A case of taxonomic inflation in coccoid algae: *Ellipsoidion parvum* and *Neocystis vischeri* are conspecific with *Neocystis* (=*Nephrodiella*) *brevis* (Chlorophyta, Trebouxiophyceae)

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Abstract

Determining the actual phylogenetic diversity of unicellular coccoid algae, and developing a biologically appropriate classification system for them, pose challenges. We studied the phylogenetic and morphological diversity of a series of algal strains identified as *Nephrodiella* spp., *Ellipsoidion parvum*, and *Neocystis* spp. By determining sequences of the 18S-ITS1-5.8S-ITS2 rDNA region we showed that the strains studied represent just two genotypes; differences in the ITS2 region of the two genotypes, including one compensatory base change (CBC) in the helix II, suggested that they may be considered as two separate species. We then employed geometric morphometrics to evaluate the extent of morphological differences among the strains. This analysis revealed that the degree of morphological variability is higher among strains of the same genotype than between the genotypes. Our results thus suggest that all the strains represent only two closely related cryptic species of trebouxiophycean algae, which may be identified as *Neocystis brevis* and *Neocystis mucosa*. Two previously described species, *Ellipsoidion parvum*, originally described as a heterokont alga, and *Neocystis vischeri*, are revealed as junior synonyms of *N. brevis*. We discuss our findings as a case of taxonomic inflation that may be a general, yet somewhat neglected, aspect of the current taxonomy of unicellular algae.

Key words: Heterokonten, morphological plasticity, phylogenetic diversity, Xanthophyceae

Introduction

The advent of molecular taxonomy has revealed that the classification of eukaryotic microbes as developed during the era of traditional (morphology-centred) systematics grossly underestimated the real diversity existing in nature (Adl *et al.* 2007, 2012, Medlin *et al.* 2007, Epstein & López-García 2008). The poor reflection of actual microbial diversity is mainly caused by the paucity of discriminating morphological characters, which may result in polyphyletic taxa lumping together distantly related organisms. Consequently, traditional species concepts often include multiple cryptic or semicryptic species. These problems can now be reassessed with the aid of appropriately variable genetic markers, such as the internal transcribed spacer 2 (ITS2) in the rRNA operon, the plastid *rbc*L gene, or the mitochondrial *cox1* gene, although the question of actual species delimitation often persists due to the ambiguous nature of species concepts (Fenchel & Finlay 2006, Medlin *et al.* 2007, Coleman 2009, Edwards 2009, Boenigk *et al.* 2012). The traditional genus *Chlorella* is a prototypical case of a failure to distinguish the actual extent of phylogenetic diversity behind a single morphotype, as the use of molecular markers, primarily 18S rDNA and the ITS2 region, has led to its separation into several genera, often very distantly related and collectively comprising many more species than distinguished before (Huss *et al.* 1999, Luo *et al.* 2010, Bock *et al.* 2011, Fučíková & Lewis 2012).

Much less attention has been paid to an opposite form of disparity between traditional taxonomic schemes and real protist diversity, i.e. taxonomic inflation. The most trivial cases include independent description of the same species by different authorities due to ignorance, or difficulties with identification of taxa resulting from poor or inadequate description by the original author. However, the varying extent of intraspecific morphological plasticity stemming from slightly different genetic or epigenetic constitutions of individuals or clones within the species may be a more important source of taxonomic inflation in protists. To properly explore such possible cases, molecular characterization must be combined with rigorous assessment of the morphological variability of the organisms studied, which can be achieved by employing various methods, such as geometric morphometrics (e.g. McManus *et al.* 2011, Nemjová *et al.* 2011).

Here we combine a geometric morphometric approach with DNA sequencing to clarify the taxonomy of several species of coccoid algae originally placed in "Heterokonten" as conceived by Adolf Pascher (largely corresponding to the modern class Xanthophyceae within heterokont algae). *Nephrodiella brevis* Vischer (1945: 489) was described as a new species of the genus *Nephrodiella* Pascher (1937–1939: 428). However, analyses of pigment composition of the authentic strain of *N. brevis* revealed the presence of chlorophyll *b*, suggesting that this species is actually a green alga rather than a xanthophycean (Šesták 1963, Whittle & Casselton 1969). Later, this species was transferred into Chlorophyta, specifically into the genus *Coccomyxa* Schmidle (1901: 23) as a new combination *Coccomyxa brevis* (Vischer) Gärtner & Schragl (1988: 513), but the strain studied by Gärtner & Schragl (1988), although also isolated by Vischer and determined by him as *N. brevis*, was not the authentic strain of the species. Reinvestigation of both strains suggested that they represent two distinct, albeit related, species; they were transferred into the green algal genus *Neocystis* Hindák (1988: 65) as a new combination *Neocystis brevis* (Vischer) Kostikov & Hoffmann (2002: 10) and as a new species *Neocystis vischeri* Kostikov & Hoffmann (2002: 14). All these revisions relied solely on pigment data and morphological features without employing molecular characters. Most recently, based on a phylogenetic analysis of 18S rDNA sequences, *N. brevis* and a new species *Neocystis mucosa* Krienitz, C. Bock, Nozaki & Wolf in Krienitz *et al.* (2011a: 887) were shown to occupy a deep lineage within the green algal class Trebouxiophyceae (Krienitz *et al.* 2011a).

