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Generic and species concepts in Microglena (previously the Chlamydomonas monadina group) revised using an integrative approach†

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Traditionally the genus Microglena Ehrenberg has been used to contain species that belong to the Chrysophyceae; however, the type species of Microglena, M. monadina, represents a green alga, which was later transferred to the genus Chlamydomonas. The taxonomic status of the genus has therefore remained unclear. We investigated 15 strains previously assigned to C. monadina and two marine species (C. reginae and C. uva-maris) using an integrative approach. Phylogenetic analyses of SSU and ITS rDNA sequences revealed that all strains form a monophyletic lineage within the Chlorophyceae containing species from different habitats. The strains studied showed similar morphology with respect to cell shape and size, but showed differences in chloroplast and pyrenoid structures. Some representatives of this group have the same type of sexual reproduction (homothallic advanced anisogamy). Three different morphotypes could be recognized. Strains belonging to type I have a cup-shaped chloroplast with a massive basal part, in which a large, single, ellipsoidal pyrenoid is located. The members of type II also have a cup-shaped chloroplast, which is partly lobed and has a thinner basal part than type I; here the pyrenoid is half-ring or horseshoe-shaped and occupies different positions in the chloroplast depending on the strain. The strains of type III have multiple pyrenoids, which appear to have developed from the subdivision of a single ring-shaped pyrenoid into several parts. We compared the results of our morphological investigations with the literature and found that 15 strains could be identified with existing species. Two strains did not fit with any described species. As a result of our study, we transfer all strains to the genus Microglena, propose 11 new combinations, and describe two new species. Comparison of the ITS-1 and ITS-2 secondary structures confirmed the species delineations. All species have characteristic compensatory base changes in their ITS secondary structures and are supported by ITS-2 DNA barcodes.

Key words: Chlamydomonas, Chlorophyceae, DNA barcode, ITS-2 DNA Barcode, Microglena, molecular phylogeny, Monadina-clade, phenotypic plasticity, species concept, systematics

Introduction

The genus Chlamydomonas Ehrenberg comprises biflagellate unicellular green algae, which are distributed in almost all habitats. More than 800 species have been described, many of them so incompletely that it is difficult or impossible to determine what they are. Therefore, Ettl (1976, 1983) recognized only around 400 species. Phylogenetic analyses have clearly demonstrated that the traditional genus Chlamydomonas is polyphyletic (Buchheim et al., 1990, 1996; Hepperle et al., 1998) and can be split into eight independent, well-supported lineages within the so-called clockwise group of the Chlorophyceae (Pröschold et al., 2001; Pröschold & Leliaert, 2007). Taxonomic revision of the genus has been initiated by the typification of Chlamydomonas reinhardtii Dangeard as the conserved type (Pröschold & Silva, 2007) and by the description of two new genera Oogamochlamys and Lobochlamys (Pröschold et al., 2001). Another of the eight well-supported lineages is the ‘Monadina’-clade, which is not related to C. reinhardtii and until

†Molecular phylogeny and taxonomic revision of Chlamydomonas (Chlorophyta). II.
now has contained only a few strains, the only named species \textit{C. monadina} (Ehrenberg) Stein (SAG 31.72) and different unidentified strains isolated from polar regions. However, the taxonomic status of the \textit{Monadina}-clade has remained unclear and is the focus of this study.

\textit{Chlamydomonas monadina} was originally described by Ehrenberg (1832) as \textit{Microglena monadina}. The second species of this genus described by Ehrenberg (1832) was \textit{Microglena volvocina} Ehrenberg, which was later transferred to the genus \textit{Trachelomonas} (Euglenophyta) as \textit{T. volvocina} (Ehrenberg) Ehrenberg (1834). Since Ehrenberg, six species of Chrysophyceae have been described under the name \textit{Microglena} (see Ettl, 1978), e.g. \textit{M. arenicola} Droop. Stein (1878) transferred \textit{Microglena monadina} to \textit{Chlamydomonas}, with the consequence that the only algal species referred to \textit{Microglena} during the last century have been chrysophycean algae. In addition, the generic names \textit{Microglena} L"onnroth and \textit{Microgaena} K{"o}rber have also been used and refer to a genus of lichen, described independently twice. According to the International Code for Botanical Nomenclature (ICBN) the name \textit{Microglena} Ehrenberg has priority against both \textit{Microglena} L"onnroth and \textit{Microgaena}. This was recognized by Mayrhofer and Poelt (1985), who made both lichen generic names synonyms of \textit{Thelenella} Nylander. However, \textit{Microglena} remains a valid name for chlorophycean algae related to the type species \textit{M. monadina}.

As mentioned above, recent studies have shown that \textit{Chlamydomonas}-like species isolated from snowfields and ice of different regions belong to the \textit{Monadina}-clade (Leya, 2004; Liu et al., 2006; Eddie et al., 2008). We compared these data with our investigations of 15 strains previously designated as \textit{C. monadina} from public culture collections and two marine species (\textit{C. reginae} Ettl \\& J.C. Green and \textit{C. uva-maris} Butcher), using the integrative approach proposed by Pr"oschold \\& Leliaert (2007). Phylogenetic analyses of SSU and ITS rDNA sequences were made to establish the relationships of the strains and to establish an appropriate generic classification. Comparisons of morphology and studies of the ITS secondary structure were made to determine species limits and the newly developed, unique ITS-2 DNA Barcode was used to provide a basis for unambiguous future identifications.

Materials and methods

\textit{Cultures and light microscopy}

Strains were obtained from the Sammlung von Algenkulturen, University of G"ottingen, Germany (SAG: Schl"ossler, 1994; www.epsag.uni-goettingen.de), Culture Collection of Algae, University of Cologne, Germany (CCAC; www.ccac.uni-koeln.de) and Algal Collection of Kyiv University, Ukraine (ACKU; Kostikov et al., 2009); they are listed in Table S1 (Supplementary material). The freshwater strains were grown in modified Bold’s Basal Medium (3N-BBM + V: medium 26a in Schl"ossler, 1997), both marine strains in modified artificial seawater medium (MASM: www.ccap.ac.uk/media/documents/MASM_000.pdf). The cultures were grown in Erlenmeyer flasks at 20°C, 50 \(\mu\text{E m}^{-2} \text{s}^{-1}\) light intensity and a light:dark cycle of 14:10 h. Sexual reproduction was induced using a similar method to that described for \textit{Oogamochlamys} in Pr"oschold et al. (2001): dense cell suspensions from 14-day-old cultures in Erlenmeyer flasks were centrifuged at low speed and the pellets were resuspended in either 2 ml 3N-BBM + V or in 2 ml BBM–N + V (the same medium without sodium nitrate). These suspensions were transferred into the shallow centre of special cell culture dishes (Fa. Corning, NY, USA, 60 mm \(\times\) 15 mm, Costar No. 3260). In the surrounding part of these dishes 2–3 ml of distilled water were added to reduce evaporation. Observations were made with ZEISS Axio Imager (Zeiss, Oberkochen, Germany) and Olympus BX60 microscopes (Olympus, Tokyo, Japan), using the ZEISS and Cell'D imaging software respectively, and with an XSP-XY microscope (Ningbo Shengheng Optics \\& Electronics, Ningbo, China).

\textit{DNA isolation, PCR and sequencing}

Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen GmbH). The SSU and ITS rDNA were PCR amplified according to Luo et al. (2006), using the Taq PCR Mastermix Kit (Qiagen, Hilden, Germany) with the primers EAF3, E528F, 920F, BR, 3730 sequencer (Applied Biosystems, Foster City, CA, USA) using the primers (EAF3, E528F, 920F, BR, N920R, 536R, GF and GR; Marin et al. [2003], Pr"oschold et al. [2005]). The nucleotide sequences are available in the EMBL, GenBank and DDBJ sequence databases under the accession numbers given in Fig. 1 and Table S1.

\textit{Phylogenetic analyses}

The SSU rDNA sequences were aligned according to their secondary structure by comparison of the structure presented for \textit{Chlamydomonas reinhardtii} M32703 (Wufts et al., 2000) and included in a dataset (1524 base-pairs [bp]) containing 85 taxa of all representative clades belonging to the clockwise (CW-) group of Chlorophyceae (Gerloff-Elias et al., 2005; Pr"oschold \\& Leliaert, 2007). The sister clade of the CW-group, the \textit{Chaetophora}-clade sensu Pr"oschold et al. (2001) was used as the outgroup. The ITS-1 and ITS-2 sequences of all strains were folded by using the program mfold.
The phylogenetic tree shown was inferred by maximum likelihood method based on a dataset of 1524 aligned positions of 85 taxa using PAUP 4.0b10. For the analysis, the GTR model (base frequencies: A 0.2465, C 0.2107, G 0.2819, T 0.2609; rate...

