***Prototheca richardsi*, a pathogen of anuran larvae, is related to a clade of protistan parasites near the animal–fungal divergence**

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***Prototheca richardsi* is a protist of uncertain taxonomy which mediates growth inhibition in anuran larvae. Cells of *P. richardsi* were isolated from tadpole faeces and DNA was purified by Qiagen chromatography. Nuclear small-subunit (18S) rDNA (ssu-rDNA) was amplified by PCR using universal primers, cloned, and six clones (two from each of three separate isolates) were sequenced. All clones yielded an essentially identical sequence of 1802 nucleotides. *In situ* hybridization of fluorescent *Prototheca*-specific oligonucleotide probes, designed using the derived 18S rDNA sequence, confirmed that the sequence was indeed from *P. richardsi* cells and not from other components of tadpole faeces. The *P. richardsi* sequence was aligned with ssu-rDNA from a range of other eukaryotes, and phylogenetic analyses were carried out using several inference methods. *P. richardsi* consistently and stably grouped within a novel clade that contains rDNAs from an apparently heterodisperse group of parasitic micro-organisms assigned to the class Ichthyosporea. *P. richardsi* is evidently misplaced in the genus *Prototheca*, and the authors propose its inclusion in a new genus *Anurofeca*.**

Keywords: *Prototheca*, *Anurofeca*, Ichthyosporea, anuran growth inhibition

INTRODUCTION

Prototheca richardsi (Wong & Beebee, 1994) is an enigmatic protist that has been implicated as a gut parasite of anuran larvae (e.g. Richards, 1962; Beebee, 1991). It mediates growth inhibition of tadpoles in the laboratory by means of interference competition (Beebee & Wong, 1992) and may play a role in determining the structure of natural anuran assemblages (Wong *et al.*, 1994; Bardsley & Beebee, 1998). Beebee & Wong (1993) showed that cells of *P. richardsi* are stimulated to replicate by passage through tadpole digestive systems, and that their occurrence in natural ponds is correlated with the presence of anuran larvae (Wong *et al.*, 1994).

The colourless, highly vacuolate unicellular eukaryote currently known as *Prototheca richardsi* was discovered in faeces of larval *Rana pipiens* by Richards (1958). Its

cells were later characterized as looking ‘remarkably like chlorococcalean algae’ (Richards, 1962), and H. J. Pfaff (personal communication to C. M. Richards) provisionally identified the organism as a member of the genus *Prototheca*. Algae of the genus *Prototheca* (Kruger, 1894) have spherical unpigmented cells 2–20 µm in diameter, distinctive cell walls, and refractive, inclusion-bearing vacuoles that restrict the cytoplasm to the edges of the cell (Pore, 1985, 1986; Beebee, 1991). Mean cell size, utilization of carbon sources, and other growth parameters were consistent with classification of the anuran parasite in the genus *Prototheca* (Wong & Beebee, 1994). Furthermore, DNA–RNA hybridization studies (Beebee, 1991) showed stronger heterologous binding with nucleic acids from a green alga (genus *Dunaliella*) than with those from yeast or protozoa (genera *Saccharomyces* and *Acanthamoeba* respectively). Wong & Beebee (1994) thus established for this organism the status of a new species, *P. richardsi*.

Nonetheless, some inconsistencies remained with this identification. Antigenic cross-reactivity with cell preparations from four species of *Prototheca* was weak or

Abbreviation: ssu-rDNA, small-subunit (18S) ribosomal gene DNA.

The GenBank accession number for the sequence reported in this paper is AF070445.