The genus *Nephrodiella* has been classified in the family Pleurochloridaceae (order Mischococcales in the class Xanthophyceae; Ettl 1978), which has been demonstrated as polyphyletic with many of its former members now placed elsewhere (Hibberd 1981, Darienko *et al.* 2010, Přibyl *et al.* 2012). Algae morphologically similar to some *Nephrodiella* species are also included in the genus *Ellipsoidion* Pascher (1937–1939: 326, 407, 408), likewise classified in Pleurochloridaceae. No species of the latter genus has been characterised by molecular methods and its real identity and affinity remain obscure. Here we provide 18S rDNA and ITS sequences of the species *Ellipsoidion parvum* Reisigl (1964: 441) and of *N. vischeri* and demonstrate that these two species cannot be distinguished from *N. brevis*. We also show that some other strains maintained in public culture collections under the labels *Nephrodiella minor* Pascher (1937–1939: 431) or *Nephrodiella* sp. are conspecific with either *N. brevis* or *N. mucosa* (Krienitz *et al.* 2011a). Finally, we have employed a recently developed geometric morphometric analysis based on a combination of fixed and sliding landmarks placed along the outline of individual cells (Neustupa 2005, Neustupa & Nemjová 2008) to rigorously assess the morphological variability of the algal strains subject to molecular characterization.

Material & methods

Algal culturing and microscopic observations

Seven algal strains were obtained from public culture collections (Table 1). *N. mucosa* KR 1989/14 (= CCAP 204/1) was kindly provided by Lothar Krienitz (Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Stechlin-Neuglobsow). The strains were cultivated on BBM agar medium (Bischoff & Bold 1963) at 15°C with continuous illumination of 20 µmol m^{-2} s^{-1} provided by 18W cool fluorescent tubes (Philips TLD 18W/33). The cultures were observed after 30 days of cultivation using an Olympus BX51 light microscope (Olympus Corporation, Tokyo, Japan) with differential contrast and Olympus Z5060 microphotographic equipment.

TABLE 1. Attributes of algal strains investigated in this study.

Strain	Origin[*]	Original identification[*]	Taxonomic identity as redefined in this study	18S-ITS-5.8S-ITS2 rDNA sequence (GenBank accession number)
CAUP D 802	Vischer, 1941/267. Soil. Switzerland, Unterengadin.	*Nephrodiella brevis* (Vischer, authentic strain)	*Neocystis brevis*	JQ920360
CCALA 393	Vischer, 1941/267. Acid humus layer in soil near Fuorn. Switzerland, Unterengadin.	*Nephrodiella minor* (*CCALA* staff?)	*Neocystis brevis*	JQ920362
ASIB BS 319	Vischer, 1941/319. Material unknown. Switzerland, Unterengadin .	*Neocystis vischeri* (Kostikov and Hoffmann, authentic strain)	*Neocystis brevis*	JQ920361
SAG 40.86	Reisigl (about 1960). Soil from Kreuzspitze, 3457 m. Austria, Ötztaler Alpen.	*Ellipsoidion parvum* (Reisigl, authentic strain)	*Neocystis brevis*	JQ920364
CCALA 341	Vischer, 1940/241. Material and locality unknown.	*Ellipsoidion parvum* (*CCALA* staff?)	*Neocystis brevis*	JQ920363
KR 1989/14	Krientz, 1989/14. Material unknown. Germany, Lake Feldberger Haussee.	*Neocystis mucosa* (Krienitz *et al.*, authentic strain)	*Neocystis mucosa*	JQ920365
CAUP D 801	Flint, 1964/3. Soil. Antarctica, probably from theMcMurdo Sound region.	*Nephrodiella* sp.	*Neocystis mucosa*	JQ920366
SAG 40.88	Broady, ca. 1973. Material unknown. Antarctica, Signy Island.	*Ellipsoidion* sp. (Broady), *Nephrodiella minor* (*SAG* staff?)	*Neocystis mucosa*	JQ920367

[*]Information on the origin of the strains and their original identification comes from the literature (ASIB BS 319—Kostikov & Hoffmann 2002; KR 1989/14—Krienitz *et al.* 2011a), from personal communication with P.A. Broady, and from strain-specific web pages at the Internet sites of respective culture collections: *CAUP*—http://botany.natur.cuni.cz/algo/caup.html, *CCALA*—http://www.butbn.cas.cz/ccala/index.php, *SAG*—http://www.epsag.uni-goettingen.de.

DNA isolation, PCR and sequencing

DNA extraction, PCR reactions and sequencing essentially followed Přibyl *et al.* (2012). Sequences of the 18S rDNA-ITS1-5.8S rDNA-ITS2 region were obtained via amplification of two overlapping segments. The first segment was amplified using the forward (F) primer of Katana *et al.* (2001) or the forward 34F primer (Pažoutová *et al.* 2010) in combination with the reverse (R) primer of Katana *et al.* (2001) or the reverse 1650Rvivi primer (Kipp 2004). Sequencing of the first segment was done using the amplification primers and the sequencing primers according to Katana *et al.* (2001). The second segment was amplified using the green algal-specific forward primer 1500af (Helms *et al.* 2001) in combination with the reverse primer ITS4 (White *et al.* 1990); it was sequenced using the amplification primers plus the ITS1 (White *et al.* 1990) primer. Sequences were assembled and edited using SeqAssem (version 09/2004; Hepperle 2004). GenBank accession numbers of the newly obtained sequences are provided in Table 1.

Modelling the secondary structure of the ITS2 region
The secondary structures of the ITS2 rRNA regions were initially modelled using the mfold computer program (version 2.3; Zuker 2003) on a minimum free energy basis, with folding temperature set to 25°C. The final secondary structures were obtained by homology modelling (Selig *et al.* 2008) in the ITS2 Database v.3.0.5 (Koetschan *et al.* 2010). The predicted ITS2 structure was drawn using VARNA (Darty *et al.* 2009) and used to identify compensatory base changes (CBCs).

