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Re-analysis of the 18S rRNA gene phylogeny of the ciliate class Colpodea

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Abstract

We critically re-analyzed the 18S rRNA gene phylogeny of the ciliate class Colpodea where four main lineages have been recognized: (1) Bursariomorphida including bryometopids, (2) Platyophryida including sorogenids, (3) Cyrtolophosidida, and (4) Colpodida including bryophryids and grossglockneriids. The Platyophryida branched off first and the Cyrtolophosidida and Colpodida were classified as sister groups. On basis of multiple statistical tests, we unraveled three problematic issues in colpodean phylogenies: the positions of the Bursariomorphida and Platyophryida are unstable and depend on alignment masking; a sister relationship of the Platyophryida and Cyrtolophosidida cannot be excluded by any statistical tree topology test; and clustering of bryophryids and grossglockneriids outside the Colpodida are also statistically valid possibilities. Natural classification of the highly diverse order Colpodida remains puzzling, possibly due to the lack of a phylogenetic signal and morphostasis of the oral ciliature in several *Colpoda*-like lineages. According to the "Ur-*Colpoda*" hypothesis, *Colpoda* represents the stem lineage from which both *Colpoda*-like and morphologically more derived taxa might have branched off. This evolutionary concept preserves not only information on morphology, ecology, and evolutionary processes of colpodid ciliates, but also aids practicability because the connection to the traditional literature is optimally maintained.

Keywords: Ciliophora; Evolutionary taxonomy; Morphological evolution; Morphostasis; Oral ciliature; Paraphyly

Introduction

Ciliates of the class Colpodea Small and Lynn, 1981 are widespread all over the globe in a broad range of terrestrial and semiterrestrial habitats, such as mosses, leaf litter, soil, bark of trees, astatic puddles, hay infusions and tree holes (for a review, see Foissner 1993). Few colpodeans live in limnetic environments, ranging from ponds and lakes to running waters (Foissner et al. 1991, 1999). Only very few species have been reported from marine environments (Dunthorn et al. 2009) although molecular surveys suggest that saltwater taxa may not be so rare (Dunthorn et al. 2014; Gimmler et al. 2016). Terrestrial species have an r-selected survival strategy that is typically characterized by a small body size, high reproductive capacity and production of dormant cysts which are very resistant to desiccation and various other physical influences of the environment (Foissner 1987). Although the structure of the somatic cortex of colpodeans is conserved, their oral apparatus is highly diverse (Foissner 1993; Lynn 2008). This was not only the reason for misclassification of several species in the past but it is also a reflection of the broad food spectrum and ecological function of these ciliates in nature. Many colpodeans with a small buccal cavity are important grazers of bacteria (Foissner 1993), others with a huge buccal cavity are rapacious predators feeding on other

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ciliates and even rotifers (e.g., Foissner 1987, 1990; Lynn 1979), while those with a distinct cyrtos are diatom eaters (Buitkamp et al. 1989; Foissner 1993) and those with a special tube are fungi feeders (Aescht et al. 1991; Foissner 1980; Petz et al. 1985, 1986).

In the late 70's and early 80's of the past century, the structural conservatism of the somatic cortex helped to recognize this monophyletic group of ciliates as a distinct class (Lynn 1976, 1978, 1979; Small and Lynn 1981). However, only recently have colpodeans found their phylogenetic home within the CONThreeP super-cluster of the subphylum Intramacronucleata (Gentekaki et al. 2017; Lynn and Kolisko 2017; Lynn et al. 2018). Our view on the intraclass classification and evolution of colpodeans significantly changed in the light of 18S rRNA gene phylogenies. Foissner et al. (2011) made the first steps towards a natural phylogeny-based systematics and taxonomy of colpodeans and tried to reconcile morphological and molecular frameworks. The most important changes occurred at the order level. The order Bursariomorphida Fernández-Galiano, 1979 was found to include also the order Bryometopida Foissner, 1985. The name-bearing order Colpodida Puytorac et al., 1974 was recognized as the crown group of the whole class, containing also members of the orders Grossglockneriida Foissner, 1980 and Bryophryida Puytorac et al., 1979. The order Cyrtolophosidida Foissner, 1978 was recognized to be biphyletic and all its families, except for the nominotypical one, were transferred, together with the order Sorogenida Foissner, 1985, into the reactivated order Platyophryida Puytorac et al., 1979.

The steadily growing amount of new 18S rRNA gene sequences indicated that phylogenetic relationships within the Colpodida are very complex (Bourland et al. 2011, 2012, 2014; Dunthorn et al. 2012; Foissner et al. 2014; Quintela-Alonso et al. 2011), which led to resurrection of multiple synonymized genera and establishment of new ones: *Tillina* Gruber, 1879; *Repoma* Novotny, 1970; *Paracolpoda* Lynn, 1978; *Exocolpoda* Foissner et al., 2002; and *Emarginatophrya* Foissner, 2016. However, such an approach did not completely erase the paraphyly problem of the genus *Colpoda* Müller, 1773 and its nominotypical family Colpodidae Bory de St. Vincent, 1826. This has stimulated us to (1) critically re-analyze the 18S rRNA gene phylogