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Intraclass Evolution and Classification of the Colpodea (Ciliophora)

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ABSTRACT. Using nine new taxa and statistical inferences based on morphological and molecular data, we analyze the evolution within the class Colpodea. The molecular and cladistic analyses show four well-supported clades: platyophryids, bursariomorphids, cyrtolophosidids, and colpodids. There is a widespread occurrence of homoplasies, affecting even conspicuous morphological characteristics, e.g. the inclusion of the micronucleus in the perinuclear space of the macronucleus. The most distinct changes in the morphological classification are the lack of a basal divergence into two subclasses and the split of the cyrtolophosidids into two main clades, differing mainly by the presence vs. absence of an oral cavity. The most complex clade is that of the colpodids. We partially reconcile the morphological and molecular data using evolutionary systematics, providing a scenario in which the colpodids evolved from a *Bardeliella*-like ancestor and the genus *Colpoda* performed an intense adaptive radiation, giving rise to three main clades: Colpodina n. subord., Grossglockneriina, and Bryophryina. Three new taxa are established: Colpodina n. subord., Tillinidae n. fam., and Ottowphryidae n. fam. Colpodean evolution and classification are far from being understood because sequences are lacking for most species and half of their diversity is possibly undescribed.

Key Words. *Bardeliella*, cladistics, classification, evolutionary systematics, molecular taxonomy, ontogenesis.

WHEN Small and Lynn (1981) recognized the colpodeans as a monophyletic taxon, molecular data were not yet available. They based their ''Class Colpodea'' on the unique arrangement of the cortical fiber system, specifically the left kinetodesmal (LKm) fiber composed of transverse microtubule ribbons extending posteriorly at the left side of the ciliary rows. Unfortunately, this character can usually be revealed only by transmission electron microscopy. Indeed, the features recognizable in vivo, especially the oral structures, are often so diverse that their colpodean nature is neither immediately nor easily recognizable (Foissner 1993).

When molecular methods became available, they initially confirmed the monophyly of the Colpodea (Lynn et al. 1999; Stechmann, Schlegel, and Lynn 1998). However, when taxon sampling increased both within the colpodeans and in potential close outgroups, the genes showed no node support for or against the monophyly (Dunthorn, Foissner, and Katz 2008; Lasek-Nesselquist and Katz 2001), possibly because the molecular signatures of the ribosomal genes are too weak for the deepest nodes of the ciliate tree of life. Likewise, morphological features questioned the monophyly of the colpodeans, for instance, the ciliary plaques (Bardele 1981).

Foissner (1993) prepared an exhaustive monograph on the colpodeans, supporting the classification of Small and Lynn (1981) and investigating the intraclass relationships. This monograph stimulated molecular taxonomists to investigate the colpodeans in greater detail because it provided a detailed guide to the taxa that should be sequenced (Dunthorn et al. 2008, 2009, 2011; Lasek-Nesselquist and Katz 2001; Lynn et al. 1999; Stechmann et al. 1998). These molecular analyses supported Foissner's classification only partially and suggested a much greater diversity within the core colpodeans (~ order Colpodida).

These and other discrepancies stimulated us (i) to analyze the small subunit (SSU) rDNA of nine new colpodean taxa, especially the marynid colpodids; (ii) to use PhyloBayes, besides the standard methods, to evaluate the present and former molecular data; (iii) to perform supplementary morphological investigations on some species, especially *Bardeliella pulchra*, which branches at the base of the order Colpodida in the molecular trees; (iv) to analyze in detail the intraordinal relationships of the Colpodida, from which sequences are available for comparatively many species; and (v) to discuss the morphological and molecular evolution, providing testable hypotheses that could reconcile morphology and molecules (Dunthorn and Katz 2008).

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MATERIALS AND METHODS

Taxon sampling, identification, and terminology. Nine new colpodeans were sampled and sequenced for this study (Table 1). These isolates were collected, using the nonflooded Petri dish method following Foissner, Agatha, and Berger (2002). Identification used live observation and silver impregnation procedures (Foissner 1991). New species will be described and discussed elsewhere.

Other colpodean and outgroup sequences are from GenBank, based on the studies of Lynn and Sogin (1988), Stechmann et al. (1998), Lynn et al. (1999), Lasek-Nesselquist and Katz (2001), Dunthorn et al. (2008, 2009, 2011), Foissner and Stoeck (2009), Foissner and Wolf (2009), and Foissner (2010). They have been added to the tree shown in Fig. 38.

In most cases, we use a rankless classification in the text to simplify and facilitate communication because ranking highly depends on character priorization and the existing ranking. For instance, the unique ability of *Sorogena* to form slime

Table **1.** Taxon sampling of the Colpodea in this study.

Taxon	Sampling site
Bursaria n. sp. #2	Soil from floodplain of the Darling River near the town of Adelaide, Australia
Bursaria n. sp. #3	Mud and aeolian soil from top of Ayers Rock in the Red Centre of Australia
Exocolpoda augustini	South Africa, soil from a floodplain in the Krueger National Park
Colpoda maupasi	Soil from a rice field in the surroundings of the Lake Biwa museum, Japan
Tillina n. sp.	Soil from floodplain of the Chobe River, Botswana, Africa
Maryna umbrellata	Plankton of a meadow pond in the surroundings of Salzburg City (Krauthügel), Austria
Maryna n. sp.	From the tanks of *Guzmania monostachia*, a bromeliad in the surroundings of the village of Quick Step, Jamaica
Pseudomaryna n. sp.	As *Tillina* n. sp., i.e. from Botswana, Africa
Woodruffiides metabolicus	As *Colpoda maupasi*, i.e. from Japan

Table **2.** The four different alignments analyzed.

Alignment	Taxon inclusion	Characters masked by	# base pairs	# parsimony-informative characters
1	All Colpodea	Eye	1,626	314
2	All Colpodea	gBlocks	1,641	339
3	Just Colpodida	Eye	1,686	158
4	Just Colpodida	gBlocks	1,676	159

Ambiguously aligned characters were either masked by eye in MacClade or by the program gBlocks.

mould-like aerial sorocarps suggests family or even ordinal rank while the small subunit (SSU) rDNA shows only small differences to some ''ordinary'' platyophryid genera (Dunthorn et al. 2008, 2011; Lasek-Nesselquist and Katz 2001). Likewise, we simplify the complex oral terminology to four terms: right oral ciliary field, left oral ciliary field, oral cavity when the ciliary fields are in a more or less deep concavity, and roof kineties when the right surface of the oral cavity is ciliated. ''PBC'' denotes three molecular clades: platyophryids, bursariomorphids, and cyrtolophosidids (see Lynn 2008 for an explanation of further terms).

Amplification, sequencing, and alignment. Genomic DNA was extracted, and the nuclear SSU-rDNA locus was amplified and sequenced, following Foissner and Stoeck (2009). Up to three cloned inserts were sequenced bi-directionally. Base calling and sequence assembly was done, using CondonCode Aligner 3.0 (CodonCode Corporation, Dedham, MA). Vector and primer nucleotides were trimmed off. Sequences were added to the alignment of Dunthorn et al. (2009) and edited by eye in MacClade v4.08 (Maddison and Maddison 2005).

Four final alignments were then compiled (Table 2). First, all Colpodea and two outgroups, with ambiguously aligned regions masked by eye in MacClade. Second, all Colpodea and two outgroups, with ambiguously aligned regions masked by gBlocks v0.91b set to default parameters (Castresana 2000; Talavera and Castresana 2007). Third, just the Colpodida with *Cyrtolophosis mucicola* as outgroup, masked by eye. Fourth, just the Colpodida and *C. mucicola* as an outgroup, masked by gBlocks.

Genealogical analyses. The GTR-I-Γ evolutionary model for all alignments was the best fitted model selected by the Akaike Information Criterion as implemented in jModeltest v0.1.1 (Guindon and Gascuel 2003; Posada 2008). Maximum likelihood (ML) analyses were carried out in RaxM-HPC v7.2.5 (Stamatakis, Hoover, and Rougemont 2008). Node support came from a majority rule consensus tree of 1,000 multiparametric bootstrap replicates. Bayesian inference was carried out, using two different algorithms. First, with MrBayes v3.2.1 (Huelsenbeck and Ronquist 2003), using the GTR-I-Γ model. Posterior probability was estimated, using four chains running 20 million generations, sampling every 1,000 generation; hereafter referred to as the ''MrBayes tree.'' Second, to account for the possibility of model and rate variation, PhyloBayes v3.2e (Lartillot, Lepage, and Blanquart 2009; Lartillot and Philippe 2004) was used with the QMM model (Dirichlet processes of GTR matrices). Posterior probability was estimated, using one chain running at least two million generations, sampling every cycle, hereafter referred to as the ''PhyloBayes tree.'' For both methods the first 25% of sampled trees were considered burn-in trees and were discarded before constructing a 50% majority rule consensus tree. Trees were visualized with FigTree v1.3.1 (Rambaut 2006). For the ML bootstraps, we consider values <70 as low, 70–94 as moderate, and ≥ 95 as high, following Hillis and Bull (1993). For the MrBayes and PhyloBayes posterior probabilities, we consider support values <0.70 as low, 0.70–0.94 as moderate, and ≥ 0.95 as high. The trees were rooted with *Furgasonia blochmanni* (X65150) and *Obertrumia georgiana* (X65149) because the

Table **3.** Character states and coding used for the construction of the cladograms of the main colpodean clades (Fig. 39, 40).

	Plesiomorphic	Apomorphic
1	Somatic kinetids not colpodid (coded 0)	Colpodid dikinetids (coded 1)
2	Ciliary plaques present (coded 0)	Ciliary plaques absent (coded 1)
3	Ordinary LKm fiber (coded 0)	LKm fiber reduced (V-shaped pattern; coded 1)
4	Supraepiplasmic microtubules absent (coded 0)	Supraepiplasmic microtubules present (coded 1)
5	Cortical alveoli typical (coded 0)	Alveolocysts (coded 1)
6[a]	Silverline pattern kreyellid (coded 000)	Silverline pattern colpodid (coded 1; coded 100), secondarily kreyellid (coded 2; coded 110), platyophryid (coded 3; coded 101)
7	Nuclear apparatus ordinary (coded 0)	MA–MI complex (coded 1)
8	Oral structures ordinary (coded 0)	Oral structures elaborate (coded 1)
9	Oral ciliary fields on cell surface (coded 0)	Oral ciliary fields in more or less deep oral cavity (coded 1), in secondarily flattened oral cavity due to presence of a feeding tube (coded 2)
10[a]	Right oral ciliary field a single row of dikinetids (coded 0000)	Right oral ciliary field multiplied and fragmented (coded 1; coded 1000), partially reduced (coded 2; coded 0001), crescentic (coded 3; coded 0100), more or less reduced due to the presence of a feeding tube (coded 4; coded 0110)
11	Left oral ciliary field composed of brick-shaped polykinetids (coded 0)	Left oral ciliary field composed of equidistantly spaced rows of monokinetids, forming a ribbon (coded 1)
12	Oral basket (cyrtos) indistinct (coded 0)	Cyrtos polymerized (''nasse''; coded 1)
13	Stomatogenesis pleurotelokinetal (coded 0)	Stomatogenesis mixokinetal (coded 1)
14	Cell division in freely motile condition (coded 0)	Cell division in reproductive cysts (coded 1)
15	Oblique kinety in oral apparatus absent (coded 0)	Oblique kinety in oral apparatus present (coded 1)
16	Sex present (coded 0)	Sex absent (coded 1)
17	Resting cyst without escape apparatus (coded 0)	Resting cyst with escape apparatus (coded 1)
18	Aerial sorocarps absent (coded 0)	Aerial sorocarps present (coded 1)
19	Roof kineties absent or ordinary (coded 0)	Roof kineties a main component of the right oral ciliary field (coded 1)

The coding is mainly based on outgroup comparison with the nassulids. If not stated otherwise, the characters are additive (ordered; Wagner/Farris optimization).

[a]Binary coding of character state trees (first code for Hennigian argumentation scheme, second code for computer analyses).

LKm fiber, left kinetodesmal fiber.

Table **4.** Distribution of character states over the taxa cladistically analyzed with PAUP* (Fig. 40).

					5					10						15					20				
Nassulids	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
Sorogena	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
Platyophrya	1	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Bursaria	1	1	0	0	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Bryometopus	1	1	1	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0
Cyrtolophosis	1	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0
Grossglockneria	1	0	0	0	0	1	0	0	0	0	2	0	1	1	0	1	0	0	1	0	1	0	0	0
Colpoda	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	1	0	0	1	0	1	0	0	0
Bryophryids	1	0	0	0	0	1	0	1	0	0	1	0	1	0	0	1	0	0	1	0	1	0	0	1

Note that the character state trees of characters 6 (silverline pattern) and 9 (paroral membrane) were converted into an additive binary coding.

nassulids are possibly the nearest relatives of the colpodeans (Foissner 1993; Gong et al. 2009).

Hypothesis testing. Several constrained analyses have already been carried out on both nuclear and mitochondrial SSU-rDNA sequences in the Colpodea (Dunthorn et al. 2008, 2011). Here, we performed additional constrained analyses on alignment 1, which was compiled, using all Colpodea sequences and masked by eye (Table 2): (i) Colpodida and Grossglockneriida monophyletic, excluding the Bryometopida; (ii) Colpodida and Bryometopida monophyletic, excluding the Grossglockneriida; (iii) Colpodida monophyletic, excluding Grossglockneriida and Bryometopida; and (iv) *Maryna* monophyletic, excluding *Pseudomaryna*. The constrained topologies were compared with the nonconstrained ML topology, using the AU test (Shimodaira 2002) as implemented in CONSEL v0.1j (Shimodaira and Hasegawa 2001).