Morphometric analyses
In total, twelve landmarks spanning the outline of individual cells were analysed by means of morphometric methods. Thirty randomly chosen cells of each strain were digitised for the morphometric analysis. Landmarks at the poles of cells were in fixed positions, whereas the other ten points were semi-landmarks, i.e. they were allowed to slide along the cell outlines (Bookstein 1997, Zelditch *et al.* 2004). Morphometric analyses were conducted using the TPS software package for geometric morphometrics (Rohlf 2011). Subsequent statistical analyses were done in PAST, ver. 2.10 (Hammer *et al.* 2001). Individual cells were superimposed using generalised Procrustes analysis (Zelditch *et al.* 2004). Cell size was evaluated using the centroid size measure, i.e. by the square root of the sum of squared distances from the landmarks to their centroid (Zelditch *et al.* 2004). The centroid size acquired from landmarks placed along cell outlines was recently shown to be closely correlated with the traditional linear measurements of oval-shaped green coccoid algae (Neustupa & Nemjová 2008). The relation of cell shape and size was evaluated by multivariate regression of geometric morphometric data on the centroid size measure in TpsRegr, ver. 1.37. Similarly, TpsRegr was used for multivariate regression analyses separately evaluating effects of strain and species affiliation of individual cells on their shape. Significance of the regression models was assessed by the permutation tests on Wilk's λ with 9999 randomizations. A principal component analysis (PCA) of the geometric morphometric data was performed in TpsRelw, ver. 1.49, and the theoretical cell shapes of different morpho-space positions were reconstructed in TpsSuper, ver. 1.14. Shape differences between all pairs of strains were evaluated using two-group permutation tests on the Mahalanobis distance with 999 permutations, whereas the differences in cell size between pairs of individual strains were evaluated using permutation *t*-tests (9999 permutations).

Results

Molecular diversity of *Neocystis* spp., *Nephrodiella* spp. and *Ellipsoidion parvum* strains
We investigated eight algal cultures that are thought to represent seven independently isolated strains (Table 1). In order to assess the genetic diversity of these strains, we sequenced the 18S-ITS2-5.8S-ITS2 rDNA region of all strains. Differences were found from previously determined 18S rDNA sequences for strains CAUP D 802 (*N. brevis*) and KR 1989/14 (*N. mucosa*) (Krienitz *et al.* 2011a; GenBank accession numbers HQ287929.1 and HM565928.1, respectively). HQ287929.1 has a deletion of one nucleotide close to the 5' end and a single nucleotide substitution close to the 3' end. HM565928.1 (erroneously maintained in GenBank with the species name "*Kirchneriella phaseoliformis*") differs from our sequence by several single-nucleotide indels and by several undetermined nucleotides (specified by "N"). We checked our raw sequencing data and confirmed that our assembled 18S rDNA sequences are correct, suggesting that the previously published sequences most likely suffer from sequencing errors (particularly HM565928.1 appears to be of rather low quality). Despite these differences, the phylogenetic position of the two updated 18S rDNA sequences remained essentially the same as reported by Krienitz *et al.* (2011a), i.e. they formed (together with a sequence from the "Uncultured Dunaliellaceae clone Amb_18S_930", GenBank accession number EF023670.1) a tight cluster in an unresolved position within the radiation of the Trebouxiophyceae (not shown).

According to the 18S-ITS2-5.8S-ITS2 rDNA sequences, the eight strains could be partitioned into two distinctly different genotypes (Table 1, column "Taxonomic identity as redefined in this study"). The first

genotype, characterised by identical sequences without apparent intragenomic heterogeneity, included the authentic strain of *N. brevis* (CAUP D 802) plus the following strains: CCALA 393 (*Nephrodiella minor*), ASIB BS 319 (*Neocystis vischeri*), SAG 40.86 (*Ellipsoidion parvum*), and CCALA 341 (*Ellipsoidion parvum*). The second genotype comprised the authentic strain of *N. mucosa* (KR 1989/14) plus the strains CAUP D 801 (*Nephrodiella* sp.) and SAG 40.88 (*Nephrodiella minor*). Two of the strains, KR 1989/14 and CAUP D 801, exhibit an apparent intragenomic heterogeneity in the 18S-ITS2-5.8S-ITS2 rDNA region, sharing one A/G site, one G/T site, and three C/T sites, whereas SAG 40.88 has just one of the two alternative nucleotides at the homologous positions. KR 1989/14 has three additional unique C/T sites (occupied by C in both CAUP D 801 and SAG 40.88 strains) and CAUP D 801 has one more unique C/T site (occupied by T in both KR 1989/14 and SAG 40.88 strains). All three strains of the second genotype thus exhibit effectively identical 18S-ITS2-5.8S-ITS2 rDNA sequences. When the heterogeneity is not taken into account, the sequences of the first and the second genotype differ by one substitution in the 18S rDNA region, 15 substitutions and a one-nucleotide indel in the ITS1 region, and 13 substitutions and 2 two-nucleotide indels in the ITS2 region (while the 5.8S rDNA regions are identical).

