A new concept for *Dictyostelium sphaerocephalum* based on morphology and phylogenetic analysis of nuclear ribosomal internal transcribed spacer region sequences

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Abstract: Three dictyostelid isolates were found in Spain and Argentina that are morphologically different from known species. These isolates have some features similar to *Dictyostelium sphaerocephalum* (Oudem.) Sacc., Marchal & É.J. Marchal, but differ in size and sorocarp branching pattern. We sequenced the nuclear ribosomal internal transcribed spacer region to explore phylogenetic relationships among this group of species, including the three new isolates and their closest relatives. In all phylogenetic analyses performed, sequences of all three isolates group together with sequences from "typical" *D. sphaerocephalum* samples. This result supports previous observations of the morphological plasticity in dictyostelids, especially *D. sphaerocephalum*, leading us to broaden the classical concept of this species.

Key words: cellular slime moulds, *Dictyostelium*, morphology, nuclear ribosomal ITS, taxonomy.

Résumé : Les auteurs ont récolté trois isolats de dictyostélides, en Espagne et en Argentine, lesquels diffèrent morphologiquement des espèces connues. Ces isolats ressemblent pour certains caractères au *Dictyostelium sphaerocephalum* (Oudem.) Sacc., Marchal & É.J. Marchal, mais en diffèrent par la dimension et le patron de ramification des sorocarpes. Afin d'explorer les relations phylogénétiques parmi ce groupe d'espèces, les auteurs ont séquencé la région de l'ITS ribosomique nucléique, incluant les trois nouveaux isolats et leurs plus proches parents. Ce resultat toutes les analyses phylogénétiques réalisées, les trois isolats montrent des séquences 'typiques' d'échantillons du *D. sphaerocephalum.* Cette résulte supporte les observations antécédentes sur la plasticité des dictyostélides, surtout du *D. sphaerocephalum,* et conduit les auteurs à élargir le concept classique de cette espèce.

Mots clés : myxomycètes cellulaires, *Dictyostelium*, morphologie, ITS ribosomal nucléique, taxonomie.

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Introduction

Dictyostelid species are recognized principally by the shape, size, colour, and degree and branching pattern of their sorocarps (spore-bearing structures). The three known genera are placed in two families: (*i*) Dictyosteliaceae (*Dictyostelium*, *Polysphondylium*), with cellular stalks, and (*ii*) Acytosteliaceae (*Acytostelium*), with acellular stalks (Cavender 1990). The taxonomy of dictyostelids, based mainly on morphology, has been controversial since their description (Raper 1984; Hagiwara 1989). This is not surprising given that morphological characters of this group are extremely variable not only between species but also among isolates within the same species (Raper 1984; Cavender 1990). For this reason, morphology is sometimes insufficient for making taxonomic decisions in dictyostelids. In this study we consider species of the genus *Dictyostelium*, specifically, *Dictyostelium sphaerocephalum* (Oudem.) Sacc., Marchal & É.J. Marchal, which has not been exempt from the taxonomic controversy (Hagiwara 1984).

During the National Science Foundation (NSF)-sponsored Global Biodiversity surveys for dictyostelids in Argentina (Cavender et al. 2005) we found unidentifiable isolates with relatively large and very branched sorocarps, which appeared to be morphologically similar to several isolates from different localities in Spain. All these isolates, hereafter referred to as *Dictyostelium* sp., have a morphology that does not correspond with any known species. They possess some morphological characteristics such as the size of spores, the growth habit of the sorocarps, and the absence of pigmentation in sorophores, which are also present in *D. sphaerocephalum*, but in contrast, all of these *Dictyostelium* sp. isolates have larger sorocarps and an uncommon pattern of branching (in which the sorocarps are highly branched with terminal and many lateral sori).

We have performed phylogenetic analysis using both parsimony and Bayesian methods including sequences of the nuclear ribosomal internal transcribed spacer (nrITS) region in three unknown isolates of *Dictyostelium* sp., plus *D. sphaerocephalum*, *Dictyostelium discoideum* Raper, *Dictyostelium giganteum* B.N. Singh, *Dictyostelium minutum* Raper, and *Dictyostelium mucoroides* Bref. (Raper 1984; Cavender 1990).

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Our main goals are (*i*) to study morphological plasticity among the isolates of *D. sphaerocephalum* and *Dictyostelium* sp., (*ii*) to compare nucleotide sequence diversity among closely related species of the genus *Dictyostelium*, (*iii*) to review morphological features that define the concept of *D. sphaerocephalum*, and (*iv*) to assess the utility of nrITS sequences for providing another source of data for making taxonomical decisions in the study of the order Dictyosteliales.

Material and methods

Morphological study

Sampling and isolation

Soil samples were collected in Spain in 2004 as well as from Argentina (Patagonia), and the United States (Ohio) (Table 1). To obtain wild dictyostelids, soil samples were processed with a soil dilution technique. Twenty-gram soil samples were collected in Whirl Pak® plastic bags. Samples were processed as soon as possible after their collection. Procedures described by Cavender and Raper (1965) were followed. A final soil dilution of 1/50 was used for all samples. Soil pH was measured in the laboratory before dilution. Culture plates were incubated under diffuse light at 23–25 °C.

Each plate was carefully examined at least once a day for three weeks following appearance of initial aggregations and the location of each aggregate clone marked.

Isolates of cellular slime moulds were subcultured to facilitate identification. For this, two-membered cultures were used; the bacterial food organism employed was *Escherichia coli* (ATCC 23437). Isolates of each species were cultivated for further study on non-nutrient agar (2%) with *E. coli* pregrown for 12–24 h, and preserved by freezing spores in glycerol (20%) at –20 °C. Photomicrographs were taken using an Eclipse 600 model Nikon microscope with DIC.

Media

Hay-infusion agar consisting of 8 g·L^{-1} of dried nutrient-poor grass (hay) autoclaved 20 min at 120 °C was used to obtain initial isolations of dictyostelids. The infusion was filtered, and the filtrate made up to 1 L followed by the addition of 1.5 g·L^{-1} KH_2PO_4, 0.6 g·L^{-1} $Na_2HPO_4.2H_2O$ and 15 g·L^{-1} agar. The mixture was then sterilized for 20 min. at 120 °C. Non-nutritive agar containing 1.2 g·L^{-1} KH_2PO_4, 0.48 g·L^{-1} Na_2HPO_4, and 15 g·L^{-1} agar was used to obtain pure cultures from the first isolates.

Lactose–peptone medium containing 1 g·L^{-1} lactose, 1 g·L^{-1} peptone, and 15 g·L^{-1} agar was used for species that need a richer medium to sporulate properly, and standard medium (SM) (Sussman 1987) was used to culture dictyostelids for DNA extraction. The SM contained 20 g·L^{-1} peptone, 2 g·L^{-1} yeast extract, 20 g·L^{-1} glucose, 2 g·L^{-1} $MgSO_4$, 3.8 g·L^{-1} KH_2PO_4, 1.2 g·L^{-1} K_2HPO_4, and 20 g·L^{-1} agar.

DNA phylogeny

Phylogenetic analyses included 14 ITS sequences, of which eleven were obtained by us and the remaining three were downloaded from GenBank (www.ncbi.nlm.nih.gov) (Table 1). The closest relatives of *D. sphaerocephalum* were chosen for the present study, following Raper (1984), Cavender (1990), and S.L. Baldauf (personal communication, 2006). Nomenclature used herein follows Raper (1984), thus we have not used sequences from GenBank that follow Hagiwara's taxonomic concept, i.e., *D. mucoroides* and *D. sphaerocephalum* (Hagiwara 1984).

For DNA extraction, we grew the slime-mould species on SM plates, then collected cells from the edges of the plaques and and suspended them in a DNA extraction solution (Epicentre, Madison, Wis.)). This cell solution was then heated for 30 min at 60 °C followed by 8 min at 98 °C to cause the cells to lyse. Cell lysates were used for PCR using primers that amplify the ITS region (Romeralo et al. 2007) with the following PCR program: 5 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 50 °C, and 2 min at 65 °C, with a final elongation of 10 min at 65 °C.

Sequence data were edited using SeqEd (Applied Biosystems, Foster City, Calif.). The limits of the ITS1, 5.8S, and ITS2 sequences were determined by comparison with those of *Dictyotelium discoideum* (GenBank accession number X00601 and AY171066). Clustal X 1.62b (Thompson et al. 1997) was used for the alignment of the sequences, followed by manual adjustment in MacClade version 3.08 (Maddison and Maddison 1992). Two DNA matrices were built, the first one including the 5.8S region for all taxa and the second one including the complete nrITS region for 11 samples.

Phylogenetic analyses were conducted under maximum parsimony and Bayesian inference BI). Parsimony analyses were performed using PAUP*4.0b (Swofford 2000) for the total matrix, as well as for ITS1, 5.8S, and ITS2 independently (results not shown). Analyses were conducted using Fitch parsimony with unordered and equal weighting of all characters. Heuristic searches were replicated 100 times with random taxon-addition sequence, tree bisection–reconnection (TBR) branch swapping, with the options MULPARS in effect, and accelerated transformation (ACCTRAN) character-state optimization. Relative support for clades identified by parsimony analysis was assessed by "full" bootstrapping (100 replicates, yielding parsimony bootstrap (PB) values), using the heuristic search strategy as indicated above. Pairwise-distances values were calculated using the Neighbour-Joining method (Saitou and Nei 1987) under the Kimura 2-parameter distance model (Kimura 1980).

A BI analysis was conducted using MrBayes 3.1 software (Ronquist and Huelsenbeck 2003). To determine the simplest model of sequence evolution that best fit the data, the hierarchical likelihood ratio test (hLRT) and the Akaike information criterion (AIC) were used, employing MrModeltest 1.1b software (Nylander 2002). This provided the HKY+G substitution model for the ITS total (three species) matrix and K80 for the 5.8S matrix (five species). The MrBayes routine was performed sampling 2 million generations (four MC chains, chain temperature 0.2, sample frequency 100). All analyses were run until a split frequency of ≤0.01 between the two run sets was reached. A 50% majority-rule tree and posterior probabilities were obtained after applying a burn-in of 5000 generations to both analyses, at which point runs had reached stationarity for posterior probability (pp).

Table 1:. *Dictyostelium* material used in the present study including voucher, altitude, and habitat as well as GenBank accession numbers for their ITS sequences.