Table 1. ssu-rDNAs used in phylogenetic analysis of the ssu-rDNA sequence from *P. richardsi*

Species	Accession no.	Classification
<i>Xenopus laevis</i>	X04025	Metazoa
<i>Asterias amurensis</i>	D14358	Metazoa
<i>Brachionus plicatilis</i>	U29235	Metazoa
<i>Acanthopleura japonica</i>	X70210	Metazoa
<i>Mytilus edulis</i>	L24489	Metazoa
<i>Lineus</i> sp.	X79878	Metazoa
<i>Eisenia fetida</i>	X79872	Metazoa
<i>Artemia salina</i>	X01723	Metazoa
<i>Eurypelma californica</i>	X13457	Metazoa
<i>Tripedalia cystophora</i>	L10829	Metazoa
<i>Mnemiopsis leidyi</i>	L10826	Metazoa
<i>Beroë cucumis</i>	D15068	Metazoa
<i>Microciona prolifera</i>	L10825	Metazoa
<i>Acanthocephalus unguiculata</i>	L10823	Choanoflagellida
<i>Diaphanoeca grandis</i>	L10824	Choanoflagellida
<i>Neocallimastix</i> sp.	M59761	Fungi
<i>Spizellomyces acuminatus</i>	M59759	Fungi
<i>Chytridium confervae</i>	M59758	Fungi
<i>Aspergillus fumigatus</i>	M60300	Fungi
<i>Ustilago maydis</i>	X62396	Fungi
<i>Mucor racemosus</i>	X54863	Fungi
Agent of amphibian chytridiomycosis	AF051932	Fungi
<i>Psorospermium haeckelii</i>	U33180	Fungi/Metazoa <i>incertae sedis</i>
<i>Ichthyophonus hoferi</i>	U25637	Fungi/Metazoa <i>incertae sedis</i>
Rosette agent	L29455	Fungi/Metazoa <i>incertae sedis</i>
<i>Dermocystidium</i> sp.	U21336	Fungi/Metazoa <i>incertae sedis</i>
<i>Dermocystidium salmonis</i>	U21337	Fungi/Metazoa <i>incertae sedis</i>
<i>Acanthamoeba palestinensis</i>	U07411	Acanthamoebidae
<i>Acanthamoeba castellanii</i>	U07413	Acanthamoebidae
<i>Cyanophora paradoxa</i>	X68483	Glaucocystophyta
<i>Chlorella fusca</i>	X74002	Viridiplantae
<i>Zamia pumila</i>	M20017	Viridiplantae
<i>Lagenidium giganteum</i>	X54266	Stramenophiles
<i>Rhizosolenia setigera</i>	M87329	Stramenophiles
<i>Synura spinosa</i>	M87336	Stramenophiles
<i>Tribonema aequale</i>	M55286	Stramenophiles
<i>Labyrinthuloides minuta</i>	L27634	Stramenophiles
<i>Labyrinthuloides haliotidis</i>	U21338	Stramenophiles
<i>Theileria buffeli</i>	Z15106	Alveolata
<i>Sarcocystis muris</i>	M64244	Alveolata
<i>Perkinsus</i> sp.	L07375	Alveolata
<i>Perkinsus marinus</i>	X75762	Alveolata
<i>Porphyra spitalis</i>	L26177	Rhodophyta
<i>Palmaria palmata</i>	Z14142	Rhodophyta

nonexistent (Wong & Beebee, 1994). Other protothecans can be cultured *in vitro* on *Prototheca* isolation medium (PIM) (Pore, 1973), whereas *P. richardsi* shows only weak growth. Indeed, *P. richardsi* can be cultured successfully only in the guts of anuran larvae (Wong & Beebee, 1994), although it does replicate to a lesser extent in the guts of some other aquatic organisms such as the pond snail *Limnaea stagnalis*.

Over the past few decades systematics has been revolutionized by the introduction of molecular techniques (e.g. Hillis *et al.*, 1996). Comparative analysis of sequences of rRNA genes has been particularly useful in elucidating phylogenetic relationships among microorganisms. Studies using small subunit rRNA gene (ssu-rDNA) sequences have been used to recognize new groups and reassign taxa at all levels, from domain

(Woese *et al.*, 1990) and phylum (Winnepenninckx *et al.*, 1998) to genus and species.