To further evaluate the degree of genetic separation of the two genotypes, we mapped the differences in the ITS2 sequence on a predicted ITS2 secondary structure (Fig. 1). Most of the differences were located in terminal loops or in unpaired ("bubble") regions within the helices, with the exception of a difference in two opposite nucleotides in the helix I implying a mismatch in the first genotype, and except a single compensatory base change (CBC) in helix II.
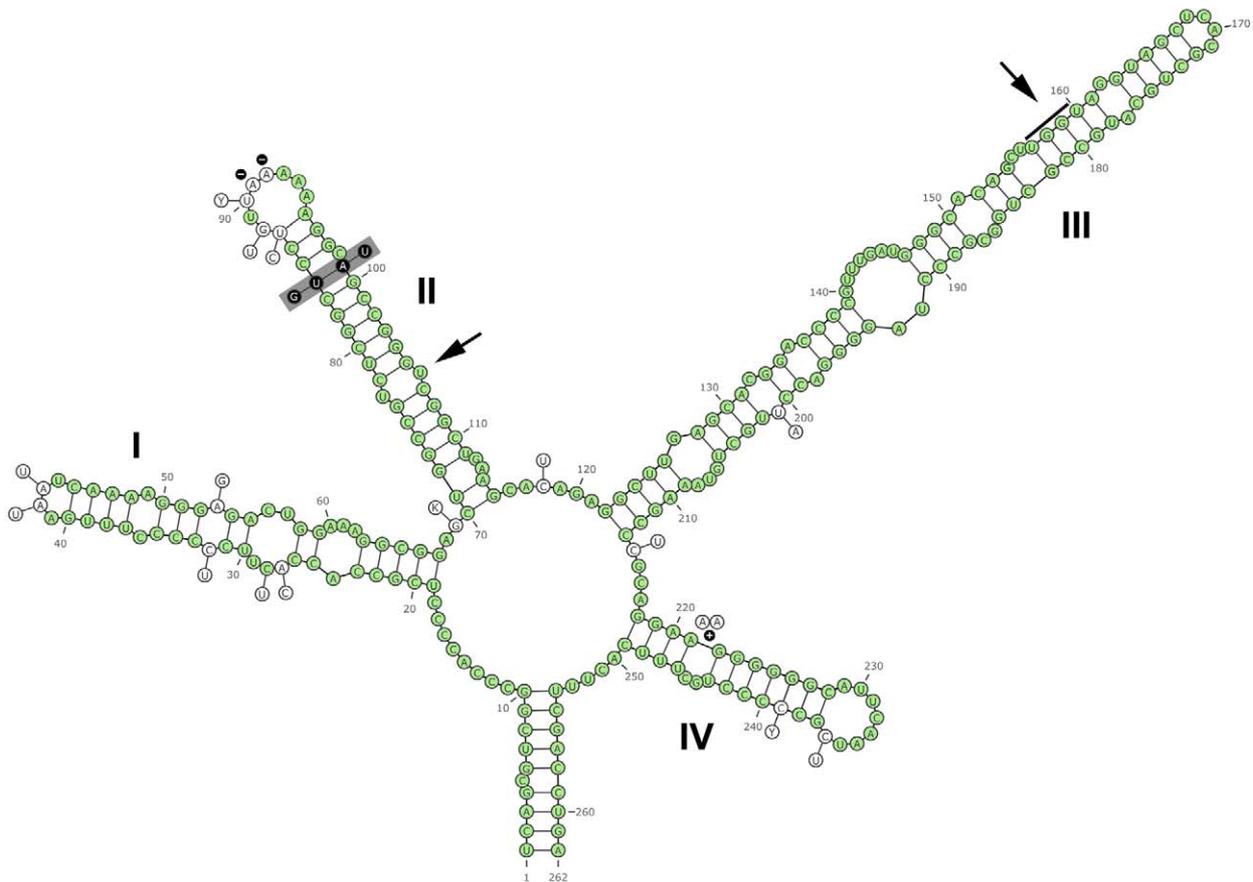
FIGURE 1: Comparison of the ITS2 sequences and predicted secondary structures of *Neocystis brevis* and *Neocystis mucosa*. Base numbering is indicated every 10 bases, and the four helices are numbered with Roman numerals. The structure shown corresponds to *N. brevis*; positions conserved in *N. mucosa* are portrayed in green, bases substituted in *N. mucosa* are shown by the structure and connected to the respective position by a short line, insertions and deletions are indicated with plus and minus symbols, respectively. The base pair marked in a grey box is a compensatory base change (CBC). The highly conserved U–U mismatch in the helix II and UGGU motif in the helix III (Schultz *et al.* 2005) are marked by arrows.
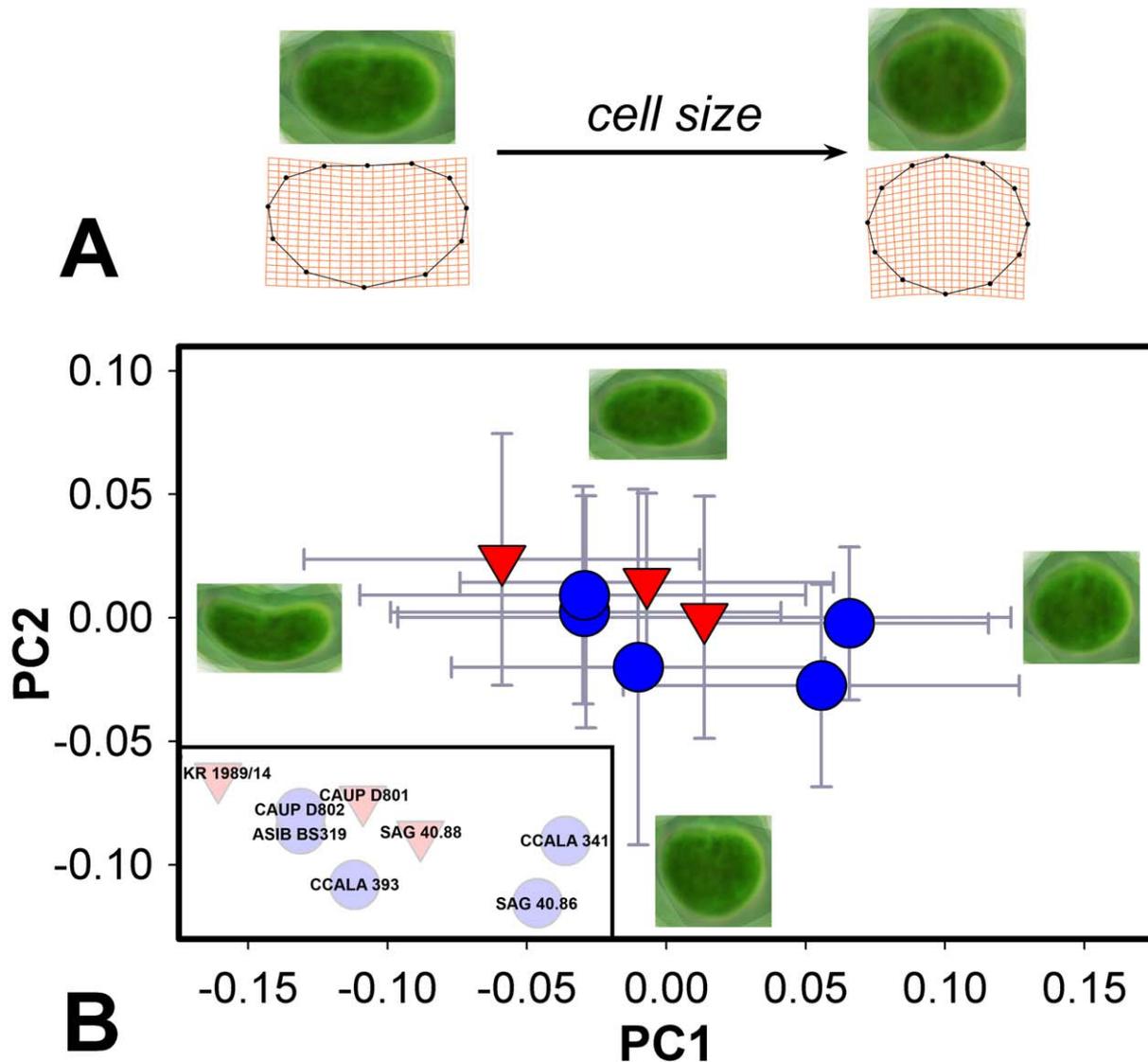
FIGURE 2: Morphometric characteristics of *Neocystis* strains. a) Visualisation of the multivariate regression model illustrating the relation of the shape and size of cells in the entire investigated dataset. b) The PCA ordination plot based on geometric morphometric data illustrating mean positions of individual strains and their standard deviations on PC1 (spanning 65.9% of the variation) and PC2 (23.2%). The theoretical cell shapes of marginal morphospace positions were reconstructed from the landmark coordinates of the original data.