Fig. 1. Molecular phylogeny of the clockwise group of the Chlorophyceae, based on SSU rDNA sequence comparisons. The phylogenetic tree shown was inferred by maximum likelihood method based on a dataset of 1524 aligned positions of 85 taxa using PAUP 4.0b10.
(Mathews et al., 1999; Zaker, 2003; mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) and their structures are summarized in Fig. S1 (Supplementary material), produced using the programs LoopDloop (Gilbert, 1992) and Adobe Illustrator CS2 (Adobe, San Jose, California). Based on these structures, ITS-1 and ITS-2 were separately aligned manually and using the program MARNA (Siebert & Backofen, 2005: www.bioinf.uni-freiburg.de /Software/MARNA/index.html) to avoid any bias in the alignments. The resulting alignments of the 17 strains were included in a concatenated dataset (2866 bp) of SSU (1775 bp), ITS-1 (435 bp), 5.8S (159 bp), ITS-2 (477 bp) and LSU (20 bp) rDNA sequences. The alignments are available via TreeBASE (http://www.treebase.org) under the number S12530.

To determine the evolutionary model that fits best for both datasets the program Modeltest 3.7 (Posada, 2008) was used. Based on the results of these tests, the best models were selected by the Akaike Information Criterion (Akaike, 1974). For both datasets the GTR model with a proportion of invariable sites (I), and a gamma shape parameter (G) was used for the phylogenetic analyses. The phylogenetic trees (Figs 1 and S2) were inferred by distance (neighbour-joining [NJ] using the GTR + I + G model), parsimony (MP), and maximum likelihood (ML; using GTR + I + G) criteria using PAUP version 4.0b10 (Swofford, 2002), by randomized accelerated maximum likelihood using RAxML version 7.0.3 (Stamatakis, 2006), and by Bayesian inference (BI) using MrBayes version 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) and the PHASE package 2.0 (Jow et al., 2002; Higgs et al., 2003; Hudelot et al., 2003; Gibson et al., 2005; Telford et al., 2005). The RAxML analyses of the concatenated dataset were performed partitioned according to their genes. For PHASE analyses, two models were used according to their secondary structure, the THREESTATE model (Tavare, 1986) being used for the unpaired regions and the RNA7D model (Tillier & Collins, 1998) for the paired regions.

To find molecular signatures (DNA barcodes) for all species of Microglena, the conserved region of ITS-2 was extracted manually from the alignment created by MARNA. According to the recommendation of Coleman (2009), the conserved region, using the 15 bp of the 5.8S-LSU stem, the first 5 bp of Helix I, the first 11 bp of Helix II (including the pyrimidine–pyrimidine mismatch), and all base-pairs of Helix III were selected for the DNA barcode (highlighted in grey boxes in Fig. 2). The resulting sequences were manually aligned again and proven automatically using Marna (Fig. 2). This alignment was used to find compensatory base changes (CBCs; see Table S2) using the program CBCAnalyzer version 1.1 (Woff et al., 2005). The corrected p-distances were calculated using PAUP. The differences (CBCs and distances) among the species are summarized in Table 1. This sequence alignment was translated into the base-pair alignment by replacing each base-pair by a number (A–U = 1; U–A = 2, G–C = 3, C–G = 4, GeU = 5, UaG = 6, mismatch = 7, deletion/unpaired or single bases = 8; Fig. 3). From this base-pair alignment a NEXUS file was created for the maximum parsimony analysis calculated in PAUP (Fig. 4).

**Results**

**Molecular phylogeny, secondary structures and DNA barcoding**

To examine whether all strains studied belonged to a monophyletic lineage, a SSU rDNA dataset was established including representatives of the clockwise group among the Chlorophyceae, with the Chaetophora-clade sensu Pröschold et al. (2001) as outgroup. The phylogenetic analyses presented in Fig. 1 showed that all strains investigated in this study belonged to the Monadina-clade (=Microglena; see below), together with several strains previously isolated and sequenced from snowfields or glaciers of polar regions or from marine habitats in Japan (the ‘Polar-subclade’ sensu Eddie et al., 2008, in Fig. 1). The principal clades of clockwise Chlorophyceae, including the Monadina-clade, have a high posterior probability (Bayesian: PHASE and MrBayes) and bootstrap proportion (ML, NJ, MP) in all analyses, with the exception of the Chlorogonium and Dunaliella clades, which have high Bayesian support but no or weak bootstrap support in the ML analysis.

In order to get better resolution within the Monadina-clade, a concatenated dataset of SSU and ITS rDNA sequences was aligned according to their secondary structures and analysed using different phylogenetic methods with the two marine strains (SAG 17.89 and SAG 19.89) as outgroup. Figure S2 shows that the 15 strains

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**Fig. 1.** Continued

matrix: A-C 1.1409, A-G 2.6738, A-T 1.4865, C-G 0.9087, C-T 5.3353, G-T 1.0000) with the proportion of invariable sites (I = 0.5213) and gamma distribution shape parameter (G = 0.5923) was chosen, which was calculated as best model by Modeltest 3.7. Bayesian values (>0.95) were calculated by MrBayes 3.1 (first value in boxes) using the covarion settings (5 million generations) and PHASE 2.0 (second value) using THREESTATE and RNA7D models for unpaired and paired nucleotides respectively. Bootstrap values (>50%) of the maximum likelihood (using the GTR + I + G model, 100 replicates; third value) using PAUP, the randomized accelerated maximum likelihood using the RAxML 7.0.3 (using the GTR + I + G model, 1000 replicates; fourth value), neighbour-joining (using the GTR + I + G model, 100 replicates; fifth value), and maximum parsimony (1000 replicates; sixth value) were marked in boxes and only given for the clades. The strains marked in bold are new sequences in this study. Strain and accession numbers are given after the species name. The clade designation follows Pröschold & Leliaert (2007). The Chaetophora-clade was used as outgroup.
Fig. 2. Comparison of the conserved DNA Barcode region of ITS-2 among species of *Microtroglo*. The upper part of the figure shows the ITS-2 secondary structure of SAG 55.72 *M. monadina*, including details of the stem of 5.8S and LSU rDNA, and helices I–III. The conserved regions are highlighted in grey boxes. The bottom part of the figure shows the conserved region of ITS-2 aligned manually or automatically using MARNA. Helices I–IV of ITS-2 are drawn with straight lines for practical reasons.
Table 1. Compensatory base changes (CBCs) and uncorrected \( p \)-distances among the ITS-2 DNA barcodes of the *Microglena* species. The upper-right half of the table shows the total number of compensatory changes (CBC/Hemi-CBCs), while the lower-left half gives the uncorrected \( p \)-distances calculated in PAUP.

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<th>( p )-distances</th>
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<td>SAG 0.18214</td>
<td></td>
<td>15 (9/6)</td>
<td></td>
</tr>
<tr>
<td>M. reginae</td>
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<td>15 (9/6)</td>
<td></td>
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<tr>
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<td>15 (9/6)</td>
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<td>15 (9/6)</td>
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<td></td>
<td>15 (9/6)</td>
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<tr>
<td>M. uva-maris</td>
<td>SAG 19.89</td>
<td>15 (9/6)</td>
<td></td>
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<tr>
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<td></td>
<td>15 (9/6)</td>
<td></td>
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Fig. 3. ITS-2 DNA Barcode of the Microglena species. The sequence alignment presented in Fig. 3 was translated into a base-pair alignment using a number coding for each base-pair. The base positions in the sequence alignment are given for comparison. The barcode for each species is named by a letter (A–M). Unique compensatory base changes and non-homoplasious synapomorphies for Microglena species are marked by asterisks.

```

Position in alignment

Barcode position

Table 3. ITS-2 DNA Barcode of the Microglena species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Barcode</th>
<th>Sequence Alignment</th>
</tr>
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<tbody>
<tr>
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<td>23421342453326 64142 6534437444 38888338881348884834454114234214341348</td>
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<td>(SAG 55.72)</td>
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<td></td>
</tr>
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<td>237421342453326 64142 6534237444 32313833826113433888484834454114234884341348</td>
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<td></td>
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<tr>
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<td>C</td>
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</tr>
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<td></td>
</tr>
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<td>M. globulifera</td>
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<td>M. skujae</td>
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<tr>
<td>(SAG 19.89)</td>
<td></td>
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</tbody>
</table>

1 = A-U  3 = G-C  5 = G+U  7 = mismatch  
2 = U-A  4 = C-G  6 = U+G  8 = deletion, unpaired or single bases
```
Fig. 4. Molecular phylogeny of the ITS-2 DNA Barcode in comparison to the morphotypes in *Microglena*. The phylogenetic tree represents a majority-rule consensus of five equal trees (120 steps) calculated by maximum parsimony in PAUP. The asterisk marks the only branch that varies among the five trees. Morphotypes I–III are characterized by chloroplast shape and the shape and position of the pyrenoid. Each species is documented by a line drawing and grouped according to morphotype.
previously assigned to *Chlamydomonas monadina* represent 11 lineages. However, the relationships among these lineages were unresolved. To gain further information about relationships and to decide if the 13 lineages in Fig. 3 are a DNA barcode (highlighted in grey boxes in Fig. 2), as described in the Materials and methods. The resulting sequences were manually aligned and checked automatically using MARNA to avoid any bias (Fig. 2). Both alignments were identical. This sequence alignment (160 bp) was translated into a base-pair alignment (80 base-pairs) by replacing each base-pair by a number. Each of the 13 lineages had a characteristic barcode sequence (unique changes are highlighted with an asterisk in Fig. 3) and were also supported by compensatory base changes (summarized in the upper corner in Table 1; a comprehensive analysis of CBCs is summarized in Table S2). Each of the 13 can therefore be interpreted as a separate species. Uncorrected p-distances between them are given in Table 1 (lower corner).