Cladistic analyses. We performed also cladistic analyses, both manually and with computer programs (Hennig86, PAUP*). Instead of using character states to build a separate cladogram, we used the topology inferred from the molecular trees, and mapped inferred character state changes onto this tree (Gong et al. 2009).

The nassulids were used as outgroup, as in the molecular analyses. Polymorphic characters (6, 9, 10; Table 4) were included, as they contribute important phylogenetic information and thus consistently increase the accuracy of the analyses (Poe and Wiens 2000). Therefore, the ''majority method'' was applied, which codes a polymorphic taxon as having the trait that is most common among the taxa considered (Wiens 2000). In characters 2 (ciliary plaques), 3 (LKm fiber), 4 (supraepiplasmic microtubules), and 14 (cell division), it appeared reasonable to assign the scattered data to the closest relatives. Characters 6 (silverline pattern) and 10 (right oral ciliary field) were translated into character state trees.

The trees derived from Henning86 are not shown because they were useable only when the characters were weightened. Even then, the trees contained polytomies and differed distinctly from the molecular ones.

For PAUP* (v4ob10; Swofford 2002), the 50% majority rule consensus tree was found by a heuristic analysis of equally weighted ordered characters and an optimization with (i) Dollo parsimony for characters 2 (ciliary plaques) and 16 (sex) and (ii) accelerated transformation (ACCTRAN). A stepwise and random addition of taxa, the tree-bisection-reconnection branch swapping algorithm, and 10 bootstrap replicates were performed. The resulting tree was imported into TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

RESULTS

Supplementary morphological and ontogenetic observations. To facilitate understanding of the discussion, we show a representative each of the four molecular clades in the scanning electron microscope and/or in silver preparations (Fig. 1–7). Further, we show some important or new morphological data (Fig. 15–22, 28–33), concentrating on the silverline patterns, the ontogenetic modes, a reinvestigation of *Ottowphrya* cf. *dragescoi*, *B. pulchra*, and *Ilsiella palustris*, and the resting cysts.

The colpodeans show three reticulate silverline patterns, named after representative genera (Foissner 1993). The kreyellid pattern (genus *Kreyella*) is an irregular, very narrowly meshed net extending throughout the cortex (Fig. 8). The platyophryid pattern (genus *Platyophrya*) consists of comparatively large and regular meshes subdivided by a median silverline (Fig. 9). The colpodid pattern (genus *Colpoda*) consists of highly ordered, comparatively large meshes extending between two ciliary rows each (Fig. 10, 11).

Two variations of telokinetal stomatogenesis occur in the colpodeans (Foissner 1993, 1996). The pleurotelokinetal mode is characteristic for three of the four molecular clades: the platyophryids, the bursariomorphids, and the cyrtolophosidids, all usually dividing in freely motile condition and producing two offspring. The new oral apparatus originates from kinetofragments produced subequatorially in some right side somatic kineties (Fig. 12). The parental oral apparatus is not or only partially reorganized. The merotelokinetal mode occurs only in the colpodid clade, whose members usually divide in cysts, producing four or more offspring. First, the cell rounds up and reorganizes the somatic and oral ciliature. Concomitantly, kinetofragments develop at the anterior end of some of the newly developing somatic kineties. These kinetofragments organize the new oral apparatus. In the small species, the right oral ciliary field is usually made by only one kinety (Fig. 13, 14), while several are involved in the larger species (Fig. 16–18). The merotelokinetal mode is typical for small colpodids, such as *Colpoda steinii* (Perez-Paniagua, Perez-Silva, and de Puytorac 1979), *Colpoda inflata* (Martin-Gonzalez, Benitez, and Gutierrez 1991), *Exocolpoda augustini* (Foissner et al. 2002), *Bromeliothrix metopoides* (Fig. 13, 14, 25), and the mycophagous grossglockneriids (Foissner 1993).

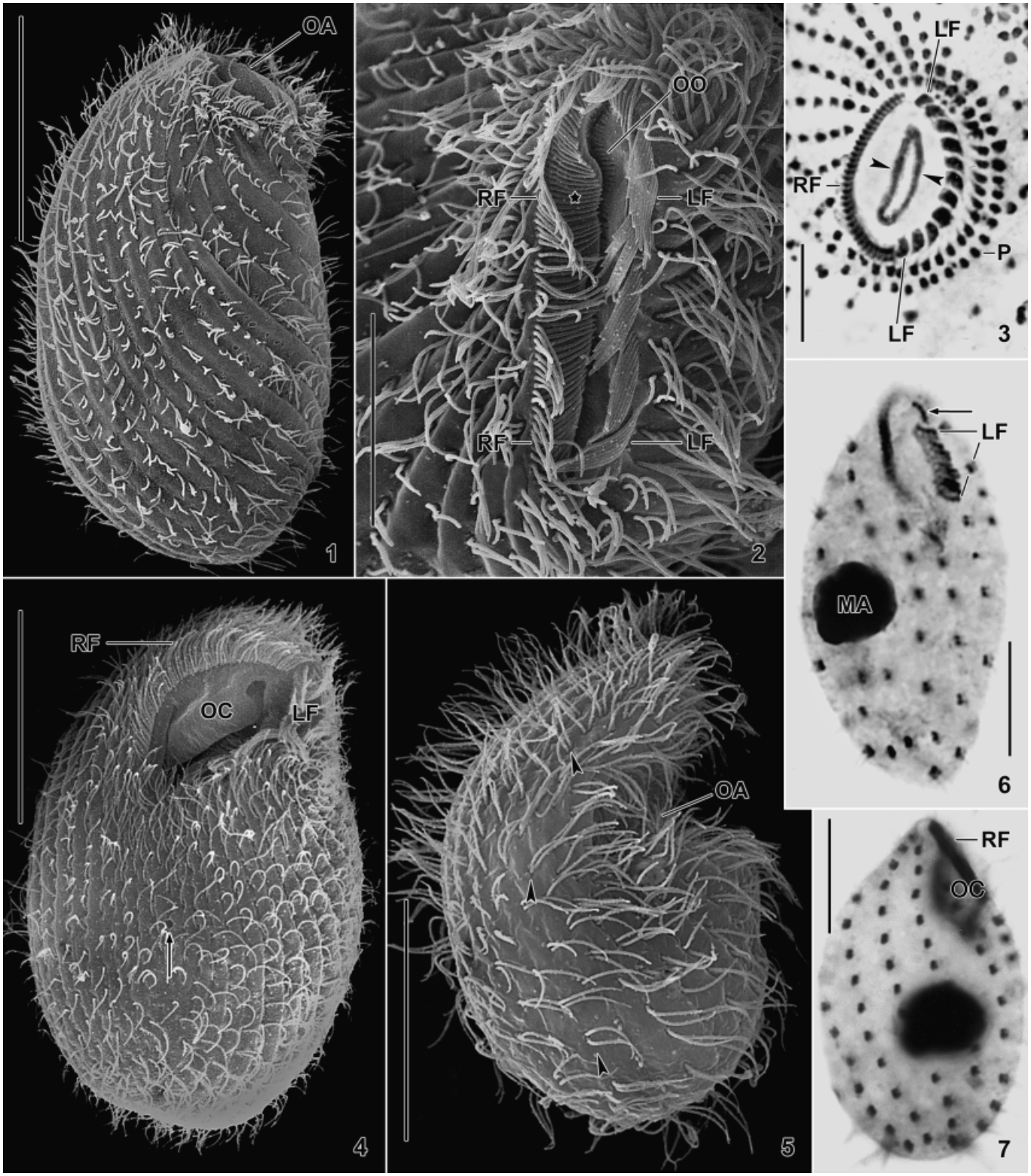
In the larger colpodids, such as *Tillina* (Perez-Paniagua and Perez-Silva 1978), *Colpoda cavicola* (Fig. 15–18), and *Bresslaua vorax* (Garcia-Rodriguez, Perez-Paniagua, and Perez-Silva 1981) occurs a combined pleuromerotelokinetal mode not recognized previously (Fig. 15–18): the kinetofragments originate preequatorially and pleurotelokinetally within the postoral somatic kineties, while the cell rounds up, resorbs the parental oral apparatus, and reorganizes the somatic ciliature, typical merotelokinetal features. Thus, stomatogenesis of the larger colpodids is basically pleurotelokinetal: they transferred the plesiomorphic mode into a cyst.

Ottowphrya forms a clade with the curious *Sorogena* in the molecular trees (Fig. 1, 2, 38). A reinvestigation of a Jamaican population showed that *Ottowphrya* makes resting cysts but does not produce aerial sorocarps. Unlike *Sorogena*, division occurs in cysts as, for instance, in *Woodruffides metabolicus* (Foissner 1993).

In the molecular trees, the ''unusual'' genera *Bardeliella* (Fig. 19) and *Ilsiella* (Fig. 27) are at the base of the colpodid clade (Dunthorn, Foissner, and Katz 2011; Fig. 38, 41), a surprising result that stimulated us to re-investigate their morphology. Three traits indicated that both are transition taxa between cyrtolophosidids and colpodids.

Both divide in cysts, as typical for most colpodids (Fig. 15–18), while most PBC ciliates divide in freely motile condition (Fig. 12).

A further strong indicator is a minute ''oblique kinety'' in the anterior portion of the oral cavity of *Cyrtolophosis* (Fig. 6), first described by Foissner (1987) and later found also in other, but not



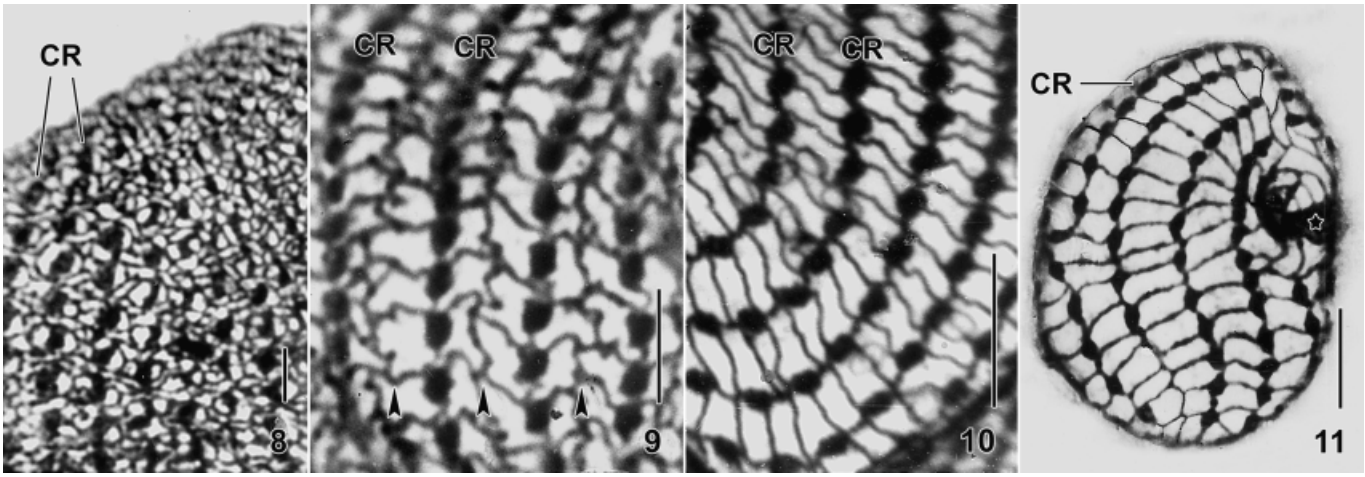


Fig. **8–11.** Main types of silverline patterns in the Colpodea (dry silver nitrate impregnation; from Foissner 1993). **8.** Irregular, fine-meshed kreyellid pattern of *Bryometopus*. This pattern occurs mainly in the bursariomorphids and rarely in the platyophryids. **9.** Platyophryid pattern of *Platyophrya*, in which the comparatively large meshes are divided by a ''median'' silverline extending longitudinally between each two ciliary rows (arrowheads). This pattern occurs in the platyophryids and bryophryids. **10, 11.** Colpodid pattern of *Colpoda cucullus* and *C. steinii*, in which the meshes are rectangular and comparatively large. This pattern occurs also in some platyophryids and cyrtolophosidids. The data available suggest that the colpodid pattern evolved from the kreyellid pattern found in the nassulid ancestor. However, the kreyellid pattern was regained in some platyophryids and the bursariomorphids. The asterisk (11) denotes the left oral ciliary field. CR, somatic ciliary rows. Scale bars: 10 μm.

all, cyrtolophosidids (Foissner 1993). This curious kinety occurs also in *Bardeliella* (Fig. 20, 21) and *Ilsiella* (Foissner 1993). Furthermore, *Ilsiella* has a platyophryid oral ciliary pattern: the right field consists of a single row of dikinetids, while the left one comprises several brick-shaped polykinetids often so close together that they appear as a continuous ribbon (Fig. 27).