Species	Isolates	Voucher	GenBank accession number
D. discoideum	Dd1	(Kuspa et al. 2003)	AY171066
D. discoideum	Dd2	(Ozaki et al. 1984)	X00601
D. giganteum	Dg1	Hong et al. (published exclusively in GenBank)	AF219102
D. giganteum	Dg3E	Spain. Ciudad Real: Retuerta del Bullaque, Embalse de Torre de Abraham, 39°33′13″N 4°14′27″W, 900 m, under *Quercus ilex* L., 23-VI-2003, Romeralo 3E.	EF66486
D. minutum	Dmi11C	Spain. Sevilla: El Pedroso, 4 km from Cazalla, 37°54′44″N 5°48′09″W, 512 m, under *Quercus ilex*, 24-VI-2003, Romeralo 11C	AM282593
D. mucoroides	Dmc1	USA. Ohio. The Wilds. Mixed forest.	EF66487
D. mucoroides	Dmc115A	Spain. Madrid: Rascafría, 40°55′53″N 3°49′55″W, 1140 m, under *Quercus faginea* L, 1-VI-2004; Romeralo 115A	AM282597
D. sphaerocephalum	Ds89G	Spain. Huelva: La Presa, sendero la Urralera, 37°53′25″N 6°39′54″W, 825 m, under *Arbutus unedo* L. in a mixed forest with *Pistacia lentiscus* L., *Quercus ilex* and *Smilax aspera* L., 4-XI-2003, Romeralo 89G	AM282604
D. sphaerocephalum	Ds89B	Spain. Huelva: La Presa, sendero la Urralera, 37°53′25″N 6°39′54″W, 825 m, under *Arbutus unedo* in a mixed forest with *Pistacia lentiscus*, *Quercus ilex* and *Smilax aspera*, 4-XI-2003, Romeralo 89B	AM282603
D. sphaerocephalum	Ds88A	Spain. Huesca: El Collado, Puerto de Alajar, 37°53′10″N 06°39′42″W, 845 m, under *Olea europaea* L., 4-XI-2003, Romeralo 88A	AM282602
Dictyostelium sphaerocephalum	Ds20B	Spain. Huelva: Jabugo, km 132, 37°54′39″N 6°43′39″W, 690 m, under *Castanea sativa* P. Mill.,, 25-VI-2003, Romeralo 20B	AM282601
Dictyostelium sp.	*Dictyostelium* sp. Cal	Argentina: Provincia Santa Cruz, El Calafate, highway RP-11, at Río Centinela 50°21′13″S 72°30′09”W, 195 m, Estepa, with *Berberis buxifolia* Calafate, 25-I-2005, Cal	EF66488
Dictyostelium sp.	*Dictyostelium* sp. 14A	Spain. Sevilla: El Pedroso, 37°54′52″N 5°48′13″W, 512 m, under *Quercus ilex* with deer dung, 24-VI-2003, Romeralo 14A	AM282600
Dictyostelium sp.	*Dictyostelium* sp. 118B	Spain. Madrid: Rascafría, 40°55′53″N 03°49′55″W, 1140 m, under *Quercus faginea*, 1-VI-2004, Romeralo 118B	AM282605

Results

Morphological description of studied species and isolates

We have analyzed the more relevant characters of these species: *D. sphaerocephalum*, *Dictyostelium* sp., and four related species of the genus: *D. discoideum*, *D. giganteum*, *D. minutum*, and *D. mucoroides* (Table 1). All of these species belong to the genus *Dictyostelium* and have elliptical spores with no polar granules (Raper 1984; Cavender 1990).

Dictyostelium *sp.*

We analyzed three isolates: D. sp.14A, D. sp.118B, D. sp.Cal.

1- D. sp.14A

Sorocarps generally clustered, with some solitary sorocarps of medium size (0.8–2 mm length), highly branched, erect, or semierect (Fig. 1A), no pigmentation. Sorophores mostly relatively thick and short, but at times longer and more branched. No collar near the tip. Sori large and globose (diameter 60–140 μm). Spores blunt-elliptical (4–8 μm × 3–5 μm, averaging 6 μm × 3 μm) without polar granules. Slugs do not migrate. Aggregations radiate, usually very large.

2- D. sp.118B

Sorocarps generally clustered, with some solitary sorocarps, of medium size (length 1–4 mm), highly branched and semiprostrate. Great diversity of size in the same culture. No pigmentation. Sorophores mostly thick and short, sometimes longer and more branched. No collar near the tip. Sori large and globose (diameter 75 μm –170 μm). Spores blunt–elliptical (5–7 μm × 2.5–3.5 μm, averaging 6 μm × 3 μm), with unconsolidated granules that occupy a central position, not polar. Slugs do not migrate. Usually large, radiate aggregations, which subdivide into various sorogens.

3- D. sp.Cal

Sorocarps generally clustered, with some solitary sorocarps, of medium size (length 1–4 mm), highly branched, erect or semiprostrate. No pigmentation. Sorophores mostly relatively thick and short, at times longer and more branched. Smaller sorocarps appear in older cultures. No collar near the tip. Sori large and globose. Spores blunt–elliptical (average 6 μm × 3 μm) without polar granules. Slugs do not migrate. Large, radiate aggregations which subdivide into various sorogens.

Dictyostelium sphaerocephalum

Four isolates from Spain: Ds20B, Ds88A, Ds89B, Ds89G were analyzed and show a great stability in their morphological characters.