In contrast to the unresolved relationships from the sequence-based SSU–ITS alignment (Fig. S2), phylogenetic analysis (by maximum parsimony) of the base-pair alignment presented in Fig. 3 showed that species with similar morphologies (morphotypes I–III; described in detail below) are more closely related (Fig. 4). Only the relationship of six species belonging to the subclade marked with an asterisk in Fig. 4 remained unresolved.

**Morphology of vegetative stages**

The strains investigated were not only phylogenetically closely related, they also had similar morphology of the vegetative cells. The vegetative cells of all strains had a broadly ellipsoidal to spherical cell shape; a wide truncate papilla (excluding the marine strain SAG 17.89); two apical contractile vacuoles; two flagella that were as long as the cell length or slightly longer or shorter; an anterior or central, ellipsoidal to rod-like or fusiform stigma; a cup-shaped chloroplast; and a large pyrenoid surrounded by several to many starch grains, which were orientated in parallel rows. The nucleus was always central (excluding SAG 67.72).

However, there were also some morphological differences among the strains studied, which could be allocated to three different morphological groups, based mainly on chloroplast shape. We refer to these as morphotypes I–III (Fig. 4).

The strains belonging to morphotype I had a cup-shaped chloroplast with a thick basal part, in which a single broadly ellipsoidal pyrenoid was located. Algae from morphotype II also had a cup-shaped chloroplast but this was without a thick basal part; the single pyrenoid had a characteristic shape (horseshoe-, sausage-like or half-to quarter-ring shaped) and was located in a lateral ring-like thickening of the chloroplast. Morphotype III comprised algae with a cup-shaped chloroplast lacking a thicker basal part, as in morphotype II, but with several spherical or ellipsoidal pyrenoids located in different places in the lateral part of the chloroplast.

Comparison of the three morphotypes with original species descriptions and several monographs (Pascher, 1927; Korshikov, 1938; Huber-Pestalozzi, 1961; Ettl, 1976, 1983) showed similarities to existing species. Details of the morphological features are summarized in Table 2 and documented in Figs 5–7.

- **Morphotype I** contains four strains: the authentic strains of both species, *C. reginae* (SAG 17.89 = *Microglena reginae*, comb. nov.; see below) and *C. uva-maris* (SAG 19.89 = *M. uva-maris*, comb. nov.), isolated from marine habitats, and two strains (ACKU 267-03 and ACKU 274-03) isolated at different times from the same freshwater body in the Ukraine. The first two strains fit the original descriptions (Butcher, 1959; Ettl & Green, 1973), while the two Ukrainian strains could be identified as *Chlamydomonas monadina var. charkoviensis* (Korshikov) Korshikov (= *M. charkoviensis*, comb. nov.; Korshikov, 1938).

- **Nine strains belong to morphotype II.** Seven of these could be clearly identified as *Chlamydomonas monadina var. monadina* (SAG 55.72 = *M. monadina*; see below: Ehrenberg, 1832; Stein, 1878), *C. monadina var. longirubra* Ettl (SAG 5.92 = *M. longirubra*, comb. nov.: Ettl, 1976), *C. opisthopyren* Skuja (SAG 54.90 and SAG 8.87 = *M. opisthopyren*, comb. nov.: Skuja, 1956), *C. monadina var. indica* Iyengar (SAG 46.96 = *M. indica*, comb. nov.: Iyengar & Desikachary, 1981), *C. nova* Skuja (SAG 16.90 = *M. skujae*, nom. nov.: Skuja, 1956), and *C. braunii* Goroschankin (SAG 50.86 = *M. braunii*, comb. nov.: Goroschankin, 1890). In contrast, two strains did not fit with any diagnoses of described species and are therefore described below as new species (SAG 31.72 = *M. lobata*, sp. nov. and SAG 67.72 = *M. basinucleata*, sp. nov.; see below).
<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
<th>Cell shape, size in μm</th>
<th>Cell wall, papilla</th>
<th>Chloroplast (morphotype)</th>
<th>Pyrenoid</th>
<th>Stigma</th>
<th>Nucleus</th>
<th>Flagellar length, contractile vacuoles</th>
<th>Reproduction</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. monadina</em></td>
<td>SAG 55.72</td>
<td>ellipsoid to wide ellipsoid, 19–23 × 12–18</td>
<td>thick with large, broad, trapezoid papilla</td>
<td>cup-shaped (II)</td>
<td>half-ring-shaped surrounded by many small starch grains</td>
<td>bright, short rod-like in anterior-medial position</td>
<td>central position</td>
<td>as long as the cell; two apical</td>
<td>2-4 zoospores</td>
</tr>
<tr>
<td><em>M. basinucleata</em></td>
<td>SAG 67.72</td>
<td>ellipsoid to wide ellipsoid, 15–22 × 12–20</td>
<td>thick with large, broad, trapezoid papilla</td>
<td>cup-shaped (II)</td>
<td>half-ring-shaped without starch grains</td>
<td>bright, elongated in anterior-medial position</td>
<td>basil position</td>
<td>as long as the cell; two apical</td>
<td>2-4 zoospores</td>
</tr>
<tr>
<td><em>M. braunii</em></td>
<td>SAG 50.86</td>
<td>ellipsoid to wide ellipsoid, almost spherical, 17–25 × 11–23</td>
<td>thick with large, broad, trapezoid papilla</td>
<td>cup-shaped (II)</td>
<td>horseshoe-like, half-ring-shaped or widely ellipsoid surrounded by many small starch grains</td>
<td>bright, short rod-like in anterior-medial position</td>
<td>central position</td>
<td>as long as the cell; two apical</td>
<td>2-4 zoospores</td>
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<tr>
<td><em>M. charkoviensis</em></td>
<td>ACKU 267-03</td>
<td>ellipsoid to wide ellipsoid, 10–16.5 × 8–14.5</td>
<td>thick with large, broad, trapezoid papilla</td>
<td>cup-shaped (I)</td>
<td>widely ellipsoid surrounded by many small starch grains</td>
<td>bright, rod-like to fusiform in anterior-medial position</td>
<td>central position</td>
<td>as long as the cell; two apical</td>
<td>2-4 zoospores</td>
</tr>
<tr>
<td><em>M. coccifera</em></td>
<td>SAG 54.91</td>
<td>wide ellipsoid, (16)22–24(2)/ × 19–23(27)</td>
<td>thick with large, broad, trapezoid papilla</td>
<td>cup-shaped (III)</td>
<td>1 to 4–5 (8); widely ellipsoid or spherical surrounded by many small starch grains</td>
<td>bright, rod-like to fusiform in anterior-medial position</td>
<td>central position</td>
<td>as long as the cell; two apical</td>
<td>2-4 zoospores</td>
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<tr>
<td><em>M. globulifera</em></td>
<td>CCAC 0015</td>
<td>wide ellipsoid to spherical, 16–22 × 10–19</td>
<td>thick with large, broad, trapezoid papilla</td>
<td>cup-shaped perforated with small fissures (III)</td>
<td>horseshoe-like or half-ring-shaped surrounded by many small starch grains, fragmented into 3–5 spherical</td>
<td>bright, rod-like to fusiform in anterior-medial position</td>
<td>central position</td>
<td>as long as the cell; two apical</td>
<td>2-4 zoospores</td>
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<tr>
<td><em>M. indica</em></td>
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<td>spherical to wide ellipsoid, (9)14–18 × (7)13–17</td>
<td>thick with large, broad, trapezoid papilla</td>
<td>cup-shaped (II)</td>
<td>half-ring-shaped surrounded by many small starch grains</td>
<td>bright, rod-like in anterior-medial position</td>
<td>central position</td>
<td>as long as the cell; two apical</td>
<td>2-4 zoospores</td>
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</table>
Table 2. Continued.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
<th>Morphology of the vegetative cells</th>
<th>Reproduction</th>
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<tr>
<td></td>
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<td>Cell shape, size in μm</td>
<td>Flagellar length, contractile vacuoles</td>
</tr>
<tr>
<td></td>
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<td>Cell wall, papilla</td>
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<tr>
<td></td>
<td></td>
<td>Chloroplast (morphotype)</td>
<td>Pyrenoid</td>
</tr>
<tr>
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<tr>
<td>M. lobata</td>
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<td>ellipsoid to wide ellipsoid, (12)17–23 × (9)13–20</td>
<td>cup-shaped (II)</td>
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<td>thick with large, broad, trapezoid papilla</td>
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<td>M. longinbrea</td>
<td>SAG 5.92</td>
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<td>cup-shaped (II)</td>
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<td></td>
<td>thick with large, broad, trapezoid papilla</td>
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<td>M. opisthopren</td>
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<td>cup-shaped (II)</td>
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<td>SAG 54.90</td>
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</tr>
<tr>
<td>M. reginae</td>
<td>SAG 17.89</td>
<td>ellipsoid to ovoid, wide ovoid to almost spherical, 15–22 × 13–18</td>
<td>cup-shaped (I)</td>
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<tr>
<td></td>
<td></td>
<td>thick with large, broad, trapezoid papilla</td>
<td></td>
</tr>
<tr>
<td>M. skujae</td>
<td>SAG 16.90</td>
<td>ellipsoid to wide ellipsoid, (12)18–23(25) × (8)14–20</td>
<td>cup-shaped dissected in several lobes (II)</td>
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<td></td>
<td></td>
<td>thick with large, broad, trapezoid papilla</td>
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<tr>
<td>M. uva-maris</td>
<td>SAG 19.89</td>
<td>ellipsoid to wide ellipsoid, 8–17 × 6-13</td>
<td>cup-shaped (I)</td>
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<tr>
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<td>thick with large, broad, trapezoid papilla</td>
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The morphology of the four strains belonging to morphotype III fits with the original descriptions of *C. monadina* var. *globulifera* Korshikov (CCAC 0015 and CCAC 0017 = *M. globulifera*, comb. nov.: Korshikov, 1938) and *C. coccifera* Goroschankin (SAG 54.91 and SAG 55.91 = *M. coccifera*, comb. nov.: Goroschankin, 1905).