We have reisolated *P. richardsi* from anuran larvae, amplified and sequenced its nuclear ssu-rDNA, and confirmed by *in situ* hybridization that the sequence is derived from the parasite. Comparative sequence analysis has demonstrated that *P. richardsi* is not an alga, but is a member of the Ichthyosporea (Cavalier-Smith, 1998), a newly recognized but previously untitled clade of protistan parasites near the animal–fungal divergence (Ragan *et al.*, 1996).

METHODS

Purification of *P. richardsi* cells. Cells of *P. richardsi* were purified from the faeces of *Rana temporaria* larvae raised in the laboratory under crowded conditions (after Beebee, 1991). The faecal isolates were then incubated in a cocktail of antibacterial and antifungal agents (2%, w/v, phthalic acid, 200 g streptomycin ml⁻¹, 200 units penicillin ml⁻¹, 0.025 mg 5-fluorocytosine ml⁻¹) at 37 °C for 24 h. The suspension was then layered over a sterile Percoll gradient (30 ml, density 1.04 g ml⁻¹) preformed by centrifugation at 48 000 g for 60 min at 10 °C. The gradient was then centrifuged at 460 g for 30 min at 4 °C. The band of protothecans thus produced was carefully removed, washed with sterile water and incubated with 1 mg DNase ml⁻¹ (Sigma) in 10 mM Tris/HCl pH 8.3, 50 mM KCl, 2 mM MgCl₂ for 1 h at room temperature. This procedure was included to remove exogenous DNA fragments from any contaminating cells, especially those lysed by the previous antibiotic treatment. The DNase was deactivated at 65 °C for 15 min and the cell suspension was then washed four times in sterile water. Finally the cells were pelleted at 4000 g and frozen at –80 °C.

DNA extraction. The frozen *P. richardsi* cell pellets (> 10⁶ cells) were ground under liquid nitrogen for 15 min with a mortar and pestle, and the resulting powder was incubated in 0.4 ml of 1 mg Protease K ml⁻¹, 0.5% (w/v) SDS, 50 mM Tris/HCl, 0.4 M EDTA pH 7.5 for 2 h at 50 °C. This cell digest was then extracted with phenol/chloroform and precipitated with ethanol (Beebee & Wong, 1993). The genomic DNA pellet was then resuspended and purified further using the Qiagen 20 kit, following the manufacturer's instructions. The final DNA pellet was dissolved in sterile water at approximately 200 ng l⁻¹.

Amplification, cloning and sequencing of genomic DNA. The nuclear ssu-rDNA was amplified from purified *P. richardsi* genomic DNA by PCR using eukaryote-specific primers (Medlin *et al.*, 1988). Amplification products from three separate faecal isolates were purified using a Qiagen PCR clean-up kit and were cloned using the Invitrogen TA cloning kit following the manufacturer's protocol. Two non-sibling clones from each faecal isolate were sequenced using the universal ssu-rRNA primers described by Elwood *et al.* (1985) on an Applied Biosystems 370A automated DNA sequencer.

***In situ* hybridization.** To ensure that the amplified DNA originated from the *P. richardsi* cells, *in situ* hybridization was conducted following the method of DeLong *et al.* (1989). Three fluorescein-labelled DNA oligonucleotides were synthesized for use as hybridization probes. The first (TB01) was a 17 bp fragment of conserved small-subunit rRNA sequence (Elwood *et al.*, 1985), which acted as a negative control to test for non-specific hybridization and fluorescence. Although this

probe would hybridize to the complementary strand of rDNA, only the rRNA transcription product is present in cells in sufficient quantity to register by *in situ* hybridization. The second probe (TB02) was a 15 bp oligonucleotide that is universally complementary to a conserved region of nuclear ssu-rRNA (Elwood *et al.*, 1985). The third probe (TB03) was an 18 bp sequence designed to be complementary to a unique region of *P. richardsi* ssu-rRNA. All probes were checked for complementarity to all rRNA sequences in the small-subunit and large-subunit databases using the Check Probe program from the Ribosomal Database Project (Maidak *et al.*, 1997).

