

## ***Elongatocystis ecballocystiformis* gen. et comb. nov., and some reflections on systematics of Oocystaceae (Trebouxiophyceae, Chlorophyta)**

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**Abstract:** Three new strains of members of the family Oocystaceae collected in inland waters of Africa were studied microscopically and by molecular phylogeny. The new genus *Elongatocystis* was described, and the new combination *Elongatocystis ecballocystiformis* was proposed. The phylogenetic position of *Oocystidium* sp., and *Quadricoccus ellipticus* within the family was shown. The SSU rRNA phylogeny of Oocystaceae recovered a need for further studies to display the generic and species concept in this monophyletic group of green algae. The essential research steps were discussed.

**Key Words:** *Elongatocystis* gen. nov., molecular phylogeny, *Oocystidium*, *Oocystis*, *Quadricoccus*, SSU, taxonomy

### **Introduction**

The family Oocystaceae is a natural lineage in the Trebouxiophyceae (Chlorophyta) proved by both ultrastructural and molecular criteria. The cell wall is multi-layered and contains crystalline cellulose fibres which are oriented in each layer perpendicular to that of the adjacent layers (ROBINSON & WHITE 1972; SACHS et al. 1976; QUADER 1983). Molecular phylogenetic analyses revealed the monophyly of the family (HEPPERLE et al. 2000; KRIENITZ et al. 2003; PAŽOUTOVÁ et al. 2010). However, the conception of genera and species within Oocystaceae remained obscure. In this study we analyse the phylogeny of three oocystacean isolates from African inland waters and discuss the systematic context of Oocystaceae. The new description *Elongatocystis ecballocystiformis* gen. et comb. nov. is given, and the phylogenetic position of *Oocystidium* sp., and *Quadricoccus* within this family is suggested.

### **Material and methods**

Three new oocystacean strains were isolated by microcapillaries directly from the water samples and grown in a modified Bourrelly medium (HEGEWALD et al. 1994, KRIENITZ & WIRTH 2006) in suspensions or on agar at room temperature under a 14 h : 10 h light–dark regime in the strain collection of the Leibniz–Institute of Freshwater Ecology and Inland Fisheries (IGB,

Stechlin, Germany). Later, the strains were deposited at the Culture Collection of Algae and Protozoa (CCAP, Oban, UK). The designations and origin of the new strains are given in Table 1.

The morphology of algae was examined using a Nikon Eclipse E600 light microscope (LM) with differential interference contrast. Microphotographs (Figs 1–12) were taken with a Nikon Digitalcamera DS-Fi1, and Nikon software NIS–Elements D (Nikon Corporation, Tokyo, Japan).

Total genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). Gene amplification by polymerase chain reaction (PCR) was performed by using the Taq PCR Mastermix Kit (Qiagen GmbH, Hilden, Germany) with the primers 18SF and 18SR (KATANA et al. 2001). Sequencing primers for the SSU were used after MARIN et al. (2003). To obtain the main part of the eight introns of *Quadricoccus ellipticus*, the additional primers OOR3, OOF3 (PAŽOUTOVÁ et al. 2010) and 1400F (OLIVEIRA & RAGAN 1994) were used.

The three new SSU rRNA gene sequences were compared with 15 other Oocystaceae, nine other members of Chlorellales, and (as outgroup) three prasinophytes (Fig. 13). These sequences were obtained from the GenBank (National Center for Biotechnology Information [NCBI] <http://www.ncbi.nlm.nih.gov/>). The accession numbers of sequences are given in Fig. 13. An alignment of 30 taxa with 1549 base positions were used for the phylogenetic analyses, introns were excluded. Four different methods were used for the tree reconstruction: maximum likelihood (ML), maximum parsimony (MP), distance (neighborjoining; NJ), and Bayesian analyses (MB) using PAUP\* version 4.0b10

Table 1. Designation and origin of three new strains of Oocystaceae used in this study.