Morphology of reproductive stages

Asexual reproduction by sporulation was observed in all of the strains studied. During sporulation, the protoplast of the mother cell divided by longitudinal division, with or without rotation (Fig. 8a, b; see Ettl, 1979, 1988). Two to four zoospores were usually formed per sporangium. Unusual motile aggregates of cells were observed in all strains (Fig. 8c–f). These cells were extremely similar to stages in a type of asexual reproduction described by Massjuk & Demchenko (2001) and referred to as ‘protocytotomy’. This type of cell division is characterized by formation of a new elastic cell wall within the old mother cell wall, which enlarges and dissolves with the production of mucilage. The new internal cell wall divides together with the protoplast and forms a new cell wall surrounding each

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product of the cell division. Unfortunately we did not observe the full course of development and cannot confirm if cell division was protocytotomy or some other process.

Palmelloid stages were not observed in any strains. Akinetes were observed in one strain only (SAG 55.72 *M. monadina*) and were similar in morphology to zygotes (data not shown).

Sexual reproduction was an advanced form of anisogamy, which is illustrated here for strain SAG 55.91 *M. coccifera* (Fig. 8g–i) and was observed also in strains SAG 55.72, SAG 50.86, SAG 54.91 and SAG 5.92. In all cases the female gamete (macrogamete) developed directly from a vegetative cell by rounding up of the protoplast and slight expansion. The macrogamete was usually surrounded by the gametangium cell wall, but was sometimes released. In contrast, each male gametangium usually contained four to eight, rarely 16 male gametes (microgametes). After release the microgametes were small (in SAG 55.91; 8.3–14.4 μm long), ovate or drop-shaped, and surrounded by a cell wall; they possessed two long flagella, which were twice as long as the cell. After attachment of the microgamete to the macrogamete, mostly at the macrogamete’s anterior...

end, the cell walls of both gametes dissolved at the point of connection. Then the protoplast of microgamete moved towards the protoplast of the macrogamete and fused with it (Fig. 8g). Sometimes the protoplasts of both gametes were released from their cell walls and fused, whereas the inner part of the microgamete cell wall was always visible (Fig. 8h). Rarely, several microgametes fused with a single macrogamete (polyspermy); however, this event was clearly recognizable by the presence of the inner parts of the empty microgamete cell walls, which are always absent in macrogametes (see also Goroschankin, 1890). During sexual reproduction, the flagella of the gametes were sometimes lost, sometimes retained, but in the latter case they were always without function. After fusion, the resulting zygote was always without flagella. Young zygotes were surrounded by a primary zygote wall, but often bore the empty cell wall of the microgamete (sometimes also of the macrogamete) (Fig. 8h). The secondary zygote walls were smooth without ornamentation. Mature zygotes contained many transparent or yellowish globules (Fig. 8i).

Fig. 7. Morphology of vegetative cells in Microglena. a–d. Microglena uva-maris (SAG 19.89). e–i. Microglena longirubra (SAG 5.92). j–m. Microglena lobata (SAG 31.72). n–q. Microglena basinucleata (SAG 67.72; arrows indicate the position of the pyrenoid, which is unclear). a–f, j, k and n–q show general views of vegetative cells; g–i, l and m, surface views. Scale bars = 10 μm.
**Discussion**

**Genetic diversity and DNA barcoding in Microglena**

Phylogenetic analyses of SSU rDNA sequences (Fig. 1) show that there is a monophyletic lineage within the Chlorophyceae that includes all of our *C. monadina* strains, together with *C. reginae*, *C. uva-maris* and various strains isolated from marine rock pools and snow/ice fields, indicating a high genetic diversity. This clade has previously been called the *Monadina-clade* (Pröschold et al., 2001) or *Monadinia* (Nakada et al., 2008). However, the correct name for a genus corresponding to the *Monadina-clade* is *Microglena*, dating from 1832. The monophyly of *Microglena* is supported by all Bayesian and bootstrap analyses and we therefore make formal transfers of all of the *Monadina clade* to *Microglena* below. As shown in Fig. 1, we also confirmed that the psychrophilic strains isolated by Eddie et al. (2008), Liu et al. (2006) and Leya (2004) form a monophyletic lineage within *Microglena*, called the Polar-subclade.

To get better resolution, we analysed a concatenated dataset of SSU and ITS rDNA sequences, aligned according to their secondary structure. Figure S2 shows that our 17 strains belong to 13 independent lineages within *Microglena*. However, the relationship among these lineages is unresolved by the concatenated SSU and ITS data (no Bayesian and bootstrap support). Therefore, to decide if the 13 lineages represent different species, the conserved region of the ITS-2 secondary structure was analysed using the CBC approach proposed by Coleman (2000, 2009), Moniz & Kaczmarska (2009) and Bock et al. (2011). Figures 3 and 4 and Table 1 show that all 13 lineages differ by at least one CBC, mostly by more (up to 18). This indicates that these lineages represent different species in accordance with Müller et al. (2007), who showed that in 93% of the cases studied independent species differ in at least one CBC. As a consequence, we propose the use of the conserved region in ITS-2 presented in Figs 3 and 4 to provide diagnostic characters to distinguish microalgal isolates at species level.

**Chloroplast morphology and phenotypic plasticity in Microglena**

The most striking character of the species in *Microglena* is the structure of the chloroplast and its embedded pyrenoid(s). All representatives of this group have a cup-shaped chloroplast but the pyrenoid varies in shape and position. Summarizing, three morphotypes could be observed in *Microglena* (Fig. 4). Morphotype I seems to be the ancestral type of chloroplast in *Microglena*. This type, in which the chloroplast has a thick basal part, where the single large pyrenoid is located, is present in *M. charkoviensis*, *M. reginae* and *M. uva-maris*. Strains of the Polar-subclade were not the focus of our study, but comparisons with published pictures show that these strains also have a chloroplast of morphotype I (Leya, 2004; Liu et al., 2006; Eddie et al., 2008).