Solitary and erect or semierect sorocarps (Fig. 1B), with the characteristic L-shape, of medium size (length 1–4 mm) by comparison with other species of cellular slime moulds. No pigmentation. Short sorophore, not usually branched although sparse, irregular branching sometimes occurs. Collar near the tip present. Globose sori, relatively large in relation to the whole sorocarp. Blunt–elliptical spores (5.5–7 μm × 3–3.5 μm, averaging 6 μm × 3 μm), without polar granules. Some slugs migrate, but not long distances. Radiate, small aggregations.

Dictyostelium discoideum

Two DNA sequences downloaded from GenBank: Dd1, Dd2.

The morphological description is based on Raper (1984).

Sorocarps solitary and erect, of medium size (length 1–4 mm) and unbranched. Sorophores with basal disk, with globose sori that become yellow with age. Spores elliptical and very refringent with no polar granules (6.5–8 μm × 2.5–3.5 μm). Slugs migrate long distances without sorophore formation (Raper 1984). Aggregations radiate. This species has been used as a model organism for cellular and molecular biology research since 1950.

Dictyostelium giganteum

One isolate from Spain: Dg3E

Sorocarps solitary, erect, or prostrate, large (length 4–10 mm), strongly phototropic. No pigmentation. Sorophores thin, very long and sinuous. Sori globose. Spores hyaline, oblong to elliptical, (5–6.5 μm × 3–3.5 μm, averaging 6 × 3 μm), without polar granules. Slugs migrate long distances. Aggregations radiate. The material studied matches the description made by Raper (1984) for this species.

Dictyostelium minutum

One isolate from Spain: Dmi11c

This is one of the smallest species in the group. *D. minutum* has small sorocarps (length 0.10–1.67 mm), solitary or clustered, irregularly branched, not phototropic. Sorophores colorless. Sori globose. Spores hyaline, oblong (5–6 μm × 3–3.5 μm) with inconspicuous consolidated polar granules. Aggregations mound-like. Its aggregation is small with rounded mounds without primary streams but subsequently developing secondary streams.