Species	Strain designation at IGB	Strain designation at CCAP	Origin
<i>Elongatocystis ecballocystiformis</i>	KR 2000/14	CCAP 274/3	Rockpool at Belvedere River, Mpumalanga, South Africa
<i>Oocystidium</i> sp.	KR 2007/14	CCAP 222/49	River Zambezi near Livingstone, Zambia
<i>Quadricoccus ellipticus</i>	KR 2005/238	CCAP 286/1	Lake George near Katwe, Uganda

(SWOFFORD 2002) (for ML, MP, NJ) and MrBayes version 3.1 (HUELSENBECK & RONQUIST 2001; RONQUIST & HUELSENBECK 2003) (for MB). Modeltest 3.7 (POSADA & CRANDALL 1998; POSADA & BUCKLEY 2004) was used to select the model of sequence evolution fitting best the data set. The TIM+I+G model was chosen as best model for the ML analyses with base frequencies: A 0.2495, C 0.2279, G 0.2709, T 0.2517; rate matrix: A–C 1.000, A–G 2.2908, A–T 1.3791, C–G 1.3791, C–T 4.7261, G–T 1.000; proportion of invariable sites (I = 0.6036) and gamma distribution shape parameter (G = 0.6096). To test the confidence of the tree topology, bootstrap analyses were performed using maximum likelihood (ML; using the TIM+I+G model; 100 replicates), parsimony (MP; 1000 replicates) and distance (NJ, 1000 replicates) criteria. Bayesian analyses were performed using MrBayes version 3.1 (HUELSENBECK & RONQUIST 2001; RONQUIST & HUELSENBECK 2003). Two runs with four Monte Carlo Markov chains (MCMC) iterations with the covarion setting were performed for 2 million generations until the average standard deviations of split frequencies between two runs was lower than 0.01 (stationary distribution was assumed). The first 25% of the generations was discarded as burn-in. A 50% majority-rule consensus tree was constructed with PAUP\* to calculate the posterior probabilities.

## Results

### Taxonomy

#### *Elongatocystis* KRIENITZ et C. BOCK gen. nov.

*Diagnosis:* Cellulae viridis, solitariae, elongatae ovalis ad polos late rotundatae. Membrana cellularum laevis, hyalina, firma, sine incrassationibus polaribus. Facultativum tegumentum gelatinosum homogeneous. Chloroplastae 1–4 parietalis per cellulam cum pyrenoideo granis amyli tecto. Reproductio asexualis autosporum ope (2, 4 vel 8 autosporae per sporangium). A generibus ceteris familiae ordine nucleotidorum in 18S rDNA differt.

Cells green, solitary, elongated oval, at apices

obtuse. Cell wall smooth, hyaline, thick and without polar thickenings. Facultatively with mucilaginous envelope. Chloroplasts 1–4 per cell, parietal, each containing a starch-covered pyrenoid. Propagation asexually by autosporulation (2, 4 or 8 autospores per mother cell). Genus differs from other genera of the family by the order of the nucleotides in SSU and ITS rDNA sequences.

Typus generis: *Elongatocystis ecballocystiformis* (IYENGAR) KRIENITZ et C. BOCK comb. nov.

Basionym: *Oocystis ecballocystiformis* IYENGAR (1932): Ann. Bot. 46: 224, fig 7 M–T.