An even stronger marker occurs in the bipartite left oral ciliary field of *Bardeliella*: it consists of a long distal portion composed of brick-shaped polykinetids (Fig. 19–22), as in species of the PBC group (Fig. 2–4, 23), while the shorter proximal portion consists of equidistantly spaced, monokinetidal rows (Fig. 21), as in *Colpoda* (Fig. 26). Likewise, the right field is similar to that of *Colpoda* (Fig. 20, 21, 26).

As concerns the resting cysts, we present new data for several species. The cyst of *Bryometopus atypicus* has an escape opening (Fig. 28), like that of *Bursaria* spp. (for a review, see Foissner 1993). Many types occur in the colpodids. For instance, lepidosomes with highly different fine structures occur in *C. inflata* (Fig. 29, 30) and *Colpoda lucida* (Fig. 31–33), but are absent from most or even all other ''true'' *Colpoda* species (Foissner 1993). Likewise, cysts are different in marynid colpodids: some have a cover of glass granules (Foissner et al. 2009; Fig. 35, 36) or sand grains, others are smooth or have a dull red color (Foissner 1993). Further cyst peculiarities are described in the explanations to Fig. 34 and 37.

Molecular phylogeny of the Colpodea (Fig. 38; Table 2). The colpodean alignment masked by eye contains 1,626 nucleotides, 314 of which are parsimony informative (Table 2).

The ML, MrBayes, and PhyloBayes trees inferred from this alignment are congruent. Here, we present the ML tree with node support from all methods (Fig. 38; individual trees in supporting information Fig. S1–S3).

Regardless of the method, the colpodeans show four well-supported molecular clades (75–100/1.00/1.00): the platyophryids, bursariomorphids, cyrtolophosidids, and colpodids. The new sequence from *W. metabolicus* is sister to the clade formed by *Rostrophrya*/*Sagittaria*/*Platyophrya*-like species with full node support (100 ML bootstrap/1.00 MrBayes posterior probability/1.00 PhyloBayes posterior probability). The new sequences from *Bursaria* n. sp. 2 and *Bursaria* n. sp. 3 form a clade with the two sequences from the previously published *Bursaria* n. sp. 1A and 1B with moderate to low node support (87/0.86/0.73).

Fig. **1–7.** Representatives of the four main molecular clades of the Colpodea in the scanning electron microscope (1, 2, 4, 5) and after silver carbonate (3) and protargol (6, 7) impregnation. **1, 2.** *Ottowphrya dragescoi*, right side overview and frontal view of oral apparatus (from Foissner et al. 2002). *Ottowphrya* belongs to the platyophryid clade, which has the oral structures on the cell surface. The right oral ciliary field is composed of ciliated dikinetids, while the left oral ciliary field is composed of five short, single rows of cilia. The asterisk (2) marks the inner row of the right oral ciliary field, whose cilia are almost motionless. **3.** *Platyophrya spumacola*, frontal view of oral apparatus, which has the basic pattern of that of *Ottowphrya*; but the left oral ciliary field consists of brick-shaped polykinetids (short rows in *Ottowphrya*) and the left side ciliary rows are condensed in the anterior portion, forming the postoral pseudomembrane (from Foissner 1993). The arrowheads mark the oral opening. **4.** *Bryometopus sphagni*, right side overview, showing the oral apparatus subapically and on the right side of the body (original). *Bryometopus* belongs to the bursariomorphids because it has a distinct oral cavity (OC) and a comparatively complex left oral ciliary field (Fig. 23). The arrow marks the pore of the contractile vacuole. **5.** Right side view of *Colpoda inflata*, a member of the colpodid clade (original). Typically, the ciliary rows extend slightly spirally and are composed of ciliated dikinetids (arrowheads). The oral ciliature is in a rather deep funnel and thus hardly recognizable in the scanning electron microscope (for details, see Fig. 26). **6, 7.** *Cyrtolophosis mucicola*, ventral and right side view, showing the rather deep oral cavity characteristic of the cyrtolophosidid clade (originals). The right oral ciliary field consists of a single row of cilia, while the left field comprises four brick-shaped polykinetids. The arrow marks a special kinety also found in *Bardeliella* and *Ilsiella*, both at the base of the colpodid clade in molecular trees. LF, left oral ciliary field; MA, macronucleus; OA, oral apparatus; OC, oral cavity; OO, oral opening; P, postoral pseudomembrane; RF, right oral ciliary field. Scale bars: 10 μm (Fig. 2, 3), 20 μm (Fig. 5–7), and 40 μm (Fig. 1, 4).

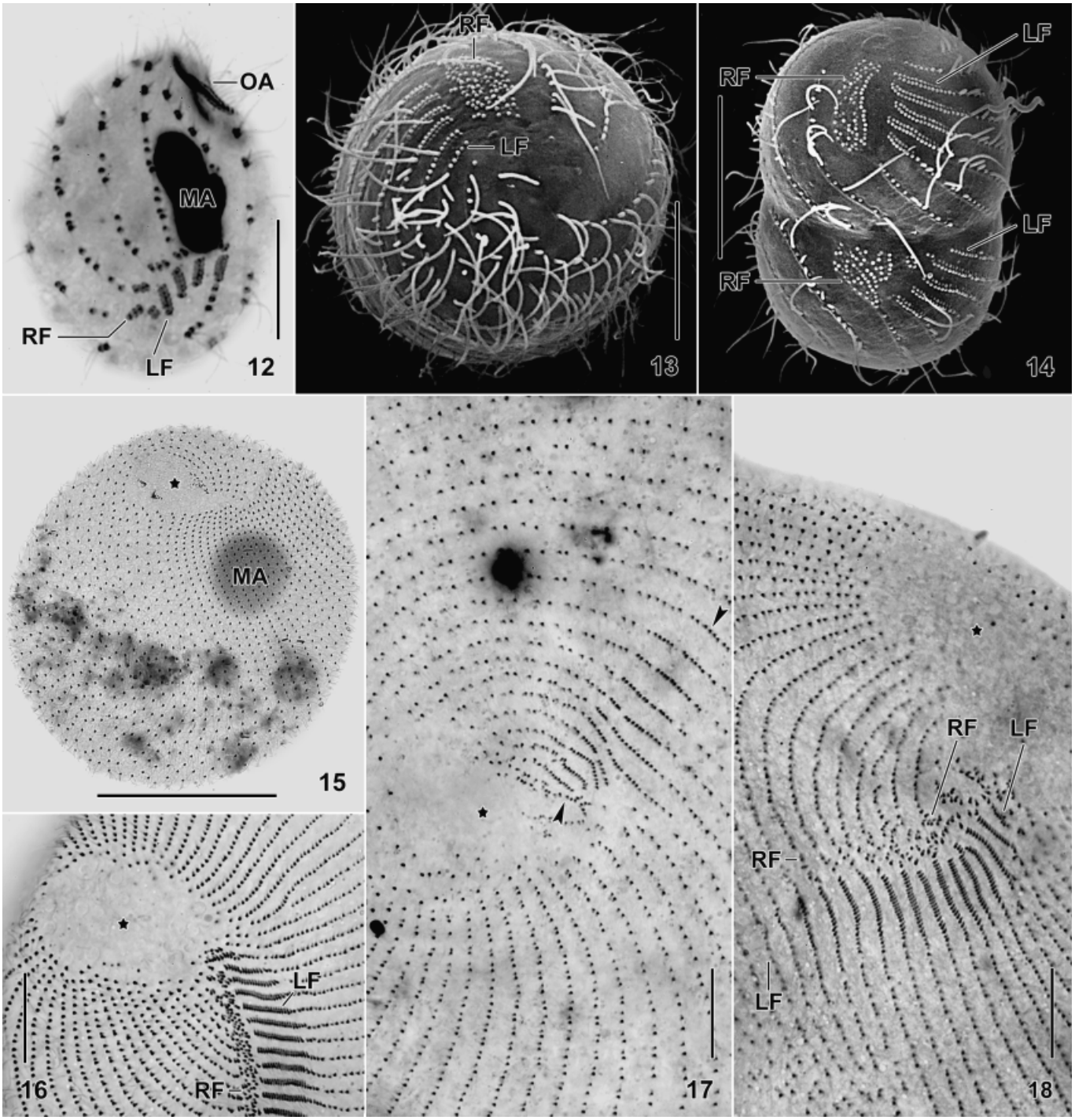


Fig. **12–18.** Stomatogenic modes in the Colpodea. **12.** Pleurotelokinetal stomatogenesis in *Cyrtolophosis mucicola* (protargol, original). For morphostatic specimens, see Fig. 6, 7. Kinetofragments develop in some right side kineties, eventually producing the right and left oral ciliary fields. This mode occurs in the platyophryids, bursariomorphids, and cyrtolophosidids. **13, 14.** Merotelokinetal stomatogenesis in *Bromeliothrix metopoides* (SEM, from Foissner 2010). For a morphostatic specimen, see Fig. 25. The specimen rounds up and reorganizes the infraciliature (13). Then, kinetofragments originate at the end of some somatic kineties and build the new oral ciliary fields (14). This mode occurs in small colpodids, e.g. *Colpoda steinii*, *B. metopoides*, and the mycophagous grossglockneriids. **15–18.** Pleurotelokinetal-like stomatogenesis in division cysts of *Colpoda cavicola* (silver carbonate, originals). Asterisks mark site of resorbed parental oral apparatus. This pattern, which occurs in the larger colpodids, is a combination of the pleurotelokinetal and merotelokinetal mode: the kinetofragments originate pre-equatorially within the somatic kineties (as in *Cyrtolophosis*), while the cell rounds up and resorbs the parental oral apparatus (as in *Bromeliothrix*). **15.** Overview. **16.** Late stage, where the left oral kinetofragments each consists of three ciliary rows and the right oral kinetids form a dense anarchic field. **17.** Very early stage, showing forming kinetofragments (deliminated by arrowheads) in the postoral kineties. **18.** Middle stage, where the left oral kinetofragments consist of two rows and the right oral kinetids form a loose anarchic field. LF, new left oral ciliary field; MA, macronucleus; OA, parental oral apparatus; RF, new right oral ciliary field. Scale bars: 15 μm (Fig. 12–14, 16–18) and 60 μm (Fig. 15).

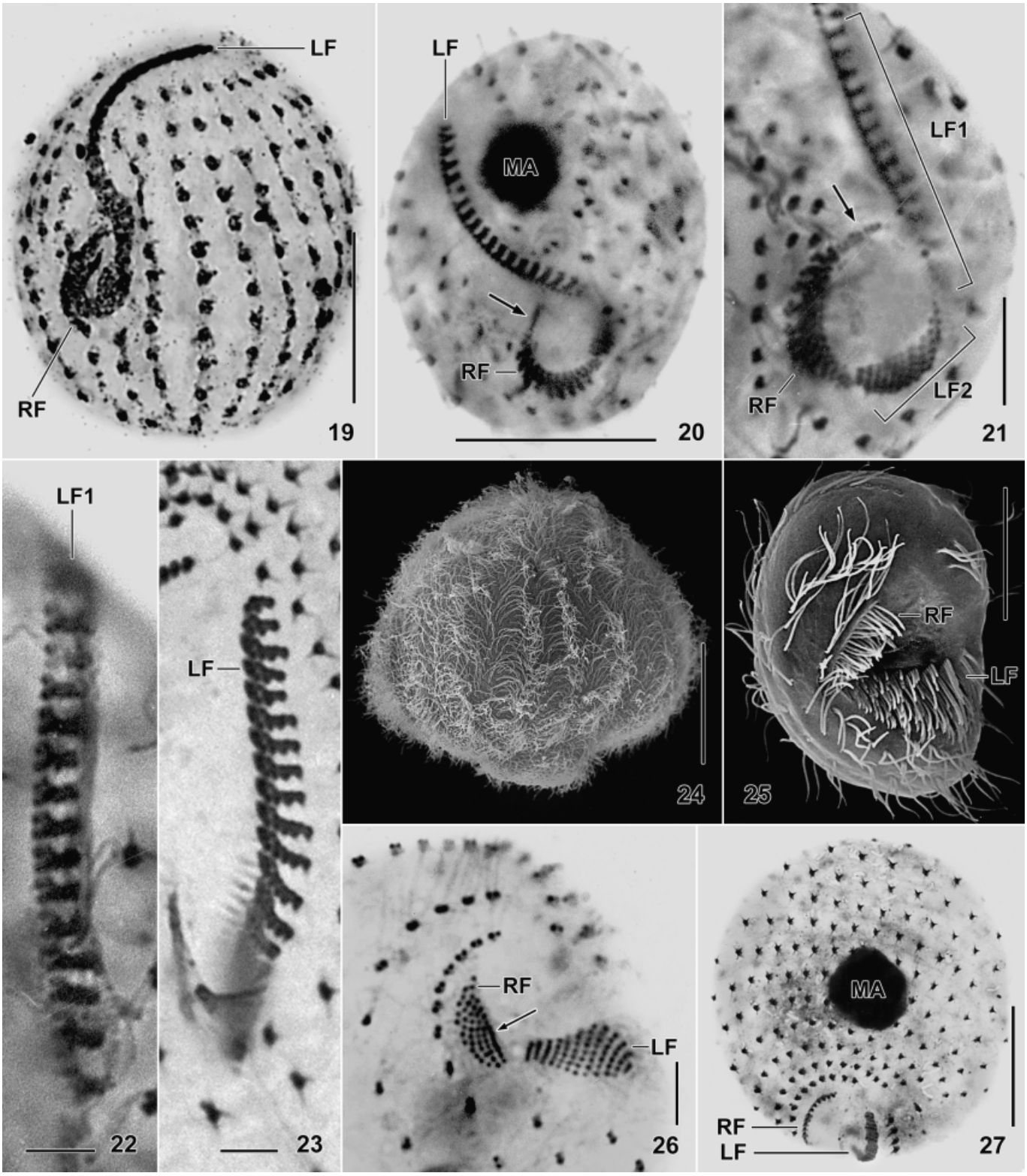


Fig. **19–27.** Representatives of several colpodean families after silver nitrate (19) and silver carbonate (20–23, 26, 27) impregnation and in the SEM (24, 25). **19–23.** *Bardeliella pulchra* (originals and from Dunthorn et al. 2011) has the left ciliary field composed of a long anterior portion (LF1) with brick-shaped polykinetids resembling those of *Bryometopus* (23), and of a small posterior portion (LF2) composed of monokinetidal rows highly similar to those of *Colpoda* (26). The arrows mark the oblique kinety. **24.** *Maryna umbrellata* (from Foissner et al. 2009). **25.** Ventral view of *Bromeliothrix metopoides* (from Foissner 2010). **26.** Oral apparatus of *Colpoda steinii* (from Foissner 1993). The arrow marks a row of dikinetids, very likely homologous to the right oral ciliature of the platyophryids. **27.** *Ilsiella palustris* has the oral apparatus in the posterior body portion (from Foissner 1993). LF, left oral ciliary field; LF1, 2, portions of the left oral ciliary field; MA, macronucleus; RF, right oral ciliary field. Scale bars: 5 μm (Fig. 21–23, 26), 15 μm (Fig. 19, 25, 27), 20 μm (Fig. 20), and 30 μm (Fig. 24).