The probes were hybridized with tadpole faecal isolates and human IB4 B cells (obtained from Dr Alison Sinclair) fixed on to glass slides (after DeLong *et al.*, 1989), and were examined by epifluorescence and phase-contrast microscopy. The relative fluorescences of *P. richardsi* cells and human cells were compared for each of the hybridizations.

Sequence analysis. The consensus sequence of the ssu-rDNA from the six clones was aligned by eye with ssu-rDNAs from representative taxa from the eukaryotic crown (Table 1). Nucleotide substitution rates were estimated for this alignment using TREECON (Van de Peer & De Wachter, 1994). Distances based on the substitution rate function were used to infer a neighbour-joining tree, and analyses were bootstrapped ($n = 100$) in TREECON. Neighbour-joining, parsimony and maximum-likelihood trees were also inferred using PHYLIP 3.53c (Felsenstein, 1989); multiple iterations ($n = 1000$) were made to increase the likelihood of finding maximally parsimonious trees, and parsimony analyses were bootstrapped ($n = 1000$; except likelihood, where $n = 100$). The maximum-likelihood trees were inferred under a range of transition/transversion ratios between 1.50 and 2.50. To root the trees, *Chlorella fusca* was set as the outgroup in TREECON analyses, and *Perkinsus marinus* was used in PHYLIP analyses. Topology within and among animals, fungi, Ichthyosporea and stramenophiles was not influenced by choice of either of these sequences as outgroup. Bootstrapped analyses were carried out on multiprocessor Sun Enterprise 4001 servers in the Canadian Bioinformatics Resource at IMB-NRC.

RESULTS

Sequence determination

The sequence of ssu-rDNA from *Prototheca richardsi* was determined for six separate clones (two from each of three isolates). Sequence variation between clones was no more than two out of a total of 1802 nucleotides. These two sites were at positions 93 and 933. The consensus sequence was deposited in GenBank (accession number AF070445).

The purification procedure used to isolate *P. richardsi* from tadpole faeces prior to DNA extraction yielded cell suspensions lacking visible contaminants (Fig. 1a). However, it was important to be sure that the DNA subsequently extracted and sequenced definitely came from *P. richardsi*. *In situ* hybridizations using specific oligonucleotide probes were therefore carried out. For these studies it was necessary to use relatively crude faecal isolates, because the long *Prototheca* purification procedure reduced signal strength substantially (probably on account of target RNA degradation). Nevertheless, the *in situ* hybridizations confirmed that the

Fig. 1. (a) Phase-contrast microscopy of *Prototheca* cells after purification from tadpole faeces; (b) fluorescence of *Prototheca* cells (two in-frame) after hybridization with *Prototheca*-specific probe TB03. Bars, 10 µm.

Table 2. *In situ* hybridization test of ssu-rDNA source

Probe	Hybridization* to:	
	<i>P. richardsi</i> in faeces	Human IB4 cells
TB01 (negative control) 5'-CTGGTTGATCCTGCCAG-3'	—	—
TB02 (positive control) 5'-AGAATTTTCACCTCTG-3'	++	+++
TB03 (<i>P. richardsi</i> specific) 5'-ATGCTTGGAGTGCCGGCT-3'	+++	—

* —, Weak non-specific fluorescence signal; ++, strong signal; +++, very strong signal.

DNA amplified and sequenced was derived from the *P. richardsi* cells. A low background of nonspecific fluorescence was observed in all hybridizations, including those with the negative control probe. *Prototheca* and human cells fluoresced when hybridized with the universal positive control probe (data not shown), but only *P. richardsi* cells fluoresced when hybridized with the *P. richardsi* probe (Fig. 1b). Table 2 summarizes results from three replicated experiments.