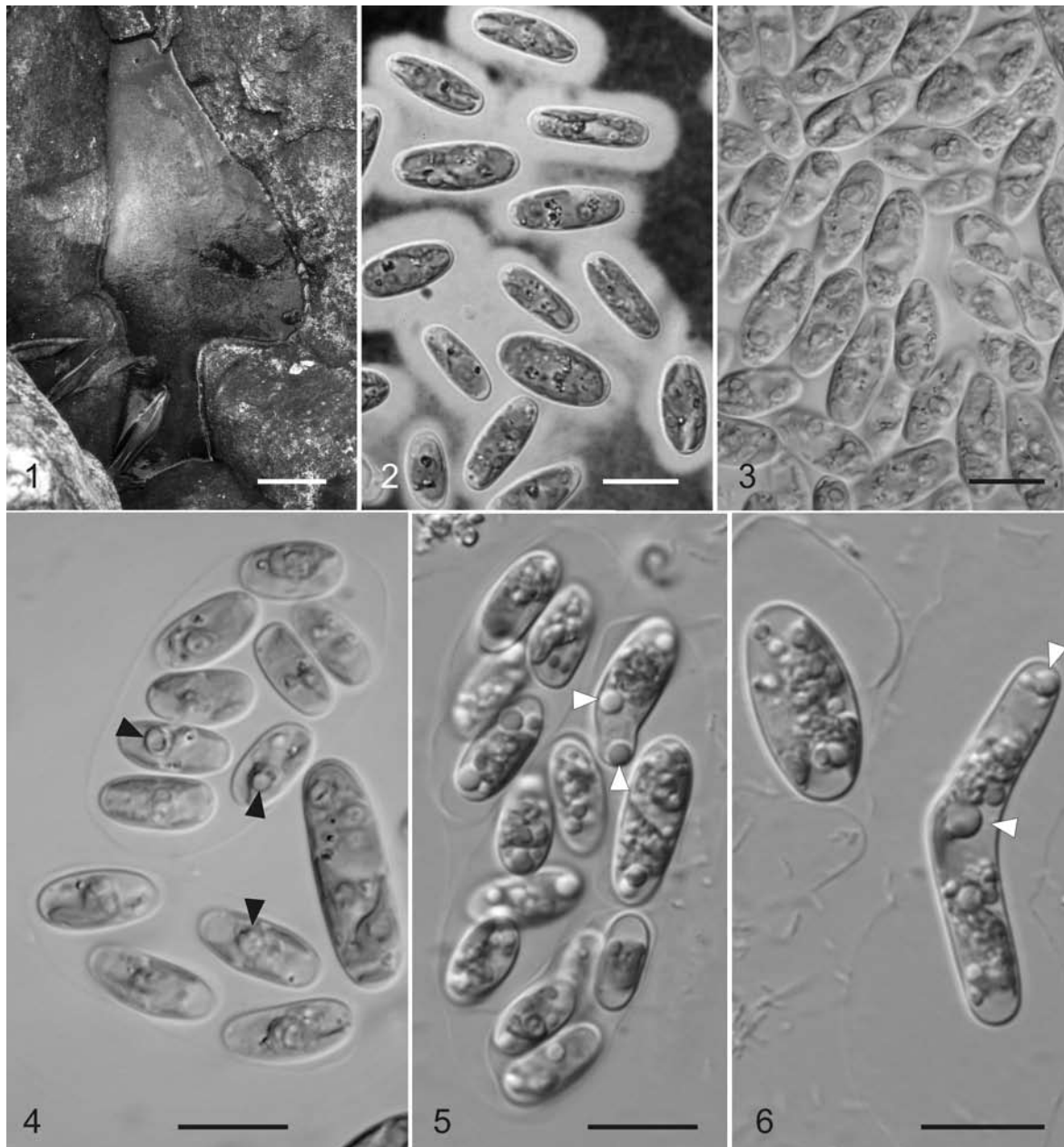
Etymology: from latin: elongatus = elongated; cysta = cyst.

Epitype (designated here): Strain CCAP 274/3, cryoconserved at the Culture Collection of Algae and Protozoa, Oban, Scotland.

### Morphology

#### *Elongatocystis ecballocystiformis* (Figs 1–6)

In a rockpool (Fig. 1) of the Belvedere River (South Africa) this alga established a mass development of vivid green colour and of jelly consistence. The cells were covered by a thick mucilaginous envelope (Fig. 2). In culture, no jelly was observed (Fig. 3). The vegetative cells were broad oval, without polar thickenings,  $8\text{--}16 \times 3\text{--}8 \mu\text{m}$  in size. The cells contained one or two parietal pyrenoid-bearing through-shaped chloroplasts which were often thickened in the central part looking like interconnections from one elongated part of the chloroplast to the other. Inside the cell numerous assimilate particles and oil droplets were contained. The mother cells produced two, four or eight autospores (Fig. 4). These autospores again propagated inside the mother cell forming large “grandmother cells” which contained several mother cells of different developmental stages (Fig. 5). Frequently, elongated, bented cells were



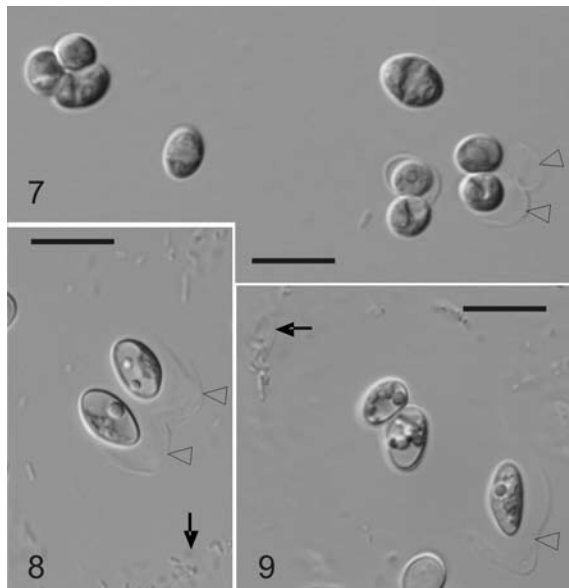
Figs 1–6. *Elongatocystis ecballocystiformis* gen. et comb. nov., in field (Figs 1, 2) and culture (Figs 3–6) : (1) sampling site, rockpool; (2) vegetative cells in field sample. The negative staining with Indian ink reveals the colourless mucilaginous envelope; (3) vegetative cells and young mother cells in culture. The cells are laying close together indicating the missing mucilaginous envelope; (4) dividing cell and mother cells with four and eight autospores, respectively. Black arrowheads indicate pyrenoids; (5) grandmother cell with several mother cells. The daughter cells contain large droplets of oil (white arrowheads); (6) elongated, bummerang-shaped cell with starch-grained chloroplasts and droplets of oil (white arrowheads). Scale bar 10 cm (Fig. 1), 10 mm (Figs 2–6).

observed with divided nuclei and chloroplasts but not producing autospores (Fig. 6).

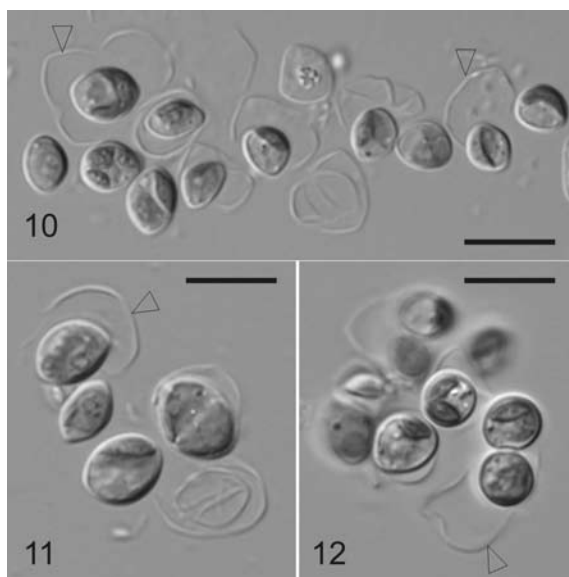
#### ***Oocystidium* sp. (Figs 7–9)**

In a water sample collected at the shoreline of the river Zambezi near the waterfront area at Livingstone (Zambia), shortly before the Victoria Falls, few specimens of *Oocystidium* were found. In wide mucilage mostly couples of broad oval

cells attached to bipartited remnants of the mother cell wall were observed. Sometimes, within the wide mucilage solitary cells or arranged in pairs and tetrads were found (Fig. 7). Several cells were irregularly ovoid (Fig. 8) or elongated (Fig. 9). The vegetative cells were  $5\text{--}6 \times 3\text{--}5 \mu\text{m}$ , the mother cells  $6\text{--}9 \times 4\text{--}5 \mu\text{m}$  in size. The hyaline cell wall did not possess polar thickenings. The cells contained one or



Figs 7–9. *Oocystidium* sp. in culture: (7) solitary cells or pairs and tetrads of cells within a joint extended mucilaginous envelope; (8) a diverging pair of cells near the remaining mother cell wall remnants (empty arrowhead). The border of the wide mucilage (arrows) is attached by bacteria; (9) a couple and a solitary cell inside the wide mucilage. Scale bar 10 µm.



Figs 10–12. *Quadricoccus ellipticus* in culture: (10) solitary cells and mother cell wall remnants (empty arrowheads) irregularly associated; (11) four cells of different stage form a group associated with cracked mother cell walls; (12) three groups of four cells. Scale bar 10 µm.

two parietal chloroplasts with pyrenoid.

#### *Quadricoccus ellipticus* HORTOB. (Figs 10–12)

In the field sample from Lake George (Uganda), typical four-celled colonies were observed. The cells were arranged along the periphery of the bowl-shaped mother cell wall remnants. During isolation with a microcapillary it was taken care to catch such typical colony. In the successful fresh unialgal culture these typical *Quadricoccus*-shape was reflected, however, was lost during the years of maintenance under culture conditions. The ovoid or citriforme cells were not arranged on the edge of the cell wall remnants but were irregularly associated with multiformed, ruptured cell wall remnants (Fig. 10). Frequently, four cells were arranged in close affinity to empty mother cell walls (Figs 11 and 12). A joint mucilaginous envelope was very fine and surrounded narrowly the cells and colonies. The cells contained one or two girdle-like chloroplasts with pyrenoid. Cell size: autospores  $5\text{--}6 \times 4\text{--}5$  µm, mother cells  $7\text{--}12 \times 6\text{--}9$  µm.