Morphotypes II and III are also characterized by a cup-shaped chloroplast, but without a thick basal part (Fig. 4). However, during ontogenesis intermediate stages can be observed among the different morphotypes. For example, mature cells of *Microglena braunii* (SAG 50.86) and *M. opisthopyren* (SAG 8.87 and SAG 54.90) have type II chloroplasts, but the young cells have a cup-shaped chloroplast with a thick basal part containing a horseshoe-like pyrenoid; it is only later that the basal part of the chloroplast becomes thinner and the pyrenoid moves into a parietal position, creating the type II morphology. This ontogenesis was not indicated in the accounts of *Microglena braunii* (as *Chlamydomonas braunii*) by Goroschankan (1890), Pascher (1927) and Ettl (1983). Such morphological variability makes it very difficult to give a clear species identification through comparison with the original description or using the identification keys presented in Pascher (1927), Korschikov (1938), Huber-Pestalozzi (1961) and Ettl (1976, 1983). In addition, most species have small dissections of the chloroplast (Figs 5–7), which are largely ignored in published descriptions and indeed, they are difficult to observe and may need long investigation to discover. In *Microglena lobata* (SAG 31.72), *M. skujae* (SAG 16.90) and *M. basinucleata* (SAG 67.72), however, the incisions are easily visible and create a clearly lobed chloroplast. As Ettl & Green (1973) have already noted, *M. reginae* (SAG 17.89) has a perforated chloroplast with regular longitudinal fissures.

The main character of the vegetative cells is the easily visible pyrenoid and its shape. Among *Microglena* species the pyrenoid is very variable in shape, but all strains have in common that the pyrenoid is surrounded by several or many starch grains arranged in parallel rows. This was also reported by Ettl & Green (1973) for *M. reginae*, by Rosowski & Hoshaw (1988) for *M. longirubra*, and Eddie et al. (2008) and Liu et al. (2006) for two unidentified psychrophilic *Chlamydomonas* strains, based on electron microscopical investigations. Pyrenoid shape varies according to morphotype (Fig. 4): spherical to widely ellipsoidal in morphotype I, elongate to horseshoe-like in morphotype II, and fragmented and nearly spherical in morphotype III. These different pyrenoid shapes have been recognized and used by many authors as diagnostic features to describe different species (see...
details in Ettl, 1976, and below). However, like the chloroplast itself, pyrenoid shape undergoes several transformations during ontogenesis. For example, shortly after release of the sporangia, young cells of all strains mostly have an ellipsoidal pyrenoid in the base of the chloroplast, which is typical for morphotype I. On the other hand, old cells of *M. opisthopryn*, *M. braunii* and *M. skujae*, which belong to morphotype II, sometimes have fragmented pyrenoids, which are characteristic for morphotype III. The morphological transformation of the pyrenoid within single populations of *Chlamydomonas monadina sensu lato* has also been recorded by Ettl (1965, 1976), Gerloff (1940), Skuja (1949, 1956) and Pascher (1927). The pyrenoid of *M. basinucleata* (SAG 67.72) is difficult to distinguish because the pyrenoid has no starch layer around the pyrenoid. Therefore *M. basinucleata* has previously been identified wrongly as *Chloromonas subdivisa* (Pascher & Jahoda) Gerloff and Ettl ex Ettl (Schlösser, 1994). Interestingly, despite the absence of a pyrenoid, true *Chloromonas subdivisa* is similar in cell morphology to species of *Microglena* (Pascher & Jahoda, 1928), which may indicate that it too should be transferred.

The stigma has a similar shape (elongated or rod-like) in all strains and is located in the anterior half of the cell. A very elongated eyespot, occupying almost a third of the cell, has been recorded for *Chlamydomonas monadina var. longirubra* (Ettl, 1976), and we found a similar eyespot in strain SAG 5.92 (*M. longirubra*). However, elongated eyespots can also be observed sometimes in other strains (for example CCAC 0015 and CCAC 0017) and the stigmata of all strains can vary in size: even in *M. longirubra*, shorter eyespots like those typical for other species can be observed. Ettl & Green (1973), Rosowski & Hoshaw (1988) and Eddie *et al.* (2008) respectively have reported that the ultrastructure of the stigma is the same (with a single thylakoid between two layers of pigmented globules) in *M. reginae* (SAG 17.89), *M. longirubra* (SAG 5.92) and an unidentified *Chlamydomonas* strain (ARC).

In contrast to the chloroplast, other organelles show little variation among strains. For example, all freshwater strains have two anterior contractile vacuoles, which can be also observed in the marine strain SAG 19.89 of *M. uva-maris*, if cultivated in freshwater (3N-BBM + V) medium. *Microglena reginae* (SAG 17.89) is unable to grow in freshwater conditions and its contractile vacuoles do not function in seawater. The nucleus is located in a central position in the cell in all strains except SAG 67.72, where it is in basal position (hence its name, *M. basinucleata*). A papilla can be observed in all species and is mainly wide and trapezoidal. Only *M. reginae* has a special, crown-like papilla, which is mentioned in the description by Ettl and Green (1973). Ultrastructural studies have shown that strains SAG 17.89, SAG 5.92 and ARC have a branched mitochondrion, which is located only around the nucleus and not between cell wall and chloroplast (Ettl & Green, 1973; Rosowski & Hoshaw, 1988; Eddie *et al.*, 2008).

Asexual reproduction by sporulation has been observed in all strains of *Microglena* (Fig. 8a, b). The cell division during sporulation occurred longitudinally after protoplasm rotation of 90° (false transverse division *sensu* Ettl 1988), which corresponds to Ettl's (1965) documentation for *Chlamydomonas monadina*. However, in our study we observed that the first cell division can occur also longitudinally without rotation of the protoplasm; this was also shown in Ettl (1979). Interestingly, some stages in cell division similar to another type of reproduction (protophytum) for *Microglena* were found also by Ettl & Green (1973) in *M. reginae*. These authors referred to such cell aggregations as being somewhat similar to gamete fusion during isogamy, but they mentioned that this was only a superficial impression and that it was more realistic to suppose that the aggregations were a monstrosity resulting from incomplete division (Ettl & Green 1973). According to our data *Microglena* aggregates arise in a manner similar to protophytomy, but unfortunately, the further development of the cell division and the further development of the daughter cells could not be observed.

The sexual reproduction of *Microglena* showed two variations, which are known under different terms in the literature: advanced anisogamy (also called heterogamy) and oogenioamy. The main differences between these types lie in the presence or absence of flagella in both gametes and the behaviour of the macrogamete during fusion with the microgamete. If the protoplasm of the macrogamete remains in its cell wall and has no flagella, sexual reproduction is termed oogenioamy; otherwise, anisogamous. However, both types can be observed in the same species. Therefore, we designate the sexual reproduction of *Microglena* as homothallic, advanced anisogamy, because both gametes usually have flagella (though without function for the macrogametes), but sometimes the flagella are discarded before fusion. It seems that this mode is characteristic for the genus *Microglena* and has been documented by Goroschankin (1890) for *M. braunii*, Goroschankin (1905) and Skuja (1949) for *M. cocifera* and Rosowski & Hoshaw (1988) for *M. longirubra*. In addition, Korshikov (1938) mentioned that *Chlamydomonas monadina* and all its
varieties, despite their variable morphology, have the same type of sexual reproduction and therefore belong to the same species. The same type of sexual reproduction was described by Pascher (1927) for Chlamydomonas cingulata (= Microglena monadina; see below), by Pascher (1943) for C. praecox, and by Gerloff (1940) for C. heterogama. Although C. praecox and C. heterogama have a different cell morphology, both probably belong to Microglena based on sexual reproduction. Unfortunately, no strains of these species are available for further studies.

Summarizing: Microglena species have a highly variable cell morphology but, so far as is known, the same type of sexual reproduction. However, the large, ellipsoidal to horseshoe-like pyrenoid and the homothallic advanced anisogamy are two characters that make it easy to recognize this genus.
in water samples. The identification at species level in contrast needs comprehensive studies on cultured material.

Biodiversity and ecology of Microglena

The 13 known species of Microglena occur in three habitats: freshwater ponds, marine rock pools, and on snow and ice fields (Fig. 1). However, more species will have to be transferred to this genus, for example when the strains of the Polar-subclade sensu Eddie et al. (2008) have been investigated in a comparative study. In addition, several varieties of Chlamydomonas monadina and its relatives have been described that may represent independent species or may be synonyms of present species. These include Chlamydomonas anulata Nygaard (1949), C. scutula Pascher (1932 [= C. braunii var. scutula (Pascher) Gerloff]), C. cingulata var. perforata Vlk (1939/40) [= C. monadina var. perforata (Vlk) Ettl], C. cingulata var. seligeriana Korshikov ex Pascher (1927 [= C. monadina var. seligeriensis (Korshikov) Korshikov: Korshikov, 1938]), C. monadina var. separatus H.J. Hu (Hu & Wei, 2006), C. nova var. minor L.S. Peterfi (1968) and C. sichuanensis H.J. Hu & S. Chen (Hu & Wei, 2006): all have similar vegetative cell morphology, but differ in shape and the position of some cell organelles. Unfortunately, no cultures are available of these species. Whether C. monadina var. ovalis Playfair (1915) and C. braunii f. elliptica Moewus (1940) belong to Microglena is doubtful: it is impossible to decide because the descriptions and illustrations are too poor (see Ettl, 1976). As shown in Fig. 1, several psychrophilic strains isolated from snow and ice fields of Svalbard (Norway: Leya, 2004) and Antarctica (Liu et al., 2006; Eddie et al., 2008) belong to Microglena. Broady (1979) described an isolate called Chlamydomonas sp. from South Georgia (Antarctica), which probably belongs to Microglena and represents a new species.