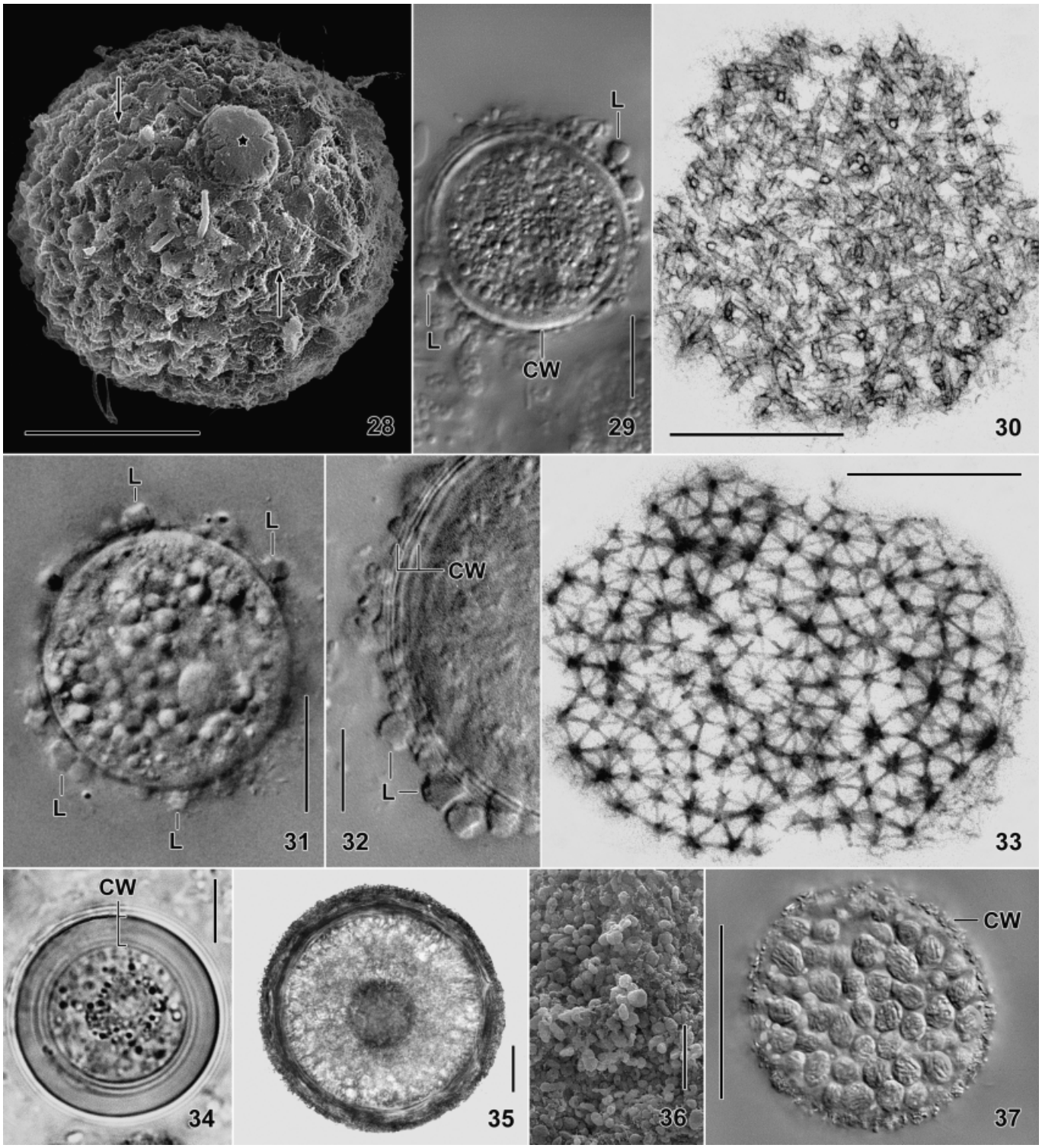


Fig. **28–37.** Resting cysts of various colpodeans in the light microscope (29, 31, 32, 34, 35, 37) and the scanning (28, 36) and transmission (30, 33) electron microscope. **28.** The cyst of *Bryometopus atypicus* has an escape apparatus (asterisk), thus resembling *Bursaria* spp. (original). The arrows mark minute spines most covered by slime and environmental debris. **29, 30.** The cyst of *Colpoda inflata* is covered by 1–3-µm-sized spheres (lepidosomes) composed of long, intertwining tubules (originals). **31**–**33.** The resting cyst of *Colpoda lucida* is covered with up to 5-µm-sized globules (lepidosomes), showing a fibrous, crystal-like fine structure (originals). **34.** Desert populations of *Exocolpoda augustini* have a very thick cyst wall (from Foissner et al. 2002). **35, 36.** The thick cyst wall of *Maryna umbrellata* is covered with glass granules about 1 µm in size (from Foissner et al. 2009). **37.** *Pseudomaryna australiensis* is an adversity strategist storing food vacuoles in the resting cyst (from Foissner and Stoeck 2009). When it divides in the cyst, the contents of the food vacuoles are digested. CW, cyst wall; L, lepidosomes. Scale bars: 0.5 µm (Fig. 30, 33), 5 µm (Fig. 36), 10 µm (Fig. 28, 29, 32, 34), and 20 µm (Fig. 31, 35, 37).

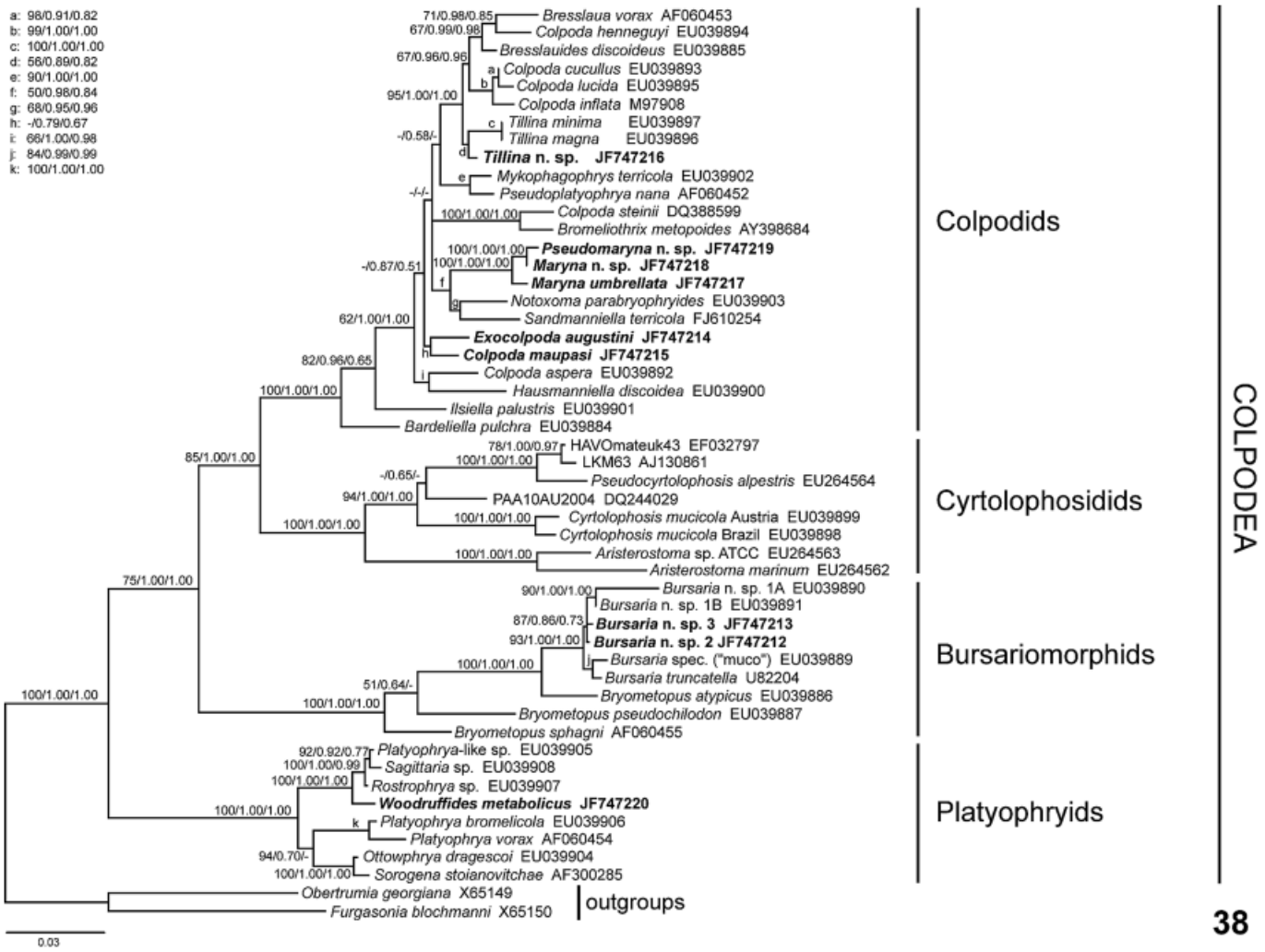


Fig. **38.** Nuclear small subunit rDNA phylogeny of the Colpodea from the alignment masked by eye. The most likely tree is shown. Node support: ML bootstrap/MrBayes posterior probability/PhyloBayes posterior probability. Values <50 are shown as ''-.'' New sequences in bold.

The colpodean alignment masked by gBlocks contains 1,641 nucleotides, 339 of which are parsimony informative (Table 2). The ML, MrBayes, and PhyloBayes trees inferred from this alignment are congruent with the trees inferred from the alignment masked by eye (ML tree with node support from all methods in supporing information Fig. S4–S7), with two exceptions (see ''Molecular phylogeny of the Colpodida'').

Cladistic phylogeny of the Colpodea (Fig. 39, 40; Tables 3, 4). The analyses of the data matrix (Table 4) produced three equally parsimonious trees with Hennig86 and four trees, using PAUP*. Of the 24 characters, 14 were parsimony uniformative. The trees created by Hennig86 have a length (length = 30), consistency index (CI = 83), and retention index (RI = 68) similar to those obtained with PAUP* (length = 29; CI = 86; RI = 76).

We could substantiate the molecular tree (Fig. 38) cladistically by both, manual and computer-assisted parsimony methods (Fig. 39, 40). However, it was unexpectedly difficult to find meaningful apomorphies. The greatest surprise was the importance of an oral cavity, being the sole apomorphy uniting bursariomorphids, cyrtolophosidids, and colpodids, which are supported by three meaningful apomorphies each. The platyophryids, in contrast, are merely defined by the potential loss of sex and the loss of ciliary plaques. This probably causes the polytomy in the computer trees. Generally, the cladogram is studded with losses, homoplasies and, possibly, parallelisms, viz., the loss of sex and ciliary plaques, the independent occurrence of a MA–MI complex (micronucleus within the perinuclear space of macronucleus), the partial reduction of the right oral ciliary field, and the silverline patterns.

Molecular phylogeny of the Colpodida (Fig. 41; Table 2). The Colpodida alignment masked by eye contains 1.686 nucleotides, 158 of which are parsimony informative (Table 2). The ML, MrBayes, and PhyloBayes trees inferred from this alignment are congruent. The phylogeny also matches those from the full Colpodea alignments (Fig. 38) and previous nuclear and mitochondrial analyses in the well supported nodes (Dunthorn et al. 2008, 2011; Foissner and Stoeck 2009). Here, we present the ML tree with node support from all methods (Fig. 41; individual trees in supporting information Fig. S8–S10).