#### Phylogeny

The topology of the phylogenetic tree (Fig. 13) was determined by the well supported main clade, the Oocystaceae. Eighteen strains were comprised under the monophylum of Oocystaceae including members of 13 different genera, 12 of them of coccoid and one (*Planktonema*) of filamentous morphology. The node that joins the Oocystaceae and the relationship to Chlorellaceae and a consortium of filamentous (*Gloeotila* and *Catena*) as well as bubbling (*Marvania*) chlorophytes was not well supported.

*Elongatocystis ecballocystiformis* established a sister to a lineage which contained *Crucigeniella rectangularis* (NÄG.) KOMÁREK and *Makinoella tosaensis* OKADA. *Quadricoccus ellipticus* clustered in close relationship to *Amphikrikos* sp. whereas *Oocystidium* sp. established a sister to *Ooplanktella planoconvexa* (HINDÁK) PAŽOUTOVÁ, ŠKALOUD et NEMJOVÁ, and *Oocystis* sp.

#### Discussion

*Oocystis ecballocystiformis* IYENGAR was found for the first time in a rockpool near the Jog Falls, Mysore Province, South India by IYENGAR (1932).

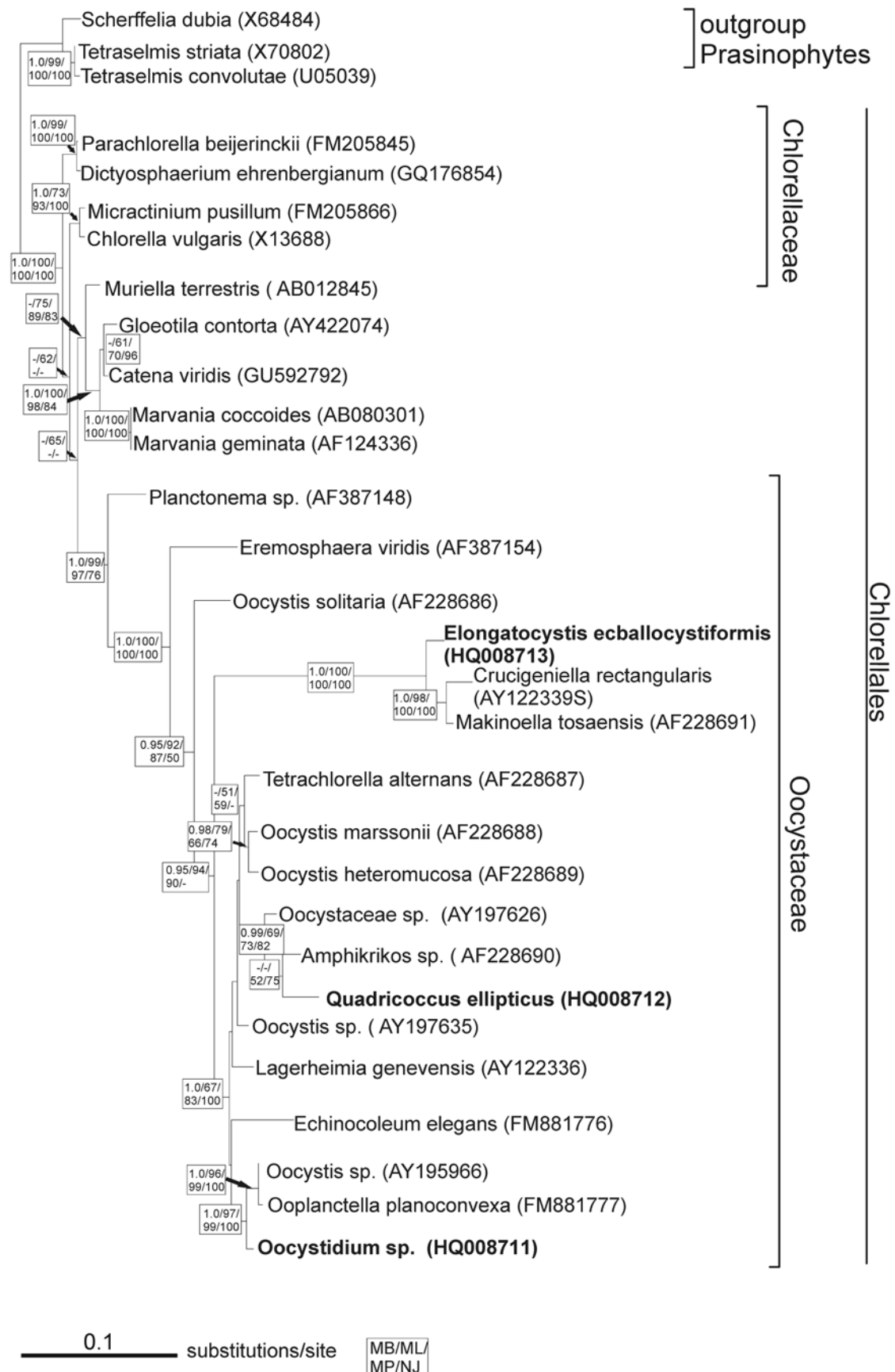


Fig. 13. Phylogenetic analyses of SSU rRNA gene sequences of members of Oocystaceae with members of prasinophytes as outgroup. Number at the branches indicate Bayesian posterior propabilities (MB) and bootstrap support from maximum likelihood (ML, 100 replicates), maximum parsimony (MP, 1000 replicates) and neighbour joining (NJ, 1000 replicates). Hyphen indicate support below 50 % for ML, MP, NJ and below 0.95 for MB.

This locus classicus is comparable to the habitat where we found the material in South Africa, a rockpool near the fast flowing pristine Belvedere River. The morphology of our strain CCAP 274/3 corresponds widely to the findings of Iyengar. The phylogenetic position of this alga near the coenobial genera *Crucigeniella* and *Makinoella* is distant from other *Oocystis* species. Therefore, we excluded this taxon from the genus *Oocystis*, described the new genus *Elongatocystis* and established a new combination *Elongatocystis ecballocystiformis* (see chapter Taxonomical measures).