Morphometric and morphological reassessment of the strains

The strains investigated shared a common ontogenetic pattern of asexual reproduction via autospores. Mature vegetative cells, embedded in amorphous mucilage, transformed into autosporangia. Typically, there were 4–16 autospores in individual sporangia (Fig. 3). They were 5.5–9.5 µm in length and 3.0–5.0 µm in width. The cell walls of mature autosporangia ruptured and their remnants then gradually dissolved to form mucilage surrounding the freshly released autospores. Mature cells were mostly about 9.5–15.0 µm in diameter (Fig. 3). Significant differences in cell size among individual strains were observed (Fig. 4, Table 2), but they did not correlate with the assignment of the strain into the two separate genotypes. The cells of all strains uniformly possessed a single plastid, which was multiplied before autospore formation. A pyrenoid was not observed in any of the strains.

In parallel to the size change during the life cycle, the shape of the cells also depended on the ontogenetic stage of the cells. This allometric change in the shape of the cells related to their growth was strongly significant (Wilk's $\lambda = 0.7$, $p = 0.0001$, 10.9% of the total variation explained by the regression model of the entire dataset). According to this model the autospores freshly released from maternal autosporangia were

mostly elongated and bean-shaped, whereas mature cells had almost circular outlines with only slight shape heteropolarity (Fig. 2). This allometric trend was highly significant and it was almost identical in all investigated strains (data not shown).