As we note above, according to their sexual reproduction, C. heterogama (Gerloff, 1940), and C. praecox (Pascher, 1943) probably belong to Microglena, and for similar reasons also C. upsa-liensis Skuja (Skuja, 1949) and C. goroschankii Chmielewski (Pascher, 1927), but the cell morphology of these species differs from the species we describe above and no cultures are available for further studies.

Whereas the freshwater and psychrophilic species probably have a monophyletic origin (Fig. 1), the marine species Microglena reginae, M. uva-maris and two strains designated as C. kuwadae Gerloff (1940), and an unidentified species of Chlamydomonas represent three different lineages within Microglena, indicating the origin of Microglena species in marine habitats. Chlamydomonas kuwadae was originally described by Kuwada (1916) as an unidentified Chlamydomonas species from a marine habitat. The strain NIES 968 identified as C. kuwadae by Nozaki et al. (2002) belongs to Microglena (see Fig. 1), but needs further investigation. All strains studied so far have in common that they are adapted to low temperature. Our study and the studies of Eddie et al. (2008), Liu et al. (2006) and Leya (2004) have shown that Microglena strains have a maximal growth temperature at or below 20°C, and most grow optimally below 15°C. Some psychrophilic strains are also halotolerant (Eddie et al., 2008).

Systematics and taxonomic revision of the genus Microglena

As shown in Fig. 1, the genus Chlamydomonas (in the traditional sense) is polyphyletic (see also Pröschold et al., 2001, and references therein). Pröschold & Silva (2007) proposed Chlamydomonas reinhardtii as the conserved type of Chlamydomonas; with the consequence that all species not closely related to Chlamydomonas reinhardtii have to be transferred to other or new genera. The taxonomic revision was initiated by Pröschold et al. (2001), who established two new genera, Oogamochlamys and Lobochlamys. In this study, we have demonstrated the monophyly of the Monadina-clade sensu Pröschold et al. (2001) and suggest that this group should be recognized as an independent genus. As discussed earlier (see Introduction), the name Microglena is available for any genus containing M. monadina, which is the green alga we describe here. As a consequence of our study, and according to the ICBN, we emend the diagnosis of the genus Microglena and propose 11 new combinations and two new species as below. The later-described species of Microglena summarized in Ettl (1978) are chrysophytes and need to be transferred to another genus.

**Microglena** Ehrenberg emend. Demchenko, Mikhailyuk & Pröschold.


**Emended description:** unicellular green alga with two equal flagella and a clockwise basal body orientation. Cells ellipsoidal to widely ellipsoidal or spherical; cell wall thick; papilla broad, trapezoidal or crown-like. Chloroplast cup-shaped, with or without a thick basal part; pyrenoid variable, from one wide ellipsoidal to elongated quarter-half-ring- or ring-shaped, or sausage-like body, to
fragmented into several round pyrenoids; pyrenoid surrounded by many small or several big starch grains oriented in parallel, located in the thick basal part or in lateral thickenings of the chloroplast in basal, medial or upper positions. Stigma bright, ellipsoidal, rod-like or fusiform (sometimes drop- or patch-like) and in an anterior to medial position. Two apical contractile vacuoles. Nucleus central or basal.

Asexual reproduction by sporulation into two or four zoospores, cell division with or without rotation of proplast by 90°. Akinetes spherical, with layered smooth cell wall, yellowish.

Sexual reproduction by homothallic advanced anisogamy. Macro- and microgametes covered by cell wall. During copulation microgametes attach to macrogametes by the papilla or laterally near the anterior part; both gametes lose their flagella during copulation. Mature zygotes with layered smooth or corrugated cell wall, yellowish.

**Type species:** *Microglena monadina* Ehrenberg *emend.* Demchenko, Mikhailyuk & Pröschold.

**Comments:** *Microglena* differs from other *Chlamydomonas*-like genera by its characteristic pyrenoid, which is surrounded by many or several starch grains oriented in parallel rows, and which varies from ellipsoidal to characteristic horseshoe- half-ring- to almost ring-shapes; its variably perforated cup-shaped chloroplast; its trapezoidal or crown-like papilla; and sexual reproduction by advanced anisogamy. Unique ultrastructural characters of *Microglena* are the structure of pyrenoid (single thylakoids or thylakoid pairs penetrate the pyrenoid body), stigma (a single thylakoid or thylakoid band lies between two layers of pigmented globules), and mitochondrion (located around the nucleus and not between cell wall and chloroplast: Rosowski & Hoshaw, 1988). According to the ICBN, the name of *Microglena* is the oldest of any applicable to monadoid green flagellates and will have priority if further studies show that other genera are closely related to *Microglena.*

*Microglena monadina* Ehrenberg *emend.*

Demchenko, Mikhailyuk & Pröschold

(Fig. 5a–d)


Asexual reproduction by sporulation into two or four zoospores, cell division with or without rotation of proplast by 90°. Akinetes spherical, with layered smooth cell wall, yellowish.

Sexual reproduction by advanced anisogamy: macrogametangium with a single macrogamete, morphologically similar like the vegetative cell; 8–16 microgametes formed in the microgametangium; microgametes 8.5–15.7 × 6.0–11.2 µm, drop-shaped, with cell wall and two flagella that are twice as long as the cell; during copulation microgametes attach to the macrogamete by the papilla or any part of the anterior; both gametes lose their flagella during copulation; young zygotes immobile, formed inside macrogametangium or sometimes released; mature zygotes with layered smooth cell wall, yellowish.

**ITS-2 DNA Barcode:** Barcode A in Fig. 3.

**Lectotype:** Ehrenberg (1832), pl. I: fig. 1, designated in Kusber et al. (2004).

**Epitype (designated here to support the lectotype specified above):** The strain SAG 55.72 (proposed here as the authentic strain of *M. monadina*) permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.

**Comments:** Kusber et al. (2004) designate an epitype (a sample of dried specimen II Polygastrica No. CXVIII: 1 in BHUPM, shown as fig. 6 in Kusber et al. [2004]). Their figure shows high similarity to our micrographs (Fig. 5a–d). Therefore we designate the cryopreserved strain SAG 55.72 as epitype, because this strain provides essential additional information to typify the species. The epitype strain generally corresponds to the descriptions of *C. monadina* in Ehrenberg (1832), Stein (1878) and Ettl (1983). According to these publications the cells are widely ellipsoidal to almost spherical, and 18–35 µm in diameter. The cells of SAG 55.72 are slightly smaller and more elongated.

The proposal to transfer *Chlamydomonas sphaerica* and *C. cingulata* to this species is based on the strong morphological similarity to *M. monadina*
and was previously proposed by Pascher (1927) and Ettl (1976, 1983). The pyrenoid shape and size of *C. sphaerica* and *C. cingulata* is variable (Troitskaya, 1923; Pascher, 1927), but we think that they are within the limits of morphological variability of *M. monadina*.

**Microglena braunii** (Goroschankin) Demchenko, Mikhailyuk & Pröschold, comb. nov.

(Fig. 5e–h)


**Synonym:** *Chlamydomonas monadina* Stein sensu Korshikov (1938): p. 75, fig. 28.

**Emended Description:** Cells 17–25 × 11–23 μm, ellipsoidal to widely ellipsoidal and almost spherical, with two flagella about as long as the cell. Papilla trapezoidal. Chloroplast of young cells cup-shaped with a thick basal part; in mature cells cup-shaped without a basal thickening. One horseshoe-like, half-ring-shaped or wide-ellipsoidal pyrenoid, in a near-basal lateral thickening of the chloroplast. Stigma bright, short rod-like or ellipsoidal, anterior to medial. Nucleus central.

Asexual reproduction by sporulation into two or four zoospores, cell division with or without rotation of protoplast by 90°.

Sexual reproduction by advanced anisogamy; macrogametangium with a single macrogamete, morphologically similar to vegetative cells; eight microgametes formed in the microgametangium; microgametes (7.6–) 8.3–14.4 (–15.7) × 5.0–12.2 μm, elongated oviform or drop-shaped, with a cell wall and two flagella twice as long as the cell; during copulation microgametes attach to the macrogamete at the papilla or near the anterior; both gametes lose their flagella during copulation; young zygotes immobile, formed inside macrogametangium or sometimes released; mature zygotes with a layered smooth cell wall, yellowish.