The trend in developing large, colony-like “grandmother cells” by *Elongatocystis* reflect their phylogenetic position near large celled and colonial Oocystaceae such as *Makinoella*. The related *Oocystis solitaria* containing numerous chloroplasts could eventually after further studies considered as member of a new genus. *Eremosphaera viridis* DE-BARY, the oocystacean member with the highest number of chloroplasts (about 50 per cell) is known for its fibrous cell wall rich in pectin and hemicellulose (DE BOER et al. 1994). STOYNEVA et al. (2006) reported on a new species of *Eremosphaera*, *E. tanganyikae* from Lake Tanganyika. STOYNEVA et al. (2009) assumed based on ultrastructural similarities a close relationship of *Oocystis*, *Eremosphaera* and *Neglectella*.

The elongated filament-like cells of *Elongatocystis ecballocystiformis* (Fig. 6) found several times in cultures could be interpreted in two different ways: (i) as deformity or (ii) as link to the filamentous relatives of Oocystaceae such as *Planctonema* which evolved at the top of the oocystacean clade (Fig. 13). Microscopical studies revealed the facultative mucilaginous envelope and, interestingly, the layered character of apical cell wall thickenings bringing the cells within the filaments in a distant position (SKUJA 1956; BOURRELLY 1962; HEYNIG 1988). The position in the phylogenetic tree makes *Planctonema* a candidate to be included into Oocystaceae. The ultrastructural proof showing the fibrillar pattern of cell wall of *Planctonema* is unaccounted until now.

Our strain of *Oocystidium* sp. (CCAP 222/49) is clustering at the lower end of the tree as sister to *Ooplanctella planoconvexa* and *Oocystis* sp. *Oocystidium* is considered as a monotypic genus represented by *Oocystidium ovale* Korshikov. It is characterized by a wide mucilaginous envelope

containing persistent bipartited mother cell wall remnants (KORSHIKOV 1953; HINDÁK 1988). Our strain differs from the type species by smaller (~50%) and more elongated cells. However, we refrain from description of a new species before the taxonomical placement of *Oocystidium*, *Oocystis* and *Oocystella* is generally resolved. Microscopical findings showed *Oocystidium*-like extended mucilaginous envelope containing mother cell wall remnants also in several species of *Oocystella* such as *O. oogama* HINDÁK and *O. parva* (W. et G.S. WEST) HINDÁK (HINDÁK 1988).