Phylogenetic analyses

On the basis of rRNA sequence analysis, *P. richardsi* was clearly unrelated to the other protothecans, all of which cluster with *Chlorella* (Huss & Sogin, 1990). We confirmed the separation of *P. richardsi* from other

protothecans in preliminary analyses which included *P. wickerhamii*, *P. zopfii* and *Chlorella protothecoides* rDNA sequences. Trees inferred by neighbour-joining clustered the three above protothecans together in a clade separate and distant from one including *P. richardsi* (data not shown). In all inferred trees (neighbour-joining in both TREECON and PHYLIP, parsimony, and maximum-likelihood) the ssu-rDNA from *P. richardsi* grouped stably with ssu-rDNAs from *Ichthyophonus hoferi*, *Psorospermium haeckelii*, *Dermocystidium salmonis*, *Dermocystidium* sp., and the rosette agent of salmon. Within this clade, provisionally termed the DRIPs clade (as an acronym for *Dermocystidium*, rosette, *Ichthyophonus* and *Psorospermium*) by Ragan *et al.* (1996) and assigned to class Ichthyosporea by Cavalier-Smith (1998), the ssu-rDNA from *P. richardsi*

Fig. 2. Phylogenetic relationship among ssu-rDNAs. The tree shown was inferred by maximum-likelihood at transition/transversion ratio 2.00 with full (43-level) optimization. GenBank accession numbers for the ssu-rDNAs are given in Table 1. Bootstrap values (upper left) reflect the number of replicate analyses in which the grouping subtended by the indicated node appeared after resampling the alignment with replacement. PARS, parsimony ($n = 1000$); NJ, neighbour-joining ($n = 1000$); ML, maximum-likelihood ($n = 100$). The scale bar represents 0.1 likelihood units as estimated by DNAML (in PHYLIP).

always grouped with those of *I. hoferi* and *Psorospermium haeckelii* (Fig. 2). Analysis of the data using a substitution calibration also yielded this clade with a bootstrap value of 99% on a neighbour-joining tree.

The Ichthyosporidia clade appeared intact as the optimal solution under all methods of inference. Bootstrap support was >97% with maximum-likelihood, >95% with neighbour-joining, and >87% in parsimony analysis. These values are comparable with the 76–99% (neighbour-joining) and 60–79% (parsimony) bootstrap values recorded by Ragan *et al.* (1996) based on a more conservative selection of sequence positions. Within the Ichthyosporidia clade, bootstrap support for the grouping of *Prototheca richardsi*, *I. hoferi* and *Psorospermium haeckelii* ssu-rDNAs was even higher, ranging from >97% in parsimony to 100% in maximum-likelihood. The *P. richardsi* ssu-rDNA specifically grouped with the ssu-rDNA from *I. hoferi* in parsimony, maximum-likelihood and rate-uncalibrated neighbour-joining analyses (Fig. 2), whereas it formed a sister clade to ssu-rDNAs from *Psorospermium haeckelii* and *I. hoferi* in a rate-calibrated neighbour-joining analysis (results not shown). In the regions (1622 nucleotides) multiply aligned for the phylogenetic inference, the ssu-rDNA of *P. richardsi* was 91.9% identical to that of *I. hoferi* and 87.8% identical to that of *Psorospermium haeckelii*, after correction for superimposed changes under the

maximum-likelihood model of Felsenstein (1981). In these same regions, the ssu-rDNAs of *I. hoferi* and *Psorospermium haeckelii* were 89.0% identical.

DISCUSSION

Prototheca richardsi occurs naturally as a gut parasite of anuran larvae, and has yet to be cultured successfully *in vitro*. Attempts to establish cultures of *P. richardsi* have been plagued by its weak growth on defined isolation media (including *Prototheca* isolation medium), and by contamination with bacteria and filamentous fungi (Wong & Beebec, 1994). As it is well known that even low levels of contaminant DNAs (e.g. from the host amphibian or from other gut organisms) can be amplified by PCR with universal primers, we performed *in situ* hybridizations to confirm the source of the ssu-rDNA sequence we obtained. Our results established that the DNA sequenced did derive from cells of *P. richardsi*. These results were reinforced by our phylogenetic analyses, which confirmed that this ssu-rDNA was not of fungal or vertebrate origin.

The phylogenetic trees inferred from ssu-rDNA sequences also indicated that *P. richardsi* is not an alga. The initial placement of this organism in the genus *Prototheca* was based on morphology, physiology and nucleic acid hybridization data. Comparative sequence

Table 3. Members of the Ichthyosporea

Species	Morphology	Life history	Hosts	References
<i>Dermocystidium</i> spp. (> 20)	(i) 5–15 spherical cells with vacuoles and refractile inclusions, and peripheral nucleus; (ii) septate hyphae; (iii) uniflagellate zoospores	Cause systemic infections, gill and skin cysts; <i>D. salmonis</i> is transmitted by zoospores	Fish, newts, frogs	Olson <i>et al.</i> (1991); Paperna & Kim (1996); Ragan <i>et al.</i> (1996)
Rosette agent	3–7 spherical cells with cell wall	Intracellular, spleen and kidney; divides by fission	Chinook salmon	Kerk <i>et al.</i> (1995)
<i>Psorospermium haeckelii</i>	Egg-shaped spores, 100 × 60 × 60 µm	Within connective tissue	Crayfish, amphipods, scyphozoans	Vogt & Rug (1995)
<i>Ichthyophonus hoferi</i>	(i) Hyphae (similar to <i>Saprolegnia</i>); (ii) binucleate bodies; (iii) multinucleate spores with thick cell walls, up to 200 µm	In multiple organs, including muscle; white lesions on liver and kidney; spores divide by fission; transmission by hyphae	80 spp. of fish, newts, reptiles, birds, crustaceans	Herman (1984); Rand (1990, 1994); Spanggaard <i>et al.</i> (1994, 1995, 1996)
<i>Prototheca richardsi</i>	1–10 spherical cells, with vacuoles and refractive inclusions, and peripheral nucleus	Within guts, and transmitted in faeces	Anuran larvae	Richards (1962); Beebee (1991); Wong & Beebee (1994)

analysis is more robust than these methods, and provides much greater resolving power for clade identification. We propose that this organism should be removed from the genus *Prototheca* and placed appropriately within the Ichthyosporea (Cavalier-Smith, 1998). This class was created to accommodate a recently recognized clade of protists having a phylogenetic position near the animal–fungal divergence. Provisionally referred to as the DRIPs clade by Ragan *et al.* (1996), it comprises five species/groups of species: *Dermocystidium salmonis*, *Dermocystidium* sp., the rosette agent, *Ichthyophonus hoferi*, and *Psorospermium haeckelii*. In most ssu-rDNA trees these organisms are further subdivided into a *Dermocystidium*/rosette group and an *Ichthyophonus*/*Psorospermium* group. All five species are parasites of aquatic organisms, but very little is known about their life histories (Table 3). Based on a 520 bp partial sequence of nuclear ssu-rDNA, J. P. Joestensen, S. Johansen & R. Landfald (unpublished, see GenBank accession Y16260) have put forward *Sphaerosoma arcticus* as a sixth member of the Ichthyosporea.

The genus *Dermocystidium* has been used as a repository for a number of poorly characterized parasitic organisms (Ragan *et al.*, 1996). Members of *Dermocystidium* have been described as haplosporeans, fungi and apicomplexa (Paperna & Kim, 1996; Ragan *et al.*, 1996). The rosette agent, an obligate intracellular parasite of the chinook salmon *Oncorhynchus tshawytscha*, was discovered in the mid-1980s. Systematists previously believed the rosette agent to have phylogenetic affinities with the fungi and colourless algae (Kerk *et al.*, 1995). *Psorospermium haeckelii*, first described in 1883, has previously been described as a sporozoan, a histopathogenic stage of a fungus, and an alga. The genus *Ichthyophonus* has been used as a repository for a wide range of fish parasites, and has

been classified within or near taxa today known as Chytridiomycetes, Zygomycetes, Ascomycetes, Oomycetes, Apicomplexa, Haplosporida and Myxozoa (Ragan *et al.*, 1996; Spanggaard *et al.*, 1996). It seems unlikely that any existing genus within Ichthyosporea is appropriate to accommodate *P. richardsi*, and therefore a new genus will probably have to be created.