The allometric morphological trend was apparently closely related to the shape change spanned by the first principal component that described 65.9% of the total variation (Fig. 2). Clonal variability in cell shape within individual strains accounted for most of the total morphological variation. The multivariate regression model of shape variability explained by strain affiliation was highly significant (Wilk's $\lambda = 0.28$, $p = 0.0001$) and it spanned 18.3% of the total variation. On the other hand, shape variation was clearly less related to genotype affiliation (Wilk's $\lambda = 0.84$, $p = 0.0068$) and this multivariate regression model only accounted for 2.8% of cell shape variation. Hence, significant differences in cell shape were observed among many pairs of strains (Table 2). However, these pair differences mostly did not correlate with separation into the two genotypes. The most pronounced cross-strain morphological differences were identified between strains CCALA 393 and SAG 40.86, and between CAUP D 802 and SAG 40.86 (Table 2), which belong to the same genotype.
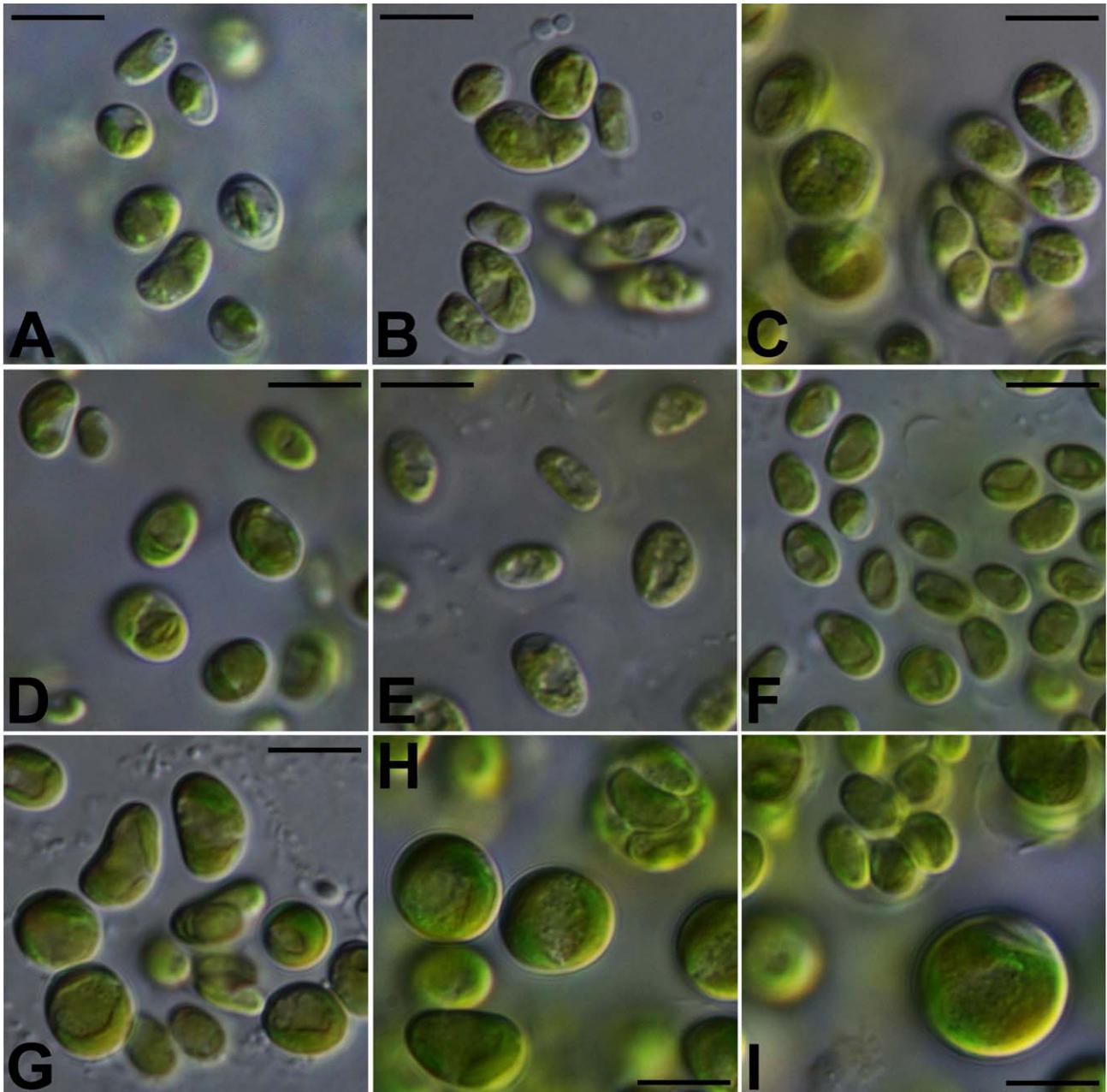
FIGURE 3: Representative images of *Neocystis mucosa* (A–C) and *Neocystis brevis* (D–I) strains. A: CAUP D 801. B: KR 1989/14. C: SAG 40.88. D: CAUP D 802. E: ASIB BS 319. F: CCALA 393. G: CALA 341. H, I: SAG 40.86. Scale bar = 10 μm.
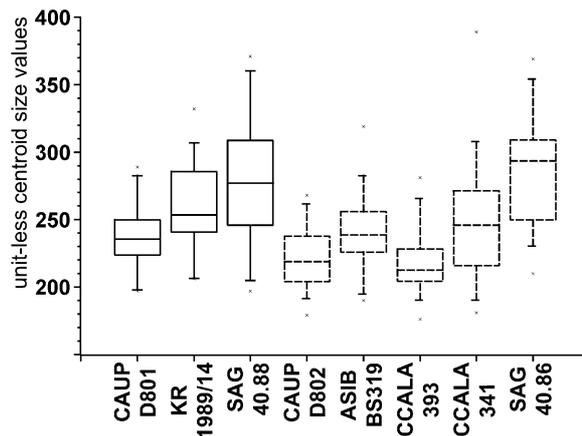
FIGURE 4: Unit-less centroid size values of strains of *Neocystis* spp. Boxplots with a full outline represent strains of *N. mucosa*. Boxplots with a dashed outline represent strains of *N. brevis*.

TABLE 2. The results of statistical tests on differentiation between strains. Size: the permutation *t*-tests on centroid size difference (CS); differences in mean centroid size values are depicted. Shape: the two-group multivariate permutation tests on the difference in the cell shape; Mahalanobis distances between groups are depicted. *****:** p value < 0.001, ****:** p value between 0.001 and 0.01, ***:** p value between 0.01 and 0.05, - : not significant.