The *Maryna*/*Pseudomaryna* clade is sister to the clade formed by *Notoxoma*/*Sandmanniella* with no ML and low MrBayes support (-/0.69); there is no support for this relationship in the PhyloBayes trees. The two *Maryna* isolates and *Pseudomaryna* form a clade with full support (100/1.00/1.00). The sister relationship between *Maryna* n. sp. and *Pseudomaryna* n. sp. with full support

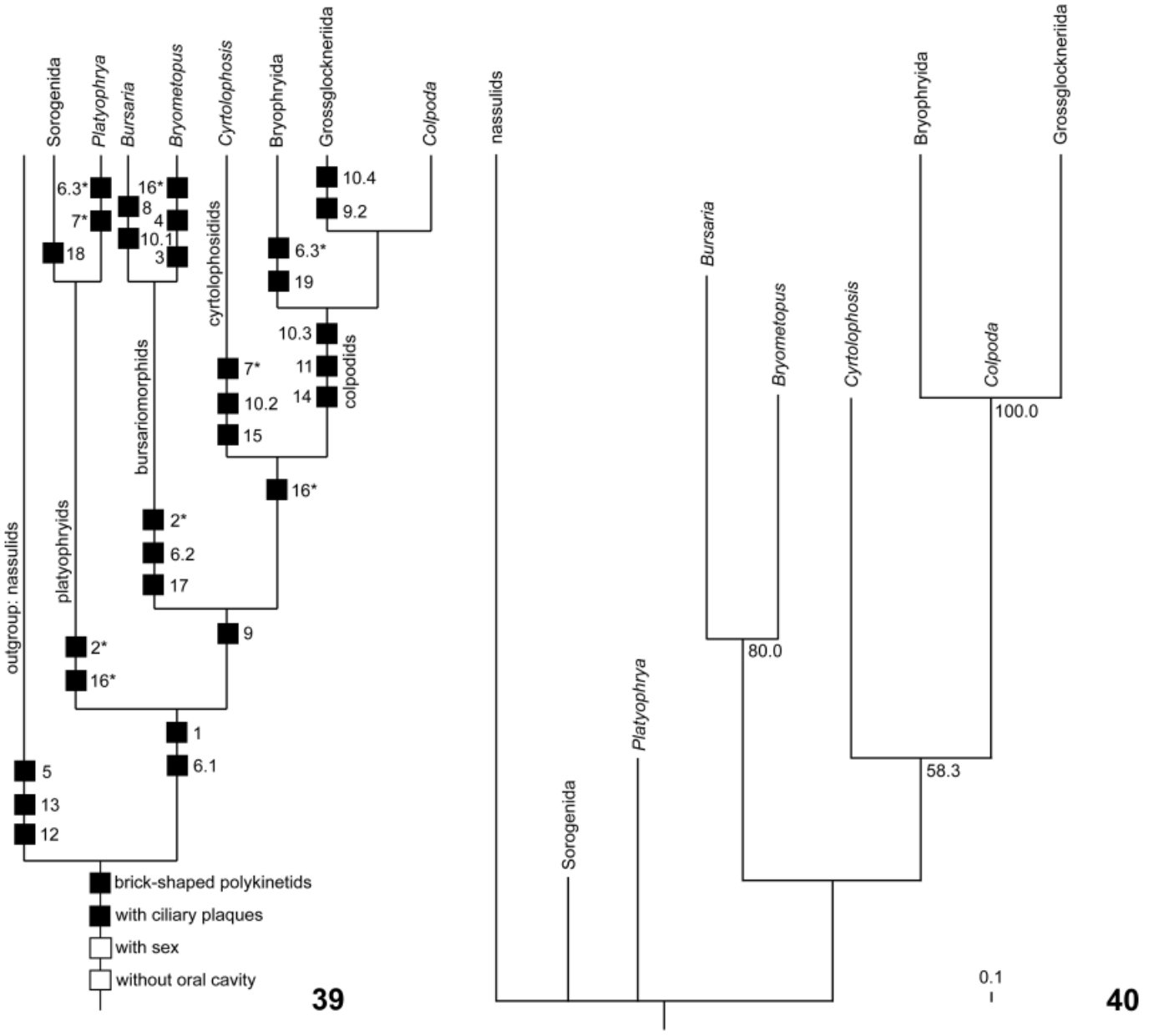


Fig. **39,40.** Manual and PAUP* cladistic tree of the four main colpodean clades. Numbers refer to Table 4; asterisks indicate homoplasies; values in Fig. 40 show bootstrap support. For characters and character states, see Table 3.

(100/1.00/1.00), renders the genus *Maryna* paraphyletic. There is no support for the clade formed by *E. augustini/Colpoda maupasi*. The placement of the clade formed by *E. augustini/C. maupasi* within the Colpodida is also unsupported. *Tillina* n. sp. is sister to the clade formed by *Tillina minima/Tillina magna* with low to high node support (61/0.95/0.83).

The Colpodida alignment masked by gBlocks contains 1,676 nucleotides, 159 of which are parsimony informative (Table 2). The ML, MrBayes, and PhyloBayes trees inferred from this alignment are congruent and match the trees inferred from the Colpodida alignment masked by eye. Here, we present the ML tree with node support from all methods (Supporting Information Fig. S11–S14). However, there are two exceptions to this overall congruence. First, in the ML and MrBayes trees, the clade formed by *E. augustini/C. maupasi* is sister to the clade formed by *Pseudoplatyophrya/Mykophagophrys/Bresslaua/Bresslauides/Colpoda*, but there is no or only low node support (-/0.71), while in the PhyloBayes tree the clade is in the large, central polytomy (supporting information Fig. S14). Second, while *Bresslaua* is sister to *Colpoda henneguyi* with low to high node support in the tree from the alignment masked by eye (Fig. 38), it is sister to the clade formed by *Colpoda cucullus/C. lucida/C. inflata* with low node support (67/0.82/0.87) in the tree from the gBlock alignments (Supporting Information Fig. S4–S7).

Constrained molecular analyses (Table 5). The following constrained topologies could be rejected: Colpodida and Bryometopida monophyletic, excluding the Grossglockneriida; Colpodida monophyletic, excluding Bryometopida and Grossglockneriida; and *Maryna* monophyletic, excluding *Pseudomaryna*. These rejections support the topologies from the unconstrained alignments. On the other hand, the constraint that Colpodida and Grossglockneriida are monophyletic, excluding Bryometopida,

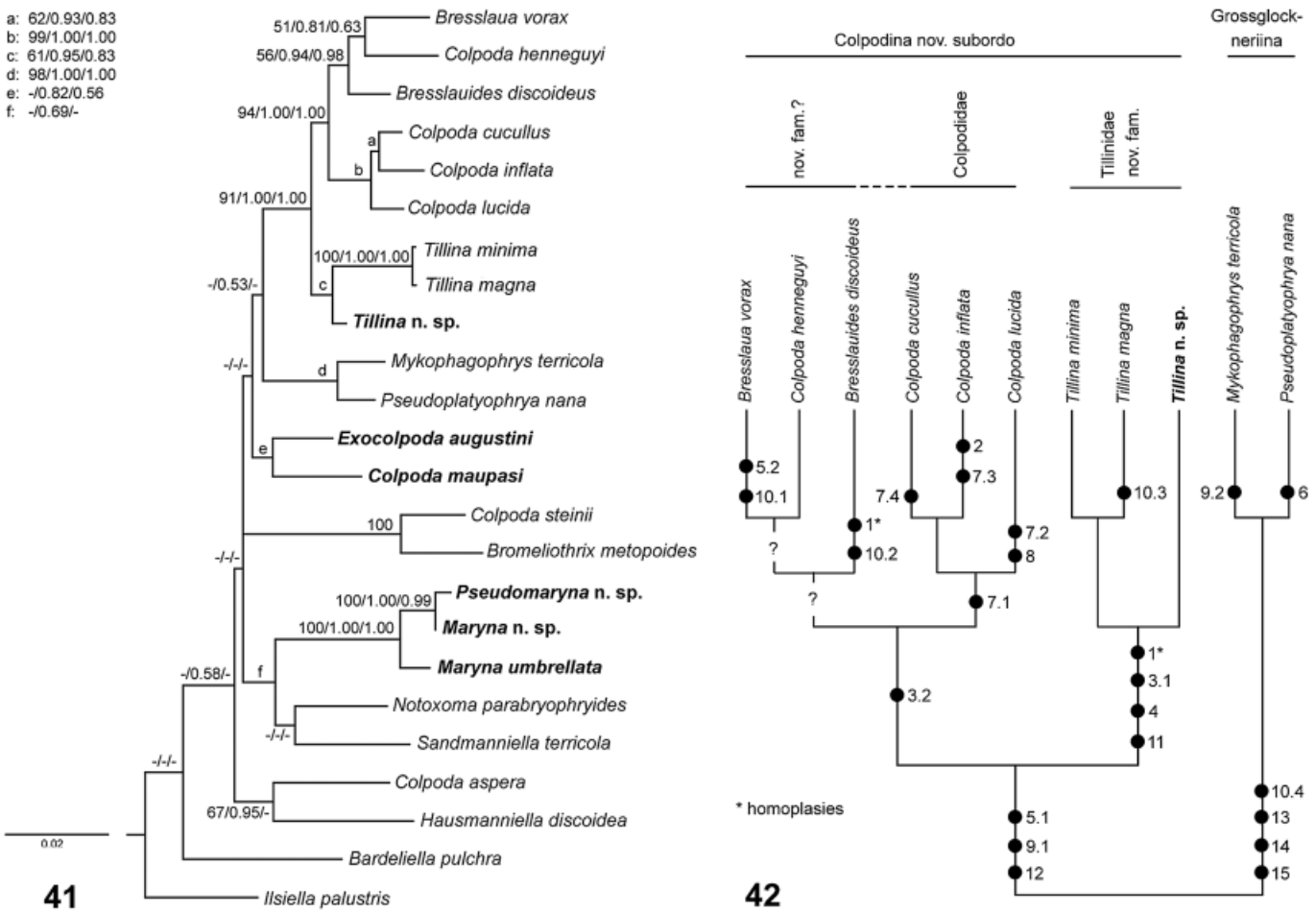


Fig. **41,42.** Figure 41 shows the nuclear small subunit rDNA phylogeny of the Colpodida from the alignment masked by eye. The most likely tree is shown. Node support: ML bootstrap/MrBayes posterior probability/PhyloBayes posterior probability. Values <50 are shown as ''-.'' New sequences in bold. Figure 42 shows the upper portion of the molecular tree (Fig. 41) mapped with the cladistic apomorphies described in Table 6.

Table **5.** Approximately unbiased test results.

Topology constraints	lnL	AU values (*P*)
None	− 8,867,845	0.951
Colpodida and Grossglockneriida monophyletic excluding Bryometopida	− 8,891,779	0.154
Colpodida and Bryometopida monophyletic, excluding Grossglockneriida	− 8,898,236	0.036
Colpodida monophyletic, excluding Bryometopida and Grossglockneriida	− 8,910,628	0.007
Maryna monophyletic, excluding *Pseudomaryna*	− 8,877,037	0.049

The unconstrained topology was able to reject all constraints, except for the monophyly of the Colpodida and Grossglockneriida excluding the Bryometopida.

could not be rejected; this suggests that the Bryometopida may indeed fall out of the Colpodida.

Cladistic phylogeny of the Colpodida (Fig. 42; Table 6). Our cladistic attempts to reconstruct the evolution of this order, using the molecular trees as templates provided strong support for the upper third of the trees as well as for the basal position of *Bardeliella* and *Ilsiella*, but failed in the middle portion of the trees, which is poorly resolved also by the molecular data (Fig. 41, 42). This middle portion, which is not shown in Fig. 42, contains small and middle-sized colpodids and a poorly supported clade uniting marynids (*Maryna*) and bryophryids (*Notoxoma*). This curious position of the marynids will be discussed below. Here, we analyze only the upper third of the trees where the colpodas form two distinct clades with high molecular bootstrap support (91/1.0/1.0) and three apomorphies (Fig. 41, 42): a crescentic left oral ciliary field, a distinct diagonal groove, and rod-shaped extrusomes. We consider this clade as a new suborder, containing the time-honored family Colpodidae and at least one new family, the Tillinidae.

DISCUSSION

Selection of evolutionary meaningful characters. Based on the four main molecular clades, we discuss the major morphologic characters used by Foissner (1993) and others to reveal the intra-class evolution of the colpodeans.

Valuable features include stomatogenesis, most oral structures, ciliary plaques, sex, and resting cysts. The importance of ontogenesis (Foissner 1993, 1996) and ciliary plaques (Bardele 1981) is fully supported by the molecular data, but weakened by their plesiomorphic state and the new data, showing that all colpodeans basically have a pleurotelokinetal stomatogenesis. The PBC

Table 6. Character states for the manual cladogram shown in Fig. 42.

