma bovista</i>	5/10/96	U.S.A., North Carolina, Coweeta Station, Macon Co.	DQ644138
27936	<i>Scleroderma laeve</i>			DQ683003
OSC 24605	<i>Scleroderma macalpinei</i>	August 1999	Australia	DQ682999
594	<i>Scleroderma polyrhizum</i>	9/3/86	U.S.A., Oregon	DQ683000
Arora 99-193	<i>Scleroderma</i> sp.	1999	China, Yunnan, Lu Feng	DQ644136
CBS 207.76	<i>Suillus pinorigidus</i>	April 1976	U.S.A.	DQ683001
8328	<i>Veligaster columnaris</i>	6/11/04	Costa Rica, Puntarenas, Cota Brus, Sitio Tinieblas	DQ683002

primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Cycle sequencing used the primers already listed, plus LR3 and LR3R (Vilgalys and Hester 1990). Information for specimens and DNA used in this study can be accessed on the Clark Fungal Database (<http://www.clarku.edu/faculty/dhibbetti/clarkfungaldb/>).

One ITS sequence (DQ644139) and three LSU sequences (see Table 1) of *D. wrightii* were used to query the GenBank ([www.ncbi.nih.gov/Genbank/](http://www.ncbi.nih.gov/Genbank/)) and UNITE ([\[unite.ut.ee\]\(http://unite.ut.ee\)\) databases via BLAST. Searches using ITS sequence recovered multiple sequences of \*Pisolithus\* sp. and \*Scleroderma\* sp., suggesting that \*Diplocystis\* is placed in the Sclerodermatineae \(no other genera of Sclerodermatineae are represented by ITS sequences in either database\). The LSU BLAST search of Genbank recovered sequences of \*Pisolithus\*, \*Scleroderma\*, \*Gyroporus\*, and \*Astraeus\*, again indicating a placement of \*D. wrightii\* within the Sclerodermatineae.](http://</a></p>
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**Fig. 2.** Bayesian MCMCMC consensus tree using a GTR + I + G model of evolution. Numbers above branches are Bayesian posterior probabilities (*bold type*) and maximum parsimony bootstrap percentages (*nonbold type*). Branches in *bold* indicate lineages with a gasteroid morphology

Fifteen newly generated LSU sequences were aligned with 39 published boletoid sequences (see Fig. 2 for GenBank accession numbers) using Clustal X 1.81 (Thompson et al. 1997) followed by manual adjustment in MacClade version 4.03 (Maddison and Maddison 2001). The dataset consists of 43 ingroup Sclerodermatineae taxa and 11 out-

group sequences and has been deposited in TreeBASE (<http://www.treebase.org/treebase/>) as S1621.

Phylogenetic analyses were performed on a Macintosh G4 and a Linux cluster in the Clark University Center for Scientific Computing. Parsimony analysis in PAUP\* version 4.0b10 (Swofford 2002) used 1000 heuristic searches



with random taxon addition sequences, MAXTREES unlimited, and tree bisection and reconnection (TBR) branch swapping, resulting in 24 equally parsimonious trees of 1003 steps [confidence interval (CI) = 0.456]. Parsimony bootstrap analysis was performed with 1000 replicates, each with 10 random taxon addition sequences, MAXTREES set to unlimited, and branch swapping set to subtreepruning and regrafting (SPR). The model of evolution used in the Bayesian analysis was determined using MrModelTest version 1.1b (Johan A. Nylander, 2002, Uppsala University, Sweden) and Modeltest version 3.06 (Posada and Crandall 1998). Bayesian Metropolis-coupled Markov-chain Monte Carlo analysis was performed using the GTR + I + G model of evolution in MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001). The analysis used four chains, sampling every 100th tree for 2 million generations. All other parameters were used at the default settings. A “burn-in” value of 700 was determined by charting likelihoods of trees to determine when the chains converged to a stable average likelihood ( $-\ln L$  6419.25415).

The present study consolidates Kreisel's (1974) prospective outline of the placement of *Diplocystis*, which is the most comprehensive treatment of the genus to date. All three LSU sequences of *D. wrightii* were placed within a strongly supported clade containing the gasteroid Sclerodermatineae plus *Gyroporus* (PP 1.0, BS 99; Fig. 2). A clade including *Tremellogaster* and *Astraeus* (see Fig. 1) was resolved as the sister-group of *D. wrightii* in the strict consensus of equally parsimonious trees and the majority rule Bayesian consensus tree, but this placement received a weak posterior probability of 0.81 and no parsimony bootstrap support greater than 50%. ITS 1 and ITS 2 sequence regions for *D. wrightii* were not alignable to other members of the Sclerodermatineae.