Dictyostelium mucoroides

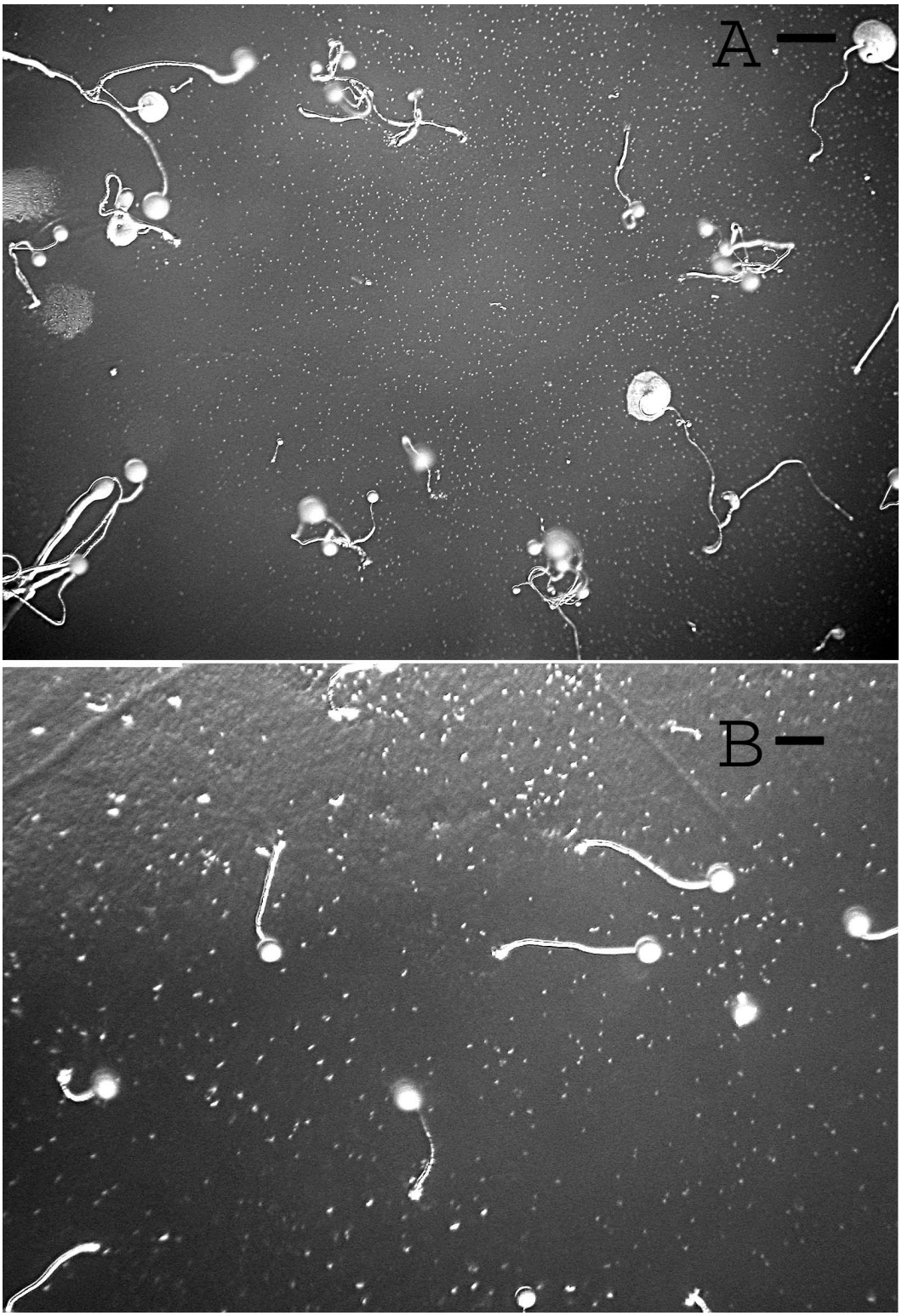
One isolate from Spain: Dmc115A and one from USA: Dmc1 (Table 1)

Sorocarps solitary and erect, of medium size (length 1–4 mm), without branching, phototropic. Sorophores colorless with clavate bases. White sori. Spores hyaline, (4.5–6 μm × 2.5–3.5 μm) without polar granules but with vesicles. Slugs migrate short distances. Small, radiate aggregations. In this species each aggregation forms one sorocarp. Hagiwara (1984, 1989) in his taxonomic study of Japanese dictyostelids, established a different concept of *D. mucoroides*, but the taxonomic criteria used herein follow Raper (1984).

Phylogenetic analyses

Values obtained in the parsimony analysis of the different data sets are summarized in Table 2. Length for the nrITS region range from 867 bases in *D. mucoroides* 115a to 905 bases in *D. mucoroides* 1; most were 876–879 bases in length (Table 2). Parsimony and Bayesian analysis produced congruent topologies (Fig. 2, 3). The main result, consistent in all analyses performed (using both Bayesian and Parsimony methods), is that all *D. sphaerocephalum* samples included in the study plus the three *Dictyostelium* sp. cluster together with high support (95% PB, 1.0 pp; Fig. 2; 100% PB, 0.92 pp; Fig. 3) Hereafter this group will be called *D. sphaerocephalum* phylogenetic complex (Figs. 2 and 3). *D. mucoroides* and

Fig. 1. Microphotographs of: A- Mature sorocarps of *Dictyostelium* sp. (D. sp.14A). Scale = 200 μm. Note the presence of branches and the clustered sorocarps.; B- Mature sorocarp of *Dictyostelium sphaerocephalum* 20B (Ds20B). Scale = 200 μm. Note the absence of branches and the solitary sorocarps.