	Plesiomorphic	Apomorphic
1	Body of ordinary size (<130 μm)	Body large (>130 μm)
2	Body shape reniform	Body L-shaped
3	Body with *Colpoda* organization	Body with *Tillina magna* (coded 1) or *Colpoda cucullus* (coded 2) organization
4	Contractile vacuole without collecting canals	Contractile vacuole with distinct collecting canals
5	Diagonal groove absent	Diagonal groove present and distinct (coded 1) or secondarily partially reduced (coded 2)
6	Somatic ciliature complete	Somatic ciliature more or less reduced
7	Lepidosomes absent	Lepidosomes present (coded 1), with tubular fine structure (coded 2), crystal-like fine structure (coded 3), or reduced (coded 4)
8	Ordinary mucocysts	Large, hyaline mucocysts
9	Extrusomes globular	Extrusomes oblong (coded 1) or with anchors (coded 2)
10	Oral cavity ordinary	Oral cavity enlarged (coded 1), very enlarged (coded 2), very large and tubular (coded 3), or reduced (coded 4)
11	No or few (1–2) roof kineties	Several to many roof kineties
12	Left oral ciliary field not crescentic	Left oral ciliary field crescentic
13	Feeding tube absent	Feeding tube present
14	Left oral ciliature complete	Left oral ciliature partially reduced
15	Right oral ciliature complete	Right oral ciliature partially reduced

group has a pleurotelokinetal stomatogenesis and lacks ciliary plaques, while the colpodids have a merotelokinetal or a mixed pleuromerotelokinetal stomatogenesis and distinct ciliary plaques forming rectangular arrays. The PBC usually divide in freely motile condition, while the colpodids usually divide in cysts, producing four or more offspring.

Sex plays an important role in understanding colpodean phylogeny because it has been lost in most of them, except for the bursariids, in which true conjugation has been proven (Foissner 1993; Raikov 1982). However, pseudoconjugation is rather frequent in flourishing environmental cultures and has been observed in *Platyophrya*, *Kreyella*, and several *Colpoda* species (Foissner 1993 and unpubl. observ.). During this process, typical conjugation pairs are produced by sticking together with the preoral cilia, while cytoplasmic bridges are never formed. This strongly suggests that the loss of sex is a rather recent event and might explain why sex is still present in the bursariomorphids (Dunthorn and Katz 2010). Thus, the loss of sex, which occurred 3 times, is an important apomorphy for the platyophryid clade and the clade uniting cyrtolophosidids and colpodids (Fig. 39).

The systematic value of resting cysts is far from being exhausted, both for higher and lower classification levels. For instance, escape openings occur only in the Bursariomorphida, which corroborates the cladistic analysis of Foissner and Kreutz (1998) and the molecular phylogenies (Lynn et al. 1999; Fig. 38) that Bursariidae and Bryometopidae have a common ancestor. On the other hand, the very different resting cysts found in the Colpodidae and Marynidae suggest a greater generic and family diversity than presently recognized. This is also supported by the sequence data (Fig. 41).

Two main features did not hold what they promised: the silverline pattern and the inclusion of the micronucleus in the perinuclear space of the macronucleus (MA–MI complex). As both have been carefully discussed by Dunthorn et al. (2008, 2009, 2011), Foissner (1993), and Lynn (2008), we refer to these studies.

The colpodean ancestor. Based on the molecular data, we prepared a Hennigian argumentation scheme, assuming that the colpodean stem species had a simple platyophryid oral apparatus, a colpodid somatic dikinetid, an ordinary ciliate nuclear apparatus, a colpodid silverline pattern, a pleurotelokinetal stomatogenesis, and sex (Fig. 39). The cladistic tree became most parsimonious under these assumptions (Fig. 40) and various morphological traits could be understood much better than before, for instance, the evolution of the oral structures described below.

The evolution of the colpodean oral structures. The colpodean oral structures are so diverse that their colpodean nature is neither immediately nor easily recognizable (Foissner 1993; Foissner et al. 2002; Lynn 2008; Small and Lynn 1981). Here, we shall show that this diversity can be explained as variations of the same plesiomorphic structures: a row of dikinetids and roof kineties in the right oral ciliary field and of brick-shaped polykinetids in the left field.

Right oral ciliary field. In the platyophryids, this is a simple row of dikinetids (Fig. 1–3). This row has been maintained in the bryometopid bursariomorphids (Fig. 4), while *Bursaria* fragmented and multiplied the row, very likely as an adaptation to the large body size (Fernández-Galiano 1979; Foissner 1993). In the cyrtolophosidids, the platyophryid pattern is maintained, but with some modifications, for instance, a partial reduction of the right oral ciliary field (Dunthorn et al. 2009; Foissner 1993). Four main patterns occur in the colpodids: (i) the platyophryid pattern in which there is a row of dikinetids (e.g. *Ilsiella*, Fig. 27) or a row of monokinetids in the mycophagous grossglockneriids; (ii) the bipartite pattern in which the dikinetidal row is accompanied by more or less disordered monokinetids, forming a roughly crescentic ciliary field, for instance, in *Colpoda* spp. and in *Maryna* spp. (Fig. 24, 26; Foissner 1993); (iii) the complex pattern in which roof kineties and ''perioral cilia condensations'' (Foissner and Stoeck 2009) are strongly involved in structuring the right oral ciliary field, for instance, in the bryophryids (Bourland et al. 2011); and (iv) the reduced pattern in which the monokinetids of the bipartite mode have been resorbed, leaving the plesiomorphic platyophryid pattern, for instance, in the colpodids *Kuehneltiella* and *Avestina* (Foissner 1993).

Left oral ciliary field. This shows three main patterns. The brick-shaped pattern is typical of platyophryids and cyrtolophosidids, in which few to many small polykinetids (adoral organelles) extend along the left margin of the oral opening (Fig. 1–3, 6, 23). A membranellar pattern occurs in the bursariomorphids in which the brick-shaped platyophryid polykinetids developed to membranelle-like assemblages (Fig. 23; Fernández-Galiano 1979; Foissner 1993; Foissner and Kreutz 1998). In the colpodids occur (i) brick-shaped polykinetids, for instance, in *Ilsiella* (Fig. 27), *Sandmanniella* (Foissner and Stoeck 2009), and *Bryophrya* (Bourland et al. 2011; Foissner 1993); (ii) small to large ''polykinetids'' composed of narrowly spaced, equidistant rows of monokinetids, for instance, in *Colpoda* (Fig. 26) and *Bromeliothrix* (Foissner 2010); and (iii) a mixed pattern composed of (i) and (ii), for instance, in *Bardeliella* (Fig. 19–22) and *Bryophrya* (Bourland et al. 2011; Foissner 1993).

Oral Cavity. An oral cavity is absent from the platyophryids, which thus have the oral ciliary fields on the cell surface (Fig. 1–3). Most bursariomorphids have a large oral cavity containing the oral ciliary fields (Foissner 1993; Foissner and Kreutz 1998). In the cyrtolophosidids, the brick-shaped polykinetids of the left ciliary field insert in a rather deep cavity, occupying about one-third of body thickness (Fig. 6, 7). However, the cavity is difficult to recognize in vivo because all described species are small or

very small ($\leq$ 50 µm). Most colpodids have a distinct oral cavity. In some genera, for instance, *Bresslauides* and *Corticocolpoda*, it is as conspicuous as in the bursariomorphids, while it is absent (likely reduced) from *Sandmanniella* and the mycophagous grossglockneriids (Foissner 1993; Foissner and Stoeck 2009).

The colpodids were classified in the Vestibulifera for a long time because of their distinct oral cavity (for reviews, see Corliss 1979; Foissner 1993; Lynn 2008). Nowadays, the Vestibulifera are an order within the Litostomatea Small and Lynn, 1981, while the colpodeans form a separate class only distantly related to the litostomateans (Lynn 2008; Vďačný, Orsi, and Foissner 2010). As far as we know, the oral cavity never played a significant role in understanding evolution within the Colpodea. The present data suggest the opposite because the oral cavity is the sole feature uniting bursariomorphids, cyrtolophosidids, and colpodids. In the litostomateans, the presence vs. absence of a oral cavity is also of great cladistic importance, separating the Trichostomatia from the Haptoria (Lynn 2008).

Our interpretation of the manifold colpodean oral ciliary patterns is strongly supported by its ontogenesis, especially of the left ciliary field which originates from two- or three-rowed precursors that either remain separate or unite to various polykinetidal patterns (Fig. 12–18). The plesiomorphic (ancient) state of the right ciliary field as a single row of dikinetids is also supported by the ontogenetic processes, for instance, in *Cyrtolophosis* and the grossglockneriids, in which the row is dikinetidal in mid-dividers and becomes partially or entirely monokinetidal in late dividers (Foissner 1993).

The four main molecular clades of the colpodeans. Four main colpodean clades have been recognized with both ribosomal and mitochondrial molecular markers (Dunthorn et al. 2008, 2011; Foissner and Stoeck 2009), but with less bootstrap support than in the present study. Likewise, these authors recognized the basal position of the platyophryids. Possibly, there are further main clades because several ''aberrant'' colpodids, such as the kreyellids and trihymenids have not yet been sequenced.

Two significant changes occurred since the monograph of Foissner (1993): (i) the cyrtolophosidids were split into platyophryids and cyrtolophosidids because the main synapomorphy, viz., the inclusion of the micronucleus in the perinuclear space of the macronucleus did not hold (Dunthorn et al. 2008, 2009, 2011); and (ii) the molecular data did not support Foissner's (1993) split of the Colpodea into two subclasses Colpodia and Bryometopia, as already noted by Lynn et al. (1999) and Lynn (2008). *Bryometopus* is nested between the platyophryids and cyrtolophosidids and forms a clade with *Bursaria*, as suggested by Foissner and Kreutz (1998), while Foissner (1993) misclassified the bursarias as closest relatives of the colpodids.

The four main molecular clades are basically supported by the cladistic analyses (Fig. 39, 40). However, only few meaningful characters were found, and thus the clades appear insufficiently supported. This failure has possibly three reasons. First, presumed significant characters, such as the nuclear apparatus and the silverline pattern, have been proven unreliable at this level of analysis. Second, the somatic and oral ciliature is very conservative. Although the latter is highly diverse, it can be derived from three ancient structures basically present in all taxa. Third, morphological characters may evolve asynchronously to molecular characters, as widely known (Hörandl 2010).

Intraclade relationships. In this chapter, we try to follow the evolution of the colpodeans at family and, where appropriate, at genus level.

The platyophryid clade (Fig. 38–40): The platyophryids were established by Puytorac, Perez-Paniagua, and Perez-Silva (1979) as a suborder (Platyophryina) within the order Colpodida Puytorac et al., 1974, using the pleurotelokinetal stomatogenesis and the special nuclear configuration (micronucleus within the perinuclear space of the macronucleus) as main diagnostic characters. Almost concomitantly, Foissner (1978, 1993) established, with the same features as Puytorac et al. (1974), the Cyrtolophosidina that now form their own clade.

In the present investigation, the platyophryid clade poses a potential problem. In the gene trees, *Ottowphrya* is more closely related to *Sorogena* than to *Platyophrya* although having the same silverline pattern and oral apparatus, but lacking the oral dome of *Sorogena* (Fig. 1, 2; Bardele, Foissner, and Blanton 1991; Foissner 1993; Foissner et al. 2002). Further, *Ottowphrya* does not produce sorocarps (see ''Results''). On the other hand, *Ottowphrya* and *Sorogena* share a typical ciliate nuclear apparatus (Bardele et al. 1991; Foissner et al. 2002; Puytorac et al. 1992), while *Platyophrya* and *Woodruffides* have a shared outer membrane of the micronucleus and macronucleus (Dragesco et al. 1977; Foissner 1993; Golder 1976). To overcome the problems, *Ottowphrya* is placed in a distinct family, Ottowphryidae n. fam.

The bursariomorphid clade (Fig. 38–40): This clade consists of *Bursaria* and *Bryometopus*. However, many more genera, not yet sequenced, may belong to the bursariomorphids, for instance, *Thylakidium*, *Paracondylostoma*, and *Bursaridium* (Foissner and Kreutz 1998). The present and previous molecular analyses (Dunthorn et al. 2011; Lynn 2008; Lynn et al. 1999) support the cladistic approach of Foissner and Kreutz (1998), showing that bursariomorphids and bryometopids have a common ancestor. The genus *Bursaria* obviously comprises at least four new species, which will be described later.

The SSU-rDNA sequences show that *Bryometopus* is paraphyletic. Morphologically, the *Bryometopus* species sequenced are rather similar, differing mainly in body shape, the location of the contractile vacuole, and the presence vs. absence of symbiotic green algae (Foissner 1993). However, the resting cysts are different: *B. atypicus* has an escape opening (see ''Results''), while *Bryometopus sphagni* has covered the wall with orange globules (lepidosomes ?) and possibly lacks an escape opening (Foissner 1993). To clarify the situation, further bursariomorphid genera and species must be sequenced.

The cyrtolophosidid clade (Fig. 38–40): The cyrtolophosidids now consist of only three genera (*Cyrtolophosis*, *Pseudocyrtolophosis*, *Aristerostoma*) because the platyophryids form their own clade. In contrast to the platyophryids, the cyrtolophosidids have a fairly distinct oral cavity (Fig. 7). Further, most have a shared outer membrane of the micronucleus and macronucleus, except for *Cyrtolophosis elongata*, for which Foissner et al. (2002) thus established the genus *Plesiocaryon*. Very recently, Quintela-Alonso, Nitsche, and Arndt (2011) published a gene sequence from *Microdiaphanosoma*, a bryometopid colpodean according to Foissner (1993), and showed its affiliation with the cyrtolophosidids. Possibly, other bryometopids will join, especially *Kreyella* and *Orthokreyella*.