The placement of *D. wrightii* within the Sclerodermatineae agrees with Kreisel's (1974) assessment based upon morphological similarities between *D. wrightii* and the gasteroid fungi in this group. *Diplocystis wrightii* has a cup-shaped mesoperidium, which Kreisel considered homologous to the corresponding peridial layers in *Calostoma* and *Astraeus*. Nevertheless, the genera in which peridia with multiple layers occur (*Astraeus*, *Calostoma*, *Diplocystis*, and *Tremellogaster*) do not form a monophyletic group. Despite some morphological similarities in this highly divergent group, there is only weak molecular support for uniting *D. wrightii* with *Astraeus* and *Tremellogaster*. The aggregation of fruiting bodies in *D. wrightii* is presumably a derived character. In contrast to other members of the Sclerodermatineae, *D. wrightii* is characterized by having clusters of fruiting bodies on a common stroma, which Kreisel (1974) interprets as a modified exoperidium. Based on its phylogenetic placement in the Sclerodermatineae, *D. wrightii* is most likely ectomycorrhizal, as has been shown for other Sclerodermatineae, including *Scleroderma* (Godbout and Fortin 1983; Buée et al. 2004), *Pisolithus* (Moyersoen and Beever 2004), *Astraeus* (Danielson et al. 1984), *Gyroporus* (Agerer 2002), and *Calostoma* (A.W. Wilson et al., unpublished data). Potential mycorrhizal hosts include *Coccoloba uvifera* (L.) L., *Pinus cubensis* Grisebach (Kreisel 1974),

*Neea buxifolia* (Hook. f.) Heimerl, and *Pisonia* L. (D.J. Lodge, personal communication). This view needs further investigation as it is not entirely supported by observations in the field, where *D. wrightii* is sometimes reported fruiting on soil in absence of potential mycorrhizal partners (Kreisel 1974).

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## References

- Agerer R (2002) *Gyroporus cyanescens*. In: Agerer R (ed) Colour atlas of ectomycorrhizae. Einhorn Verlag, Schwäbisch Gmünd, pl. 156
- Berkeley MJ, Curtis MA (1868) On a collection of fungi from Cuba: part I. J Linn Soc (Lond) 10:341–492
- Buée M, Vairelles D, Garbaye J (2004) Year-round monitoring of diversity and potential metabolic activity of the ectomycorrhizal community in a beech (*Fagus sylvatica*) forest subject to two thinning regimes. Mycorrhiza 15:235–245
- Coker WC, Couch JN (1928) The Gasteromycetes of the Eastern United States and Canada. University of North Carolina Press, Chapel Hill. (Reprint, 1974, Dover Publications, New York, pp 142–143)
- Danielson RM, Zak JC, Parkinson D (1984) Mycorrhizal inoculum in a peat deposit formed under a white spruce stand in Alberta. Can J Bot 63:2557–2560
- Gardes M, Bruns TD (1996) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. Can J Bot 74:1572–1583
- Godbout C, Fortin JA (1983) Morphological features of synthesized ectomycorrhizae of *Alnus crispa* and *A. rugosa*. New Phytol 94:249–262
- Herrera T (1972) Primer registro del género *Diplocystis* in Mexico. Bol Soc Mex Micol 6:55–59
- Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754–755
- Kreisel H (1974) Die Gattung *Diplocystis* und ihre Stellung im System der Basidiomycetes. Feddes Rep 85:325–335
- Maddison DR, Maddison W (2001) MacClade 4, version 4.03. Sinaur, Sunderland, Massachusetts, USA
- Miller OK, Miller H (1988) Gasteromycetes: morphological and developmental features with keys to the orders, families, and genera. Mad River Press, Eureka, OR
- Moyersoen B, Beever RE (2004) Abundance and characteristics of *Pisolithus* ectomycorrhizas in New Zealand geothermal areas. Mycologia 96:1225–1232
- Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818
- Swofford DL (2002) PAUP\*: phylogenetic analysis using parsimony and other methods (\*PAUP version 4.0 beta 10). Sinaur, Sunderland
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882
- Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 172(8):4238–4246
- White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols. Academic Press, San Diego, pp 315–322