	KR 1989/14	SAG 40.88	CAUP D 802	ASIB BS 319	CCALA 393	CCALA 341	SAG 40.86
CAUP D 801	CS: 24.2 **	CS: 42.2 ***	CS: 13.5 *	CS: 5.73 -	CS: 17.3 **	CS: 12.2 -	CS: 50.7 ***
	shape: 3.17 -	shape: 1.16 -	shape: 2.74 -	shape: 3.89 *	shape: 4.28 *	shape: 5.63 **	shape: 3.48 -
KR 1989/14		CS: 18.0 -	CS: 37.7 ***	CS: 18.5 *	CS: 41.5 ***	CS: 12.0 -	CS: 26.5 **
		shape: 3.76 *	shape: 1.95 -	shape: 4.75 **	shape: 4.26 *	shape: 12.29 ***	shape: 6.60 ***
SAG 40.88			CS: 55.7 ***	CS: 36.5 ***	CS: 59.5 ***	CS: 30.0 *	CS: 8.5 -
			shape: 3.19 -	shape: 3.50 -	shape: 2.93 -	shape: 2.13 -	shape: 2.35 -
CAUP D 802				CS: 19.2 **	CS: 3.8 -	CS: 25.7 **	CS: 64.2 ***
				shape: 5.03 **	shape: 5.04 **	shape: 7.58 ***	shape: 8.40 ***
ASIB BS 319					CS: 23.0 **	CS: 6.5 -	CS: 45.0 ***
					shape: 3.86 *	shape: 7.49 ***	shape: 3.67 *
CCALA 393						CS: 29.5 **	CS: 68.0 ***
						shape: 6.72 ***	shape: 4.06 *
CCALA 341							CS: 38.5 **
							shape: 3.03 -

Discussion

We provide both molecular genetic and morphological evidence for taxonomic inflation in a group of coccoid green algae currently classified in *Neocystis*. Based on the 18S-ITS1-5.8S-ITS2 rDNA region, all eight strains are closely related and belong to two distinct genotypes despite having been previously assigned to five different species. The first genotype comprises four original strains with identical sequences without apparent intragenomic heterogeneity. These strains were all isolated from a restricted area in Switzerland and Austria. In contrast, the second genotype comprises three isolates coming from widely separated locations—a lake in Germany (KR 1989/14) and the Antarctic region (CAUP D 801 and SAG 40.88, see Table 1). Two of the latter three strains exhibit slight intragenomic heterogeneity in the 18S-ITS1-5.8S-ITS2 rDNA region, but

otherwise their sequences are identical. This degree of similarity within the relatively rapidly evolving ITS1 and ITS2 regions indicates a recent common origin of the strains in each genotype. Following a practice that has been widely adopted by green algal taxonomists (Pröschold & Leliaert 2007, Darienko *et al.* 2010, Krienitz *et al.* 2011b), our strains within each genotype represent the same species. At the same time, the degree of difference in the ITS2 region between the first and the second genotype, including the presence of one CBC in the helix II, and the sharp distinction of the two genotypes, may be interpreted as evidence for their existence as two different, yet possibly cryptic, species (Müller *et al.* 2007). However, with the apparent absence of sexual reproduction in *Neocystis* spp., it is impossible to apply the most widely accepted criterion for species delimitation in eukaryotes, i.e. the "biological species" concept by Mayr (1942), and the interpretation of *N. brevis* and *N. mucosa* as two separate species must be viewed as tentative and requiring further studies of their biological properties.

Of the species names that have been applied to either of these strains, *Nephrodiella minor* has priority over *Nephrodiella brevis*, *Ellipsoidion parvum* and *Neocystis vischeri*. However, the strain CCALA 393, maintained in the respective culture collection as *Neph. minor*, is apparently a subculture of Vischer's original strain 1941/267 that he regarded (together with the strain 1941/319, i.e. ASIB BS 319) as representing the new species *Neph. brevis* (Vischer 1945). It was no longer possible to find out when and how Vischer's original identification of the CCALA 393 strain changed to *Neph. minor* in CCALA. Vischer discriminated *Neph. brevis* from the previously described *Neph. minor* on the basis of a different number of chloroplasts (one in *Neph. brevis*, one or two in *Neph. minor*) and by a less pronounced incurvation of *Neph. brevis* cells (but note that Vischer could compare his isolates only with Pascher's verbal description and a simple line drawing, not directly with biological material observed by Pascher). Vischer also pointed to possibly different habitat preferences of the two species – *Neph. brevis* was isolated from soil, whereas *Neph. minor* was reported by Pascher from freshwater (Vischer 1945). If we accept that these differences are significant enough to base delimitation of two separate species, CCALA 393 has to be treated as a strain of *Neph. brevis* rather than of *Neph. minor*. It follows that the first genotype represents a species with the basionym *Neph. brevis* and that the species names *E. parvum* and *Neph. vischeri* are its junior synonyms. However, this basionym itself is now treated as a synonym of the name *Neocystis brevis* (Vischer) I.Kostikov & L.Hoffmann (see below).

Kostikov & Hoffmann (2002) made a careful light microscopic observation of SAG 850-1, a subculture of Vischer's original strain 1941/267, and of INSIB BS-319, a subculture of Vischer's original strain 1941/319, and they were able to define some minor morphological differences (in cell shape and the number of autospores formed in autosporangia) that they interpreted as differentiating two separate species (*N. brevis* and *N. vischeri)*, even though Vischer considered both strains to represent the same species (*N. brevis*). We studied different subcultures of Vischer's original strains (CAUP D 802 and ASIB BS 319) and within the limit of our approach we found essentially no difference in the shape of the two strains (Fig. 2), which is in accord with the identity of their 18S-ITS1-5.8S-ITS2 rDNA regions. Therefore, we conclude that there is no evidence that could support the existence of *N. vischeri* as a separate species.

The demonstration that *Ellipsoidion parvum*, currently classified in the ochrophyte class Xanthophyceae (Ettl 1978; Ettl & Gärtner 1995), actually belongs to green algae, should not be considered surprising. The current circumscription of the Xanthophyceae stems from Pascher's concept of "Heterokonten" that has proven to be a very heterogeneous assemblage with some of its members subsequently moved to other groups, e.g. to Chlorarachniophyta (Hibberd & Norris 1984), Eustigmatophyceae (Hibberd 1981, Přibyl *et al.* 2012), or green algae (Gärtner & Schragl 1988, Darienko *et al.* 2010, this study). Most of the nominal xanthophycean species remain to be reinvestigated by modern methods and it is very likely that there will be many additional cases of "xanthophyceans" that need to be transferred into other algal classes. An alternative classification of *E. parvum* in the class Eustigmatophyceae by Guiry & Guiry (2012) apparently follows the fact that an alga originally identified as another species of the genus *Ellipsoidion*, *E. acuminatum* Pascher (1937–1939: 1064), was recognised as a eustigmatophycean alga (Hibberd & Leedale 1972). However, this alga is currently known as *Pseudocharaciopsis ovalis* (Braun) Hibberd (1981: 110), whereas the taxonomic identities and phylogenetic affiliations of the other described *Ellipsoidion* species remain effectively unknown.

As in the case of the first genotype, the taxonomic identity of the second genotype is not completely clear. KR 1989/14 is the authentic strain of *Neocystis mucosa*, but SAG 40.88 has been determined (by an unknown authority) as *Neph. minor*. The possibility that the second genotype represents Pascher's *Neph. minor* cannot be excluded: both *Neph. minor* and KR 1989/14 were found in a freshwater body and both were described to have a higher number of chloroplasts than *Neph. brevis*. If this was true, *N. mucosa* would be a junior synonym of *Neph. minor*. Unfortunately, the documentation available for the original *Neph. minor* is so uninformative that doubts will always remain about its identification. In addition, we could not confirm the description of *N. mucosa* by Krienitz et al. (2011a) as having 2–8 chloroplasts; instead, vegetative cells seem to typically have only one, deeply segmented, chloroplast that divides into several separate chloroplasts only before autosporogenesis. Hence, we refrain from formally proposing the synonymy of *N. mucosa* and *Neph. minor* at this point.

With this framework established on the basis of molecular genetic data, how should the results of the geometric morphometric analysis of the strain morphology be interpreted? The substantial shape variation observed in our cultures mostly did not correlate with their species assignment. There was over six times more morphological variation within strains of the same genotype than between the two species. The most morphologically dissimilar pair of strains even belonged to the same genetically defined species. Neither individual cells, nor strains of the two *Neocystis* species could be unambiguously identified to a species on the basis of cell shape. Krienitz *et al.* (2011a) differentiated *N. brevis* and *N. mucosa* by cell size and by the number of plastids in vegetative cells, but this has not been supported by our observations. Further analyses are needed to test whether there are any well-defined morphological features that can distinguish the two species.

Despite being restricted in scope, our study provides a clear-cut case of taxonomic inflation that might be a reflection of a general aspect of current taxonomic schemes of coccoid green algae. The rate of description of new species in recent literature dealing with the taxonomy of coccoid green algae (aided primarily by molecular sequence data) hugely overshadows the cases of proposed synonymy of previously described species (rare recent examples being: Škaloud & Peksa 2010; Pröschold *et al.* 2011—but see Hoshina 2011, Fučíková & Lewis 2012). We predict that the degree of taxonomic redundancy associated with the traditional taxonomic schemes for coccoid green algae is much higher than currently acknowledged, which has unfortunate consequences for disciplines relying on accurate species delimitations, such as ecology (Isaac *et al.* 2004), biogeography (Foissner 2006, Sharma & Rai 2011), or estimations of global species richness (Mora *et al.* 2011).

An open issue that could not be resolved by our study is the actual generic assignment of the two species re-defined here. Assigning them to a genus other than *Nephrodiella* rests on the assumption that the type species of this genus, designated to be *Nephrodiella phaseolus* Pascher (1937–1939: 429) by Starmach (1968), is an unrelated entity. This may indeed be the case, given the observation of zoospores with typical heterokont morphology by Pascher (1937–1939), which would place his organism in the ochrophytes (most likely xanthophyceans) rather than in the green algae. However, it is important to note that Pascher made his observations on mixed natural samples only (hence the identity of the alleged zoospores may be doubtful), that *Neph. phaseolus* has (to our best knowledge) not been observed since Pascher's report, and that two more strains identified by different authorities as *Nephrodiella* spp. (CAUP D 801 and SAG 40.88, see Table 1) both belong to green algae and even to the same lineage as *Neph. brevis* (albeit to a different species). One more former *Nephrodiella* species, *Neph. nana* Ettl (1977: 559), was transferred as a new combination to the green algal genus *Monoraphidium* by Hindák (1980), although this revision has yet to be confirmed by DNA data. Hence, the identity and taxonomic status of the genus *Nephrodiella* remain uncertain. The classification of *N. brevis* and *N. mucosa* in the genus *Neocystis* is no less questionable, as it is based on their putative relationship to the type of the genus *Neocystis*, *N. ovalis* Hindák (1988: 68), deduced from morphological characters only (Kostikov & Hoffmann 2002, Kostikov et al. 2002, Krienitz *et al.* 2011a). Sequence data are lacking from *N. ovalis* and no strain is available to make a direct morphological and molecular comparison with *N. brevis* and *N. mucosa*; hence the position of these species in the genus *Neocystis* should be viewed as tentative and awaiting additional studies.

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