We designated our strain CCAP 286/1 as *Quadricoccus ellipticus* because of its typical arrangement of four cells symmetrical on the edge of a bowl-shaped mother cell wall remnant when we isolated the strain and during its first weeks in culture. Later, this typical arrangement disappeared in culture. Probably, this symmetric position of the cells on a flat bowl is a phenotypic adaptation and supports the balance and buoyancy of the colony in the water column and is not essential under culture conditions. An other morphological criterion in *Quadricoccus*, the cell wall incrustation of the type species *Quadricoccus verrucosus* FOTT is perhaps also a phenotypic adaptation. This makes it difficult to establish an authentic strain of the type species, because the incrustations disappear in culture. Also for other coccoid green algae the incrustation was assumed to be a phenotypic character such as for *Dictyosphaerium granulatum* Hindák and *Raphidocelis* div. spec. (C. BOCK and L. KRIENITZ unpubl. results). The phylogenetic analyses showed the accommodation of *Quadricoccus* in Oocystaceae as a sister to *Amphikrikos*. Therefore, the former position of *Quadricoccus* within Dictyosphaerioideae according to KOMÁREK & FOTT (1983) is to revise.

Whereas no doubt exists about the natural delineation of the family (PRÖSCHOLD & LELIAERT 2007), the grouping inside the family on the level of genera and species is highly erratic. This is especially the case in regard of the type species of the genus *Oocystis*, *O. naegelii* A. Br. which does not possess pyrenoids. Because of uncertainties in the description of this species provided by BRAUN (1855), ŘEHÁKOVÁ (1969) suggested *O. lacustris* Chodat (which contains pyrenoids) as lectotype. However, KOMÁREK & FOTT (1983) rejected this suggestion and referred to the careful study of SKUJA (1964) who re-examined the exsiccatae of the type species *O. naegelii*. He

found on the material from the boreal zone only one or two chloroplasts per cell which does not exhibited pyrenoids. The possession of pyrenoids in members of *Oocystis* seems to be of crucial interest. LEMMERMANN (1903) established the new genus *Oocystella* for *Oocystis*-like algae with pyrenoids based on the typus *Oocystella natans* LEMM., which is only to differentiate from *O. lacustris* by the asteroid-like chloroplasts. HINDÁK (1988) came back to LEMMERMANN's conception of *Oocystella* and transferred 12 pyrenoid-bearing species of *Oocystis* to *Oocystella*.

Resuming the phylogenetic positions of different *Oocystis* species throughout the whole clade of Oocystaceae, we are unable, to decide which is the real *Oocystis* lineage, because all the strains sequenced until now possess pyrenoids. In a **first** and most important step it would be essential to isolate and sequence material from the pyrenoid-less type species *O. naegelii*. Afterwards, a decision can be made about the taxonomical relevance of pyrenoids. In recent studies, controversial experiences were made regarding the taxonomic relevance of pyrenoids. In Selenastraceae the pyrenoids are highly variable and can not be further used as taxonomical marker in this relationship (KRIENITZ et al. 2001). Out of eight genera of coccoid green algae of the *Chlorella*-clade according to KRIENITZ et al. (2004), only one genus, *Meyerella*, does not possess a pyrenoid (FAWLEY et al. 2005; BOCK et al. 2010; LUO et al. 2010). For members of the *Dictyosphaerium*-morphotype it was clearly shown that the pyrenoid is of high taxonomic relevance. So, the pyrenoid-bearing genera such as *Dictyosphaerium*, *Heynigia*, and *Hindakia* belong to the Trebouxiophyceae (BOCK et al. 2010), whereas members of the pyrenoid-less genus *Mychonastes* (formerly *Pseudodictyosphaerium*) belong to the Chlorophyceae (KRIENITZ et al. 2011). In a **second** step, after recovering of the taxonomical relevance of pyrenoids and the phylogenetic placement of the lineage containing the type species of *Oocystis* in Oocystaceae it can be decided about the question: What is the real *Oocystis*. Finally, in a **third** step, the circumscription of the remaining genera of Oocystaceae can be displayed.

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