**ITS-2 DNA Barcode:** Barcode C in Fig. 3.

**Lectotype (designated here):** Goroschankin (1890), pl. 14, fig. 1.

**Epitype (designated in support of the lectotype designated here):** The strain SAG 50.86 (proposed here as the authentic strain of *M. braunii*) permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.

**Comments:** The epitype strain generally corresponds to the diagnosis of *C. braunii* (Goroschankin, 1890), with minor differences in cell shape and details of chloroplast structure. According to the original diagnosis, cells are widely ellipsoidal to almost spherical and the chloroplast is cup-shaped with a thick bottom in which the horseshoe-like pyrenoid is situated (Goroschankin, 1890). The cells of the epitype strain are slightly more elongated. The chloroplast of young cells is as indicated by Goroschankin, but with age its basal part becomes thinner and the elongated pyrenoid occupies a lateral thickening of the chloroplast in a near-basal position.

Korshikov used the diagnosis and figure of *C. braunii* (Goroschankin, 1890) for the description of *C. monadina* in his monograph (1938, p. 75, fig. 28). Therefore we consider *C. monadina* sensu Korshikov to be a synonym of *C. braunii*.

**Microglena coccifera** (Goroschankin) Demchenko, Mikhailyuk & Pröschold, comb. nov.

(Fig. 5i–l)


**Synonym:** *Chlamydomonas coccifera var. mesopyrenigera* Skuja (1949): p. 601, fig. 3.

**Emended Description:** Cells (16–) 22–24 (–27) × (13–) 19–23 (–27) μm, widely ellipsoidal, with two flagella about as long as the cell. Papilla trapezoidal. Chloroplast cup-shaped, without a thick basal part. Pyrenoids 1 to 4 or 5 (rarely 8) separated, wide-ellipsoidal to round, in lateral thickenings of the chloroplast, irregularly distributed. Stigma large, bright, elongated rod- or drop-like or fusiform, in an anterior to medial position. Nucleus central.

Asexual reproduction by sporulation into two or four zoospores, cell division with or without rotation of protoplast by 90°.

Sexual reproduction by advanced anisogamy; macrogametangium with a single macrogamete, morphologically similar to vegetative cells but larger (to 28–35 μm in diameter), without flagella; 16 microgametes formed in the microgametangium; microgametes 7.6–9.0 × 5.0–8.0 μm, spheroidal to oviform, with a cell wall and two flagella twice as long as the cell; during copulation microgametes attach to the macrogamete by the papilla or near the anterior; microgametes lose their flagella during copulation; young zygotes immobile, formed inside macrogamete cell wall or sometimes released; mature zygotes with a layered smooth cell wall, yellowish.

**ITS-2 DNA Barcode:** Barcode E in Fig. 3.

**Lectotype (designated here):** Goroschankin (1905), pl. 3, fig. 1.
Microglena charkoviensis (Korshikov) Demchenko, Mikhailyuk & Pröschold, comb. nov.

(Fig. 5m–p)


Emended description: Cells 10–16.5 μm, rotation of protoplast by 90°, usually two or four zoospores; cell division with or without rotation of protoplast by 90°.

Sexual reproduction not observed.

ITS-2 DNA barcode: Barcode D in Fig. 3.

Lectotype (designated here): Korshikov (1938), fig. 30b.

Epitope (designated here in support of the lectotype designated here): The strain ACKU 274-03 (proposed here as the authentic strain of *M. charkoviensis*) permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.

Comments: The epitope strain generally corresponds to the diagnosis of Goroschankin (1905), with minor differences in the number and shape of pyrenoids. Cells have 5–8 separated pyrenoids according to the original diagnosis. The epitope strain often has a half-ring-shaped pyrenoid, which fragments into 4 or 5 separate pyrenoids depending on the stage of cell ontogenesis.

We propose that *C. coccifera var. mesopyrenigera* is a synonym of *M. coccifera* because there are few morphological differences between them. *Chlamydomonas coccifera var. mesopyrenigera* differs from the type variety of *C. coccifera* mainly by the location of pyrenoids in the equatorial part of the chloroplast surrounding the nucleus (Skuja, 1949). However, these differences fall within the morphological variability of the epitope strain.

Microglena globulifera (Korshikov) Demchenko, Mikhailyuk & Pröschold, comb. nov.

(Fig. 6a–d)


Asexual reproduction by sporulation, producing two or four zoospores; cell division with or without rotation of protoplast by 90°.

Sexual reproduction not observed.

ITS-2 DNA barcode: Barcode F in Fig. 3.

Lectotype (designated here): Korshikov (1938), fig. 31.
Revision of Microglena

Epitype (designated here in support of the lectotype designated here): The strain CCAC 0017 (proposed here as the authentic strain of *M. globulifera*) permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.

Comments: The epitype strain generally corresponds to the original diagnosis of *C. monadina var. globulifera* (Korshikov, 1938) and differs by only small differences in the number and shape of the pyrenoids, as well as details of chloroplast structure. Cells have up to four separated pyrenoids according to Korshikov. However, the epitype strain often has one half-ring-shaped pyrenoid, which is fragmented into 3 or 4 separate pyrenoids, depending on the stage of cell ontogenesis. The chloroplast of the epitype strain has small irregular perforations, whereas this characteristic was not mentioned, but illustrated by Korshikov (1938). This variety of *C. monadina* was previously published by Pascher (1927) as a variety of his newly described species *C. cingulata*. However, no description was provided in Pascher (1927) and this variety is therefore invalid. Korshikov (1938) described the variety as *C. monadina var. globulifera* without mentioning that his figure (fig. 31) had previously been published by Pascher (1927, fig. 230b, b).

*Microglena indica* (Iyengar in Iyengar & Desikachary) Demchenko, Mikhailyuk & Pröschold, comb. nov.  
(Fig. 6e–h)


Asexual reproduction by sporulation, with formation of two or four zoospores; cell division without rotation of protoplast by 90°.

Sexual reproduction not observed.

ITS-2 DNA Barcode: Barcode G in Fig. 3.


Epitype (designated here in support of the lectotype designated here): The strain SAG 46.96 (proposed here as the authentic strain of *M. indica*) permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.

Comments: The epitype strain generally corresponds to the original diagnosis of *C. monadina var. indica* (Iyengar & Desikachary, 1981), with small differences in cell size and papilla shape. According to the diagnosis, cells are 15×13–14 μm and have a thin blunt papilla. Cells of the epitype strain are slightly bigger and the papilla is wider.

We propose that *C. monadina var. cingulata* is synonymous because of the essential morphological similarity to *M. indica* (Korshikov, 1938); *C. cingulata* Pascher (Pascher, 1927; Korshikov, 1938), however, is a synonym of *M. monadina*.
COMMENTS: The epitype strain generally corresponds to the original diagnosis of *C. nova* (Skuja, 1956) and exhibits only small differences in cell size, pyrenoid shape and details of chloroplast structure. According to the original diagnosis, cells are 20–30 × 12–20 μm and have a spherical pyrenoid. Cells of the epitype strain are shorter; the pyrenoid varies from spherical to ellipsoidal and half horseshoe-like; and the chloroplast is dissected into lobes.

We have given a new name for this taxon because Skuja’s (1956) name ‘*Chlamydomonas nova*’ is invalid, the name having been used previously by Sörensen (1948). We do not agree with Ettl’s (1983) suggestion that *C. nova* is a synonym of *C. anulata* (Nygaard, 1949). The latter species is characterized by a completely different position of nucleus (basal) and, as discussed above, we think that *C. anulata* represents another species of *Microglena*.

*Microglena opisthopyren* (Skuja) Demchenko, Mikhail'yu k & Pröschold, *comb. nov.*

(Fig. 6m–p)


**Emended description:** Cells 10–20 × (8)11–17 μm, ellipsoidal to widely ellipsoidal, with two flagella about as long as the cell. Papilla trapezoidal. Chloroplast of young cells cup-shaped with a thick basal part, in mature cells cup-shaped but without a basal thickening. Pyrenoid single, widely ellipsoidal, horseshoe-like, or half-ring-shaped, in a near-basal lateral thickening of the chloroplast. Stigma bright, elongated rod-like, in an anterior to medial position. Nucleus central.

Asexual reproduction by sporulation, producing two or four zoospores; cell division with or without rotation of protoplasm by 90°.

Sexual reproduction not observed.

**ITS-2 DNA Barcode:** Barcode J in Fig. 3.

**Lectotype (designated here):** Skuja (1956), pl. 18, fig. 26.

**Epitype (designated here in support of the lectotype designated here):** The strain SAG 8.87 (proposed here as the authentic strain of *M. opisthopyren*) permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.