The colpodid clade (Fig. 41–43): Since the first gene trees, the colpodids have been frustrating, showing (i) the ''unusual'' genera *Bardeliella* and *Ilsiella* at the base of the clade and (ii) the species of the genus *Colpoda* scattered over the whole clade, often forming clades with species from other genera, both with the ribosomal and the mitochondrial SSU rDNA (Dunthorn et al. 2008, 2009, 2011; Foissner and Stoeck 2009). For instance, *Colpoda aspera* usually forms a clade with *Hausmanniella* although it is morphologically highly similar to *C. steinii* (Foissner 1993), which is closely related to *B. metopoides*, a species with rather different morphology and ontogenesis (Foissner 2010). Now, the marynids pose a further major problem. Although their morphology is so similar to that of *Colpoda* that some species are difficult to classify (Foissner et al. 2002), their SSU rDNA is quite different, forming a clade with the bryophryids

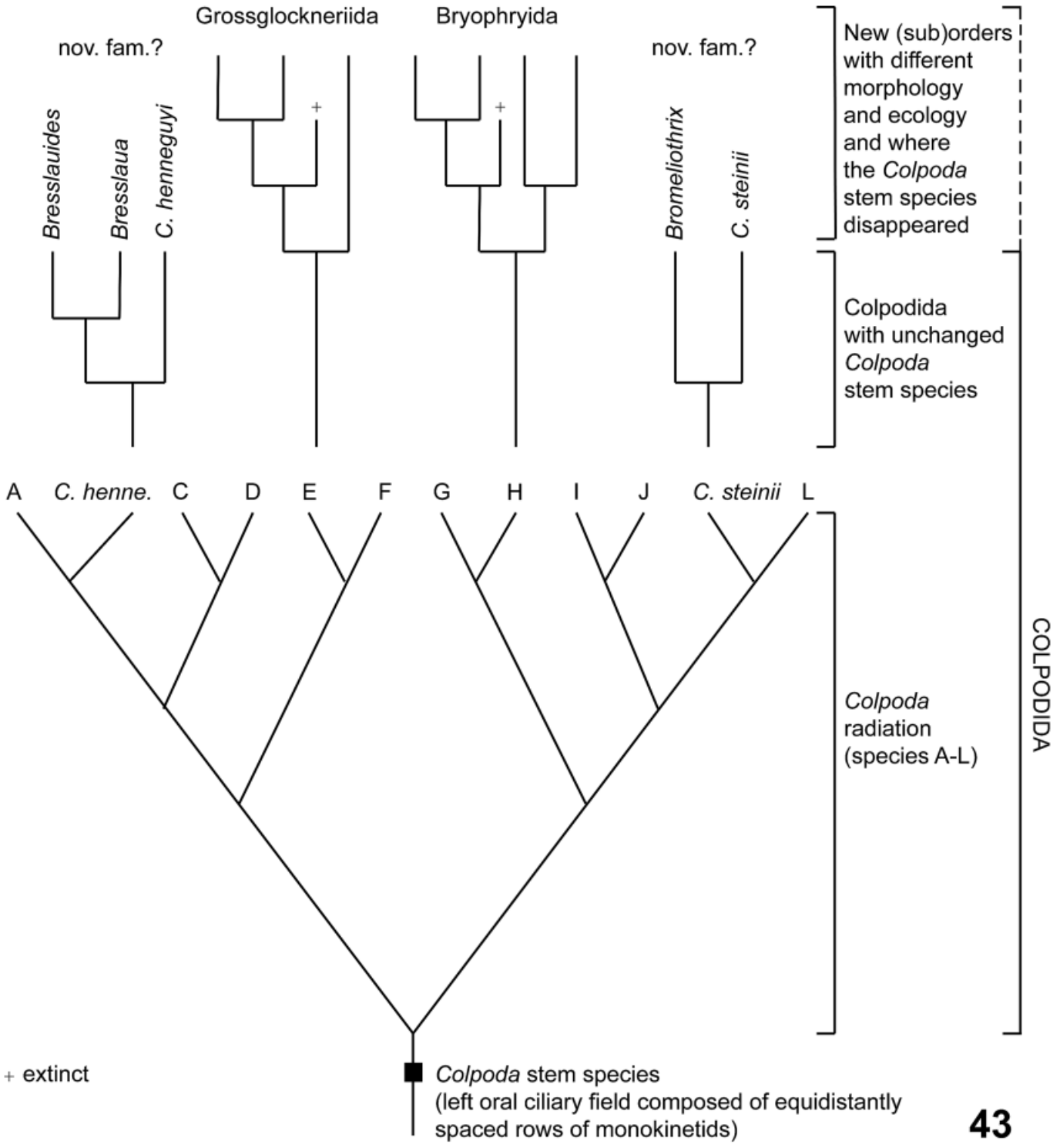


Fig. **43.** Hypothesis on evolution within the order Colpodida.

(e.g. *Notoxoma*), which have a quite different morphology (Bourland et al. 2011; Foissner 1993).

Here, we shall try to reconcile morphology and genes by adopting evolutionary systematics, which is based on phylogenetic principles and common ancestry, but considers various kinds of evolutionary processes, such as splitting, budding, and merging of lineages (Hörandl and Stuessy 2010). This concept explains the molecular ribosomal and mitochondrial data of the colpodids much better than cladistics. However, we must emphasize that only the authors W.F. and S.A. follow Mayr and Bock (2002), who developed this concept, in both the budding hypothesis and in recognizing paraphyletic taxa.

The basal position of *Bardeliella* and *Ilsiella* in the molecular trees is supported by the new morphological investigations, showing

that both have cyrtolophosidid and colpodid characteristics, especially *Bardeliella*.

In contrast to the ''large'' colpodas, the small ones are scattered over the remaining tree, forming poorly supported clades with each other or with different genera. Very likely, this is sometimes caused by the lack of data, e.g. only one of the five putative hausmanniellid genera has been sequenced (Foissner 1993, present paper). This is corroborated by the mitochondrial gene trees in which *C. aspera* and *Hausmanniella* do not form a clade (Dunthorn et al. 2011). Nevertheless, there can be no doubt that the genus *Colpoda* is paraphyletic (Fig. 41). The branching pattern and the shortness of the branches indicate an adaptive radiation of the genus *Colpoda* that currently consists of about 30 species (Foissner 1993; Foissner et al. 2002). Several, possibly most *Colpoda* species then evolved independently, forming new genera and species without significant changes in the stem species (Fig. 43), a mode of speciation that is probably quite common (Hörandl 2006). Typical examples are *C. henneguyi*, which shares a common ancestry with *Bresslaua* or *Bresslauides*; *C. steinii*, which shares a common ancestry with *Bromeliothrix*; and *C. aspera*, which shares possibly a common ancestry with *Hausmanniella* (Fig. 41). In other words, they did not evolve by ''cladistic splits,'' each producing two changed sister groups but by budding processes in which the parental branch continued essentially unchanged (Mayr and Bock 2002).

A quite similar pattern occurs, for instance, in the stichotrichine hypotrichs with the genus *Oxytricha* scattered over the whole clade (Foissner et al. 2004; Liu et al. 2010; Paiva et al. 2009; Schmidt et al. 2007). Further, such patterns are known also from multicellular organisms, e.g. fish and plants (Hörandl 2006; Koblmüller et al. 2010; Maddison 1997; Nichols 2001; Takahashi et al. 2001). In most cases, radiations result in more or less distinct discrepancies between molecular and morphological trees. The reasons may be horizontal gene transfer (including hybridization), incomplete lineage sorting, gene duplication, and extinction (Maddison 1997). None of these mechanisms can be sorted out in ciliates because both the morphological and molecular data are very incomplete, i.e. more than half of the species and genera have not yet been described (Foissner, Chao, and Katz 2008) and multigene analyses are restricted to a few model species (i.e. *Tetrahymena*, *Paramecium*). The incompleteness of data are revealed by the cladistic analysis, which largely failed in those parts of the tree where the knowledge is especially scarce, viz., between the ''large'' colpodas and *Bardeliella*.

The grossglockneriids, bryophryids, and sorogenids possibly evolved by budding from certain species of *Colpoda*-like and *Platyophrya*-like common ancestors (Fig. 43). The molecular data available reflect their morphological and ecological peculiarities very incompletely, suggesting decoupling of morphological and molecular (SSU rDNA) evolution. This is widespread, for instance, in fish from African lakes (Koblmüller et al. 2010).

Classification. The various classification systems are still hotly discussed (Hörandl 2006, 2007; Mayr and Bock 2002). However, it seems that purely cladistic (monophyletic) and/or DNA systematics has more disadvantages than evolutionary methods (Hörandl 2006; Nordal and Stedje 2005). This is supported by the present study, showing three morphologically and/or ecologically very distinct taxa within other clades (orders): the Sorogenida within the Platyophryida, and the Grossglockneriida and Bryophryida within the Colpodida (Fig. 38, 39, 42, 43). W.F. and S.A. defend a high rank of these taxa because (i) they satisfy the criteria suggested by Mayr and Bock (2002) for high ranking, (ii) the classification would be strongly distorted if the ''usual'' hausmanniellids and marynids, for example, have the same rank as the highly derived grossglockneriids and sorogenids, and (iii) these taxa will be possibly recognized as distinct orders or suborders also by their gene sequences, when more sequences are available and especially when those genes that cause the unique morphological features are investigated and compared.

The present study shows four well-supported molecular clades within the class Colpodea (Fig. 38, 39). We consider these clades as orders, as did others (Foissner 1993; Lynn 2008; Puytorac et al. 1979). To be consistent, the sorogenids, grossglockneriids, and bryophryids discussed above, should be classified as suborders at the present state of knowledge (Fig. 38, 41, 43).

Much more complex is classification within the order Colpodida, even if the grossglockneriids and bryophryids are excluded. Here ''curious clades'' emerge, consisting of *Colpoda*-like stem species and new genera budding from them. We do not expect that further investigations will change the pattern substantially because most ''curious clades'' occur in both the ribosomal and the mitochondrial SSU rDNA. Although such patterns occur also in animals and plants (Hörandl 2006; Koblmüller et al. 2010), we could not find any suggestion for a formal classification. Basically, three ways are possible: (i) establishing a new taxon for, e.g. the clade containing *C. henneguyi*, *Bresslaua*, and *Bresslauides*, including in some way the *Colpoda*-like stem species; (ii) as (i) but excluding the *Colpoda*-like stem species, or (iii) creating a new genus for each *Colpoda* stem species and including the new taxon in some way in the diagnosis of a new or existing family. We would prefer the second way because it appears otherwise impossible to make meaningful taxon circumscriptions. However, we could not force ourselves to make a decision because the data are too incomplete.

TAXONOMIC SUMMARY

Based on the data available, we suggest a revised ordinal classification of the Colpodea, using Foissner (1993) and Lynn (2008) as a guide for the diagnoses.

Class Colpodea Small and Lynn, 1981

Diagnosis. Very small to very large (~ 15–1,500 μm), holotrichously ciliated Ciliophora with slightly sinistrally spiralling kineties composed of dikinetids with well developed transverse microtubular ribbons which extend posteriorly, forming a conspicuous LKm fiber by alignment and/or overlapping with ribbons from more anterior dikinetids. Oral structures on cell surface or in a more or less deep oral cavity, highly diverse but basically composed of a right and a left ciliary field. Right field a row of dikinetids to which, in some groups, roof kineties are added; left field composed of brick- or ribbon-shaped polykinetids. Nuclear apparatus ordinary or with micronucleus in perinuclear space of macronucleus. Silverline pattern colpodid, platyophryid, or kreyellid. One or many contractile vacuoles. Extrusomes diverse, most belonging to the mucocyst type. Resting cysts highly diverse, rarely with escape opening or in aerial sorocarps, wall organic or partially inorganic (glass). Division in freely motile condition or in reproductive cysts. Stomatogenesis pleurotelokinetal or merotelokinetal, parental ciliature maintained or reorganized. With or without sex. Most terrestrial, some limnetic, few marine.

Type order. Colpodida Puytorac et al., 1974.

Remarks. The diagnosis shows the pronounced diversification of the Colpodea in all main features. For instance, it is the sole ciliate class in which silicon has been reported (Foissner et al. 2009). The strongest diagnostic feature of the class is the LKm fiber. Unfortunately, it is clearly recognizable only in the electron microscope. The class contains four orders and four suborders, all defined in the following paragraphs.

Order Platyophryida Puytorac et al., 1979 n. stat.

Diagnosis. Small to large (~ 30–300 μm), oblong to slightly reniform Colpodea with obliquely truncate, more or less projecting

anterior end bearing the oral structures on cell surface. Oral opening roundish or slit-like, apical or subapical. Right oral ciliary field a single row of dikinetids, usually forms an elliptical figure with the left oral ciliary field composed of few (~ 4) to many (~ 90) brick-shaped polykinetids arranged obliquely or in parallel with right ciliary field. Micronucleus in perinuclear space of macronucleus in some (many?) species. Some with postoral pseudomembrane consisting of short kineties with two dikinetids each along left slope of oral aperture. Silverline pattern platyophryid, rarely colpodid or kreyellid. Division in reproductive cysts or in freely motile condition. Stomatogenesis pleurotelokinetal, parental oral ciliature not reorganized. Without sex. With or without the ability to form aerial sorocarps. Most terrestrial or semiterrestrial, few limnetic.