On the basis of morphology alone, the class Ichthyosporea (including, as shown above, *P. richardsi*) seems an unlikely taxonomic grouping. Comparative analysis of ssu-rDNA sequences indicates, however, that these organisms share a common ancestry. The presence of hyphae and flagella in other members of Ichthyosporea suggests that *P. richardsi* may also have more than one life-history stage. The development of specific oligonucleotide probes complementary to unique regions of the ssu-rDNA offers one avenue for investigation of other life stages in this organism.

The current taxonomy of eukaryotes derived from ssu-rRNA sequences places the fungi, animals, choanoflagellates and several protists *incertae sedis* in a group together known as the Metazoa/fungi group or Opisthokonta (Cavalier-Smith & Allsopp, 1996). The branching order of taxa within the Opisthokonta and between other taxa within the eukaryote crown, however, remain largely unresolved (Van de Peer & De Wachter, 1997). The nodes in the eukaryotic crown are each separated by fewer than five nucleotide changes per 1000 bases, making branching order difficult to establish (Wainright *et al.*, 1993). On the basis of ssu-rRNA sequences, the Metazoa is monophyletic; its members share a most recent common ancestor with choanoflagellates, but the phylogenetic position of the choanoflagellates within the Opisthokonta is unstable (Wainright *et al.*, 1993; Van de Peer & De Wachter, 1997).

P. richardsi is a potent growth inhibitor of anuran larvae in the laboratory (Beebe, 1991) and can affect the mortality of anuran larvae in the field under certain environmental conditions (Baker & Beebe, 1997). High titres of *P. richardsi* have been found in tadpoles in natural ponds, and in pond sediments (Wong *et al.*, 1994; Bardsley & Beebe, 1998). Global declines of amphibian populations have increasingly been ascribed to pathogenic micro-organisms (e.g. Cunningham, 1996; Laurance *et al.*, 1996; Berger *et al.*, 1998), and the further study of these agents may be of critical importance in future conservation efforts. As shown in Fig. 2, the agent of amphibian chytridiomycosis characterized by Berger *et al.* (1998) is in a clade quite separate from the Ichthyosporidia. *Prototheca richardsi* is however clearly not a protothecan, and we propose that it should be renamed in a novel genus as *Anurofeca richardsi*. Apart from the ssu-rDNA sequence, *P. richardsi* is distinct from other members of the genus *Prototheca* in two other ways: (1) its cell surface is immunologically differentiated from that of other protothecans, and antibodies raised against it do not cross-react with the other protothecan species; and (2), unlike all other protothecans, *P. richardsi* will not grow on *Prototheca* isolation medium (Wong & Beebe, 1994). We further justify a novel genus because the morphology of *P. richardsi* is quite unlike that of *I. hoferi*, its apparent closest relative (Table 3). Also, the ssu-rDNA sequence differences between *P. richardsi* and *I. hoferi* (8.1%) and between *P. richardsi* and *Psorospermium haeckelii* (12.2%) are comparable with the difference between *I. hoferi* and *Psorospermium haeckelii* (11.0%), two organisms already accepted as being in separate genera.

Formal description

Proposed genus: *Anurofeca*

Diagnosis: Unicellular eukaryote; spherical, highly vacuolated cells of 3–10 µm diameter; unpigmented; non-motile.

Type species: *richardsi*. Holotype deposited in algal slide collection, British Natural History Museum, London (as *Prototheca richardsi*: slide BM/B/12899).

Full description: Mature cells are 7–10 µm in diameter, with a cell wall 0.5–1 µm thick and electron-dense vacuoles occupying at least 50% of the cell volume. A peripheral nucleus is usually visible, but mitochondria are rarely identifiable under transmission electron microscopy. No chlorophyll or other pigmentation is present. Reproduction by fission generates multicellular conglomerates (2–8 cells together), with daughter cells of 2–6 µm diameter that are otherwise identical to mature cells. All cells are non-motile, without cilia or flagella.

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