D. giganteum are placed closer to *D. sphaerocephalum* than is *D. discoideum* (97% PB, 0.95 pp; Fig. 2).

From analysis of the complete ITS region, *D. sphaerocephalum* 88A from Spain appears as a sister to all of the other samples in the *D. sphaerocephalum* complex followed by *Dictyostelium sp.* Cal from Argentina (Fig. 3). In Bayesian analyses *Dictyostelium sp.* Cal and *D. sphaerocephalum* 88A cluster together as a sister group to the other samples of the *D. sphaerocephalum* complex, but with very low pp support. The three samples that show

Table 2:. Summary statistics from the parsimony analyses of the ITS data sets.

	5.8S (14 isolates)	ITS total	ITS1	ITS2
Sequence length (bp)	165 Ds89G 164 Ds20B 162 others	905 Dmc1 867 Dmc115a 879 Ds89G 878 Ds20B 876 other	310 Dmc1 278 Dmc115a 293 Ds	433 Dmc1 427 Dmc115a 421 Ds
Tree length (steps)	49 (Fig. 2)	700 (Fig. 3)	225 (not shown)	463 (not shown)
Number of most parsimonious trees	6	1	1	4
Number of variable characters	38	540	184	346
Number of parsimony-informative characters	9	338	75	255
Consistency index	0.979	0.954	0.96	0.954
Retention index	0.96	0.932	0.922	0.938

Fig. 2. Consensus tree of the 6 most parsimonious trees obtained in 5.8S analysis (14 isolates). Numbers above branches are bootstrap values (only values >50% are shown) and numbers below are posterior probabilities. Numbers and letters on the right indicate isolate names.

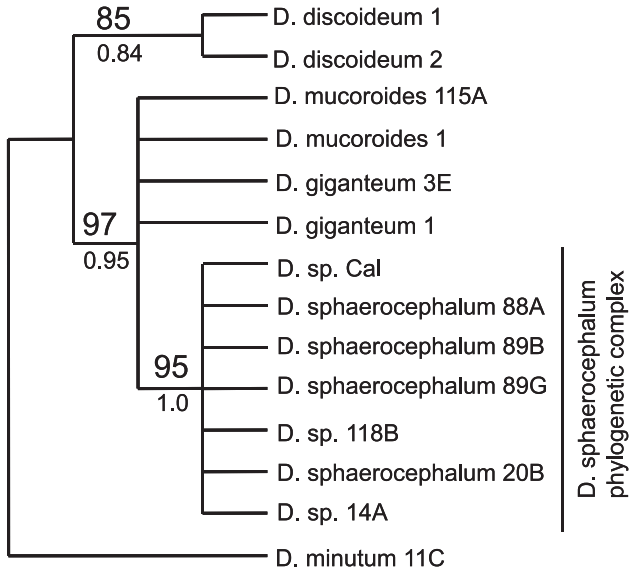
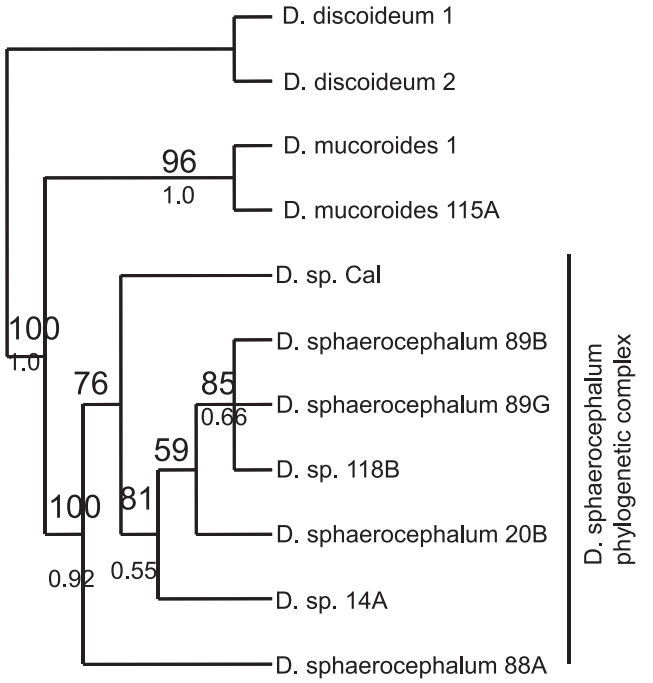