COMMENTS: The epitype strain generally corresponds to the original diagnosis of *C. opisthopyren* (Skuja, 1956) and exhibits only small differences in cell size and the shapes of the papilla and pyrenoid. According to Skuja, the cells are 12–26 × 9–21 μm and have a wide semicircular papilla and one or two spherical pyrenoids in a lateral near-basal thickening of the chloroplast. However, one of Skuja’s figures shows an elongated dumb-bell-shaped pyrenoid (Skuja, 1956, fig. 28). The epitype strain has smaller cells, a trapezoidal papilla, and an ellipsoidal to horseshoe-like pyrenoid.

*Microglena longirubra* (Ettl) Demchenko, Mikhail'yu k & Pröschold, *comb. nov.*

(Fig. 7e–i)

**Basionym:** *Chlamydomonas monadina* var. *longirubra* Ettl (1976). *Nova Hedwigia*, Beih. 49: 442, pl. 77, fig. 1.

**Synonym:** *Chlamydomonas monadina* var. *longistigma* Ettl (1976): p. 928 (invalid name).

**Emended description:** Cells (16–) 20–26 × (10–) 17–20 μm, ellipsoidal to widely ellipsoidal, with two flagella about as long as the cell. Papilla trapezoidal. Chloroplast cup-shaped without a thickened basal part. Pyrenoid single, half-ring-shaped, in a lateral medial thickening of the chloroplast. Stigma bright, large, elongated rod-like or fusiform, sometimes curved, in an anterior to medial position, a third as long as the cell. Nucleus central.

Asexual reproduction by sporulation, producing two or four zoospores; cell division with or without rotation of protoplasm by 90°.

Sexual reproduction by advanced anisogamy; macrogametangium with a single macrogamete morphologically similar to vegetative cells, with flagella; 16 microgametes formed in the microgametangium; microgametes small, 15.0 × 10.0 μm, ellipsoidal to oviform, with two flagella as long as the cell; during copulation, microgametes attach to the macrogamete near the anterior; both gametes lose their flagella during copulation; young zygotes immobile, formed inside the macrogamete cell wall or sometimes released; mature zygotes with a layered smooth cell wall, yellowish.

**ITS-2 DNA Barcode:** Barcode I in Fig. 3.

**Lectotype (designated here):** Ettl (1976), pl. 77, fig. 1.

**Epitype (designated here in support of the lectotype designated here):** The strain SAG 5.92 (proposed here as the authentic strain of *M. longirubra*) permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.
COMMENTS: The epitype strain agrees completely with the original diagnosis of *C. monadina var. longirubra* (Ettl, 1976).

**Microglena uva-maris** (Butcher) Demchenko, Mikhailyuk & Pröschold, *comb. nov.*  
(Fig. 7a–d).


**Emended Description:** Cells 8–17 × 6–13 μm, ellipsoidal to widely ellipsoidal, with two flagella about as long as the cell. Papilla trapezoidal. Chloroplast cup-shaped with a thick basal part. Pyrenoid single, widely ellipsoidal, located in the basal part of the chloroplast. Stigma bright, ellipsoidal to elongate-ellipsoidal and drop-like, in an anterior to medial position. Two apical contractile vacuoles, which do not pulse in seawater. Nucleus central.

Asexual reproduction by sporulation, producing two or four zoospores; cell division with or without rotation of protoplast by 90°. Sexual reproduction not observed.

**ITS-2 DNA Barcode:** Barcode M in Fig. 3.

**Lectotype (designated here):** Butcher (1959), pl. 9, fig. 2.

**Epitype (designated here in support of the lectotype designated here):** The strain SAG 19.89 (proposed here as the authentic strain of *M. reginae*) permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.

**Comment:** The SAG 17.89 strain agrees in morphology and reproduction with the original description of Ettl and Green (1973) and no emendations are necessary.

**Microglena reginae** (Ettl & J.C. Green) Demchenko, Mikhailyuk & Pröschold, *sp. nov.*  
(Fig. 7j–m)

**Description:** Cells (12–) 17–23 × (9–) 13–20 μm, ellipsoidal to widely ellipsoidal, with two flagella about as long as the cell. Papilla trapezoidal. Chloroplast cup-shaped without a thick basal part, dissected into several (4–6) lobes. Pyrenoid single, half-ring-shaped, located in a lateral medial thickening of the chloroplast. Stigma small, rod-like, in an anterior to medial position. Nucleus central.

Asexual reproduction by sporulation producing two or four zoospores; cell division with or without rotation of protoplast by 90°. Sexual reproduction not observed.

**ITS-2 DNA Barcode:** Barcode K in Fig. 3.

**Lectotype (designated here):** Ettl and Green (1973), text-fig. 1A.

**Epitype (designated here in support of the lectotype designated here):** The strain SAG 17.89 permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.

**Comment:** The SAG 17.89 strain agrees in morphology and reproduction with the original description of Ettl and Green (1973) and no emendations are necessary.

**Microglena lobata** Demchenko, Mikhailyuk & Pröschold, *sp. nov.*  
(Fig. 7j–m)

**Description:** Cells 15–22 × 12–20 μm, ellipsoidal to widely ellipsoidal, with two flagella about as long as the cell. Papilla trapezoidal. Chloroplast cup-shaped with a thick basal part, dissected into several (4–6) lobes. Pyrenoid single, half-ring-shaped, located in a lateral medial thickening of the chloroplast. Stigma small, rod-like, in an anterior to medial position. Nucleus central.

Asexual reproduction by sporulation producing two or four zoospores; cell division with or without rotation of protoplast by 90°. Sexual reproduction not observed.

**ITS-2 DNA Barcode:** Barcode H in Fig. 3.

**Lectotype:** The strain SAG 31.72 permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.

**Iconotype:** Fig. 7j–m.

**Type Locality:** collected by R.C. Starr, 1953, from Yellowwood fish ponds, Bloomington, IN, USA.

**Etymology:** This taxon is named after the characteristically lobed chloroplast.

**Authentic Culture:** SAG 31.72.

**Comment:** This species is the most similar to *M. monadina*, but characterized by its thinner long pyrenoid and deeply dissected chloroplast, with several distinct lobes.

**Microglena basinucleata** Demchenko, Mikhailyuk & Pröschold, *sp. nov.*  
(Fig. 7n–q)

**Description:** cells 15–22 × 12–20 μm, ellipsoidal to widely ellipsoidal, with two flagella about as long as the cell. Papilla trapezoidal. Chloroplast cup-shaped with a thick basal part, dissected into several (4–6) lobes. Pyrenoid single, half-ring-shaped, located in a lateral medial thickening of the chloroplast. Stigma small, rod-like, in an anterior to medial position. Nucleus central.

Asexual reproduction by sporulation producing two or four zoospores; cell division with or without rotation of protoplast by 90°. Sexual reproduction not observed.

**ITS-2 DNA Barcode:** Barcode I in Fig. 3.

**Lectotype (designated here):** Ettl and Green (1973), text-fig. 1A.

**Epitype (designated here in support of the lectotype designated here):** The strain SAG 17.89 (proposed here as the authentic strain of *M. reginae*) permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.

**Comment:** The SAG 17.89 strain agrees in morphology and reproduction with the original description of Ettl and Green (1973) and no emendations are necessary.
as the cell. Chloroplast cup-shaped without a thick basal part, dissected into several (4 or 5) lobes. Pyrenoid single, half-ring-shaped, naked and almost invisible, located in a lateral upper thickening of the chloroplast. Stigma bright, elongate-ellipsoidal, in an anterior to medial position. Nucleus posterior.

Asexual reproduction by sporulation, producing two or four zoospores; cell division with or without rotation of protoplast by 90°.

Sexual reproduction not observed.

ITS-2 DNA Barcode: Barcode B in Fig. 3.

Holotype: The strain SAG 67.72 permanently preserved in a metabolically inactive state (cryopreserved in liquid nitrogen) in the Sammlung von Algenkulturen, University of Göttingen, Germany.

Iconotype: Fig. 7n–q.

Type Locality: collected by W. Koch, 1958, from a pond in the Old Botanical Garden of the University, Göttingen, Germany.

Etymology: This taxon is named after its characteristically posterior nucleus.

Authentic Culture: SAG 67.72.

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Supplementary material

The following supplementary material is available for this article, accessible via the Supplementary Content tab on the article’s online page at http://dx.doi.org/10.1080/09670262.2012.678388.

Supplementary Table S1. Strains used in this study and their origins.

Supplementary Table S2. Comprehensive CBC-analysis among the species of Microglena.

Supplementary Figure S1. ITS-1 and ITS-2 secondary structures of the Microglena species.

Supplementary Figure S2. Molecular phylogeny of Microglena based on SSU and ITS rDNA sequence comparisons.