Type family. Platyophryidae Puytorac et al., 1979.

Remarks. The platyophryids were included in the cyrtolophosidids by Foissner (1993). Further, Foissner (1993) classified the sorogenids as a distinct order. However, the small molecular difference between *Sorogena* and *Platyophrya* suggests lowering the rank to suborder level, still acknowledging the unique life cycle of *Sorogena*. Accordingly, the Platyophryida are split into two suborders: Platyophryina and Sorogenina.

Suborder Platyophryina Puytorac et al., 1979

Diagnosis. Small to moderately large (~ 30–300 μm), oblong to slightly reniform Platyophryida with subapical, slit-like oral opening. Micronucleus usually in perinuclear space of macronucleus. Left oral polykinetids obliquely arranged. Most with postoral pseudomembrane.

Type family. Platyophryidae Puytorac et al., 1979.

Remarks. See also the order Cyrtolophosidida. The Platyophryina contain four families: Platyophryidae, Woodruffiidae, Sagittariidae, and Reticulowoodruffiidae. The molecular data and silverline patterns indicate many homoplasies.

Suborder Sorogenina Foissner, 1985 n. stat.

Diagnosis. Small (~ 50 μm), ellipsoidal to slightly reniform Platyophryida with apical or subapical roughly circular or slit-like oral opening. Macronucleus and micronucleus each with separate membrane. Postoral pseudomembrane absent. Silverline pattern colpodid or platyophryid. Genus *Sorogena* with the ability to aggregate and to form aerial sorocarps. On decaying plants and terrestrial.

Type family. Sorogenidae Bradbury and Olive, 1980.

Remarks. So far, the suborder was monotypic, i.e. contained only the family Sorogenidae with the single genus and species: *Sorogena stoianovitchae*. However, *Sorogena* forms a genetic clade with *Ottowphrya*, which does not form sorocarps. Thus, we classify it into a new family, the Ottowphryidae.

Family Ottowphryidae n. fam.

Diagnosis. Moderately large to large (~ 60–300 μm), broadly reniform Sorogenina with long, slit-like oral opening and left oral polykinetids in parallel with right oral ciliary field. Macronucleus and micronucleus each with separate membrane. Silverline pattern platyophryid or colpodid. Does not form aerial sorocarps. Terrestrial.

Type genus. *Ottowphrya* Foissner et al., 2002.

Remarks. We include in the Ottowphryidae also the genus *Platyophryides* Foissner et al. 2002, which differs from *Ottowphrya* by the colpodid (vs. platyophryid) silverline pattern and division in swimming condition (vs. in reproductive cysts).

Order Bursariomorphida Fernández-Galiano, 1978.

Diagnosis. Moderately large to very large (~ 60–1,700 μm), bursiform to reniform Colpodea with large apical or subapical oral opening and deep or trough-like oral cavity. Right oral ciliary field composed of one or many rows of dikinetids, left field composed of few to many oblong polykinetids, forming a conspicuous ribbon resembling an adoral zone of membranelles. Macronucleus and micronucleus each with separate membrane. Silverline pattern colpodid or mixed kreyellid-platyophryid. Resting cysts with or without escape opening. Division in freely motile condition. Stomatogenesis pleurotelokinetal. With or without sex. Most limnetic or semiterrestrial, some terrestrial.

Type family. Bursariidae Bory de St. Vincent, 1826.

Remarks. This order contains the bursariomorphids and bryometopids; the latter were classified into a separate subclass, Bryometopia, and order, Bryometopida, by Foissner (1993). However, further morphological and genetic investigations showed a common ancestry (Foissner and Kreutz 1998; Lynn 2008; Lynn et al. 1999). As both groups are rather different morphologically, the order diagnosis becomes wide, even if not all families are included. Thus, the order will be possibly split into two or more suborders, when more sequences become available. The order contains six families: Bursariidae, Bursaridiidae, Bryometopidae, Tectohymenidae, Trihymenidae, and Jaroschiidae. Formerly, the Kreyellidae were also included (Foissner 1993). However, genetic data show that a typical kreyellid, *Microdiaphanosoma*, belongs to the cyrtolophosidids (Quintela-Alonso et al. 2011). Such a transfer may also affect the Tectohymenidae, Trihymenidae, and Jaroschiidae, which were thus not included in the order diagnosis.

Order Cyrtolophosidida Foissner, 1978

Diagnosis. Small ($\leq$ 50 μm), oblong to broadly ellipsoidal Colpodea with subapical shallow oral cavity. Right oral ciliary field a single row of dikinetids, frequently partially reduced, forms an elliptical figure with left oral ciliary field comprising up to 10 brick-shaped polykinetids. Micronucleus in perinuclear space of macronucleus in some species. Silverline pattern colpodid or kreyellid. Division in freely motile condition. Stomatogenesis pleurotelokinetal, parental oral ciliature partially reorganized. Without sex. Limnetic and terrestrial, some marine.

Type family. Cyrtolophosididae Stokes, 1888.

Remarks. Foissner (1993) included six families in the Cyrtolophosidida, of which four now belong to the Platyophryida. According to the molecular data, the cyrtolophosidids comprise the families Cyrtolophosididae and Kreyellidae (Quintela-Alonso et al. 2011); the latter were classified in the Bryometopida by Foissner (1993). The family Pseudochlamydonellidae remains incertae sedis.

Order Colpodida Puytorac et al., 1979

Diagnosis. Very small to large (~ 10–600 μm), oblong, ellipsoidal, or reniform Colpodea with oral apparatus subapical, in mid-body, or in posterior body end. Oral cavity small or large, in some groups absent. Right oral ciliary field a single row of monokinetids, dikinetids, or a complex organelle including roof kineties and/or monokinetidal ciliary fields. Left oral ciliary field composed of one to several brick-shaped polykinetids and/or a comparatively large polykinetid comprising few to many rows of monokinetids. Macronucleus and micronucleus each with a separate membrane. Silverline pattern colpodid or platyophryid. Usually divide in reproductive cysts, very rarely in freely motile condition. Stomatogenesis merotelokinetal or in a pleuromerotelokinetal mode, parental ciliature usually reorganized. Without sex. Most terrestrial, some limnetic.

Type family. Colpodidae Bory de St. Vincent, 1826.

Remarks. This order, which is the most voluminous one, is split into three suborders: Bryophryina, Grossglockneriina, and Colpodina n. subord. Rather many taxa remain incertae sedis, for instance, most small colpodas and the Hausmanniellidae. Foissner (1993) considered the Bryophryina and Grossglockneriina as orders. However, this is not supported by the molecular data. Very likely, the order contains further suborders. However, more sequences are required for a formal establishment.

The Colpodida comprise five families in Foissner (1993): Colpodidae, Hausmanniellidae, Marynidae, Bardeliellidae, Grandoriidae. Here, the number increases to nine due to the inclusion of the Grossglockneriidae and Bryophryidae and two new families, the Ilsiellidae Bourland et al. (2011) and Tillinidae n. fam.

Suborder Bryophryina Puytorac et al., 1979.

Diagnosis. Small to large (~ 30–300 μm), reniform Colpodida with subapical oral apparatus. Oral cavity very small and almost flat; through like and densely ciliated; or deep, cylindroidal, and densely ciliated. Right oral ciliary field a single row of dikinetids or a complex organelle including roof kineties; left field as described for order. Silverline pattern platyophryid or colpodid. Divide in reproductive cysts. Stomatogenesis not known. Possibly without sex. Terrestrial.

Type family. Bryophryidae Puytorac et al., 1979.

Remarks. This suborder contains only two families: Bryophryidae and, possibly, Sandmanniellidae. However, the very different oral structures indicate that there could be more, for instance, the genus *Puytoraciella* possibly needs its own family. Further, the Jaroschiidae could belong to this suborder. More sequences are needed. Presently, only three are available: *Notoxoma* (Dunthorn et al. 2008), *Sandmanniella* (Foissner and Stoeck 2009), and *Bryophrya* (Bourland et al. 2011).

Suborder Grossglockneriina Foissner, 1980.

Diagnosis. Very small to small (10–60 μm), ellipsoidal to reniform Colpodida with subapical oral apparatus on cell surface, i.e. lacking an oral cavity. In center of oral field a unique feeding tube used for puncturing cell walls of fungi and yeasts and engulfing their contents. Right oral ciliary field a single row of monokinetids, left field composed of one to several brick-shaped polykinetids. Silverline pattern colpodid. Division in reproductive cysts. Stomatogenesis merotelokinetal. Terrestrial.

Type family. Grossglockneriidae Foissner, 1980.

Remarks. Members of this suborder are defined by the unique feeding tube. The ancestral oral ciliature is strongly reduced, for instance, the right oral ciliary field is dikinetidal only during stomatogenesis. Compared with Foissner (1993), the order has been lowered to subordinal rank, acknowledging their close molecular similarity with the Colpodina.

Colpodina n. subord.

Diagnosis. Small to large (~ 30–300 μm), reniform Colpodida with subapical oral apparatus. Oral cavity small to very large, in the latter case densely ciliated by roof kineties having supraepiplasmic microtubules. Right oral ciliary field composed of a row of dikinetids and a crescentic accumulation of slightly disordered monokinetids. Left oral ciliary field a crescentic polykinetid composed of many rows of monokinetids. Postorally, a more or less pronounced (diagonal) groove, which extends obliquely onto left body side. Extrusomes globular or oblong. Silverline pattern colpodid. Division in reproductive cysts. Stomatogenesis pleuromerotelokinetal. Terrestrial and limnetic.

Type family. Tillinidae n. fam.

Remarks. Based on the sequence data, the Colpodina comprise two families: Colpodidae and Tillinidae n. fam. Very likely, further families will be classified into this suborder, when more sequences become available (see text and Fig. 42 for reasoning of the suborder).

Tillinidae n. fam.

Diagnosis. Colpodina with *T. magna* organization, i.e. with large body having a distinct postoral groove; contractile vacuole with collecting canals extending to or near to anterior body end; large, conical to tubular oral cavity; several to many roof kineties, and oblong extrusomes.

Type genus. *Tillina* Gruber, 1879.

Tillina Gruber, 1879.

Improved diagnosis. As for the family.

Type species (by monotypy). *Tillina magna* Gruber, 1879.

Remarks. See Foissner (1993) for a monograph on the type species. *Tillina* has been synonymized with *Colpoda* by most authors. However, the present data suggest resurrection. Very likely, *T. magna* is a complex of morphologically rather similar but genetically different species, as indicated by the new species shown in the molecular tree (Fig. 42). Gruber (1879) did not provide a formal diagnosis of *Tillina*.

The roof kineties are an important feature in the definition of the family because they have both, a special location and fine structure, i.e. supraepiplasmic microtubules (Lynn 1976). This kind of microtubules occurs also in the ''lower'' colpodeans, viz., *Bursaridium* in which, however, they occur in all ciliary rows (Foissner 1993).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Most likely ML tree with bootstrap support of the nuclear SSU-rDNA phylogeny of the Colpodea from the alignment masked by eye.

Fig. S2. MrBayes tree with posterior probability support of the nuclear SSU-rDNA phylogeny of the Colpodea from the alignment masked by eye.

Fig. S3. PhyloBayes tree with posterior probability support of the nuclear SSU-rDNA phylogeny of the Colpodea from the alignment masked by eye.

Fig. S4. Nuclear SSU-rDNA phylogeney of the Colpodea from the alignment masked by gBlocks. The most likely ML tree is shown. Node support is as follows: ML bootstrap/MrBayes posterior probability/PhyloBayes posterior probability. Values <50 are shown as '-'. New sequences are in bold.

Fig. S5. Most likely ML tree with bootstrap support of the nuclear SSU-rDNA phylogeny of the Colpodea from the alignment masked by gBlocks.

Fig. S6. MrBayes tree with posterior probability support of the nuclear SSU-rDNA phylogeny of the Colpodea from the alignment masked by gBlocks.

Fig. S7. PhyloBayes tree with posterior probability support of the nuclear SSU-rDNA phylogeny of the Colpodea from the alignment masked by gBlocks.

Fig. S8. Nuclear SSU-rDNA phylogeny of the Colpodida (colpodids) from the alignment masked by gBlocks. The most likely ML tree is shown. Node support is as follows: ML bootstrap/MrBayes posterior probability/PhyloBayes posterior probability. Values <50 are shown as '-'. New sequences are in bold.

Fig. S9. Most likely ML tree with bootstrap support of the nuclear SSU-rDNA phylogeny of the Colpodida (colpodids) from the alignment masked by eye.

Fig. S10. MrBayes tree with posterior probability support of the nuclear SSU-rDNA phylogeny of the Colpodida (colpodids) from the alignment masked by eye.

Fig. S11. PhyloBayes tree with posterior probability support of the nuclear SSU-rDNA phylogeny of the Colpodida (colpodids) from the alignment masked by eye.

Fig. S12. Most likely ML tree with bootstrap support of the nuclear SSU-rDNA phylogeny of the Colpodida (colpodids) from the alignment masked by gBlocks.

Fig. S13. MrBayes tree with posterior probability support of the nuclear SSU-rDNA phylogeny of the Colpodida (colpodids) from the alignment masked by gBlocks.

Fig. S14. PhyloBayes tree with posterior probability support of the nuclear SSU-rDNA phylogeny of the Colpodida (colpodids) from the alignment masked by gBlocks.

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