Fig. 3. The single most parsimonious tree obtained in complete ITS analysis (11 isolates). Numbers above branches are bootstrap values and numbers below are posterior probabilities. Numbers and letters on the right indicate isolate names.



new morphological characters for this group (i.e., *Dictyostelium* sp.) do not form a natural group in any analysis. In addition, there is no sequence divergence among *Dictyostelium* sp. 118B and *D. sphaerocephalum* 89B and 89G, whereas sequence divergence within the *D. sphaerocephalum* complex ranges from 0% to 1.75% (data not shown).

Discussion

New morphological characters for *Dictyostelium sphaerocephalum*

Taxonomy in dictyostelids has been controversial for many years and is still unclear (Raper 1984; Hagiwara 1984, 1989; Cavender 1990). This is due to the fact that taxonomic identification of this group is mainly based on morphological characters. Morphological characters in dictyostelids are few and highly variable among species as well as within species (Raper 1984; Hagiwara 1989). In some cases of extreme plasticity and controversy, taxonomy has been resolved by defining some species as a morphological species complex; such is the case for *D. mucoroides* and *Polysphondylium pallidum* L.S. Olive (Raper 1984; Hagiwara 1989), and now *D. sphaerocephalum*, which also shows great morphological plasticity. This species could be divided, at first sight, into two taxa based on morphological aspects such as branching pattern, number of sori, and polar granules. However, we believe that other characters such as the size of spores and sorocarps, the absence of pigmentation in sorophores and their solitary growth, as well as the lack of molecular evidence for a possible subdivision support the inclusion of these taxa into a single species (i.e., *D. sphaerocephalum*).

In light of these results we consider *D. sphaerocephalum* as a species defined by sorocarps without pigment, of medium size (0.8–4 mm length) solitary or clustered, erect or semiprostrate. Great diversity of size in the same culture. Sorophores mostly short and relatively thick, but at times longer and highly branched, with white and globose terminal

and lateral sori. Collar of cells near sorophore tip may be present or may be absent. Some slugs migrate but not long distances. Spores blunt-elliptical, 4–8 μm × 2.5–5 μm, averaging 6 μm × 3 μm, generally without polar granules but in some cases with central granules. Aggregations usually radiate, and small, sometimes larger and more irregular.

Recently, other isolates, similar to those described here under *Dictyostelium* sp. and now considered as *D. sphaerocephalum*, have been also found in the Amazon basin of Peru and in Kenya (J.C. Cavender, unpublished data, 2006).

DNA markers as a tool in *Dictyostelium* taxonomy

The nrITS marker reveals a genetic identity for *D. sphaerocephalum*. The nrITS marker also reveals a high genetic divergence for this species, which agrees with the morphological variation observed in the present study.

No correlation between ITS genetic structure and morphology has been detected within *D. sphaerocephalum*, since each of the three samples with new morphological features (*Dictyostelium* sp.) grouped with other isolates of *D. sphaerocephalum* in all phylogenetic reconstructions. In the same way, no geographical structure can be inferred from the ITS phylogeny since *D. sphaerocephalum* Cal from Argentina and *D. sphaerocephalum* 88A from Spain are (weakly supported as) sister to all other *D. sphaerocephalum* isolates from Spain. The included isolates of *D. sphaerocephalum* and *Dictylostelium* sp. appear to form a monophyletic group, but increased sampling at the intraspecific level would be key to understanding the genetic diversity and structure of the nrITS in *D. sphaerocephalum*.

The study of morphology combined with the analyses of the nrITS sequences, allow us to propose that *D. sphaerocephalum* is a single species with considerable morphological plasticity. Phylogenetic analysis of DNA sequences has similarly provided a powerful taxonomic tool for the many other cases in which morphology was inconclusive (Spiegel et al. 1995; Baldauf et al. 2000; Acero et al. 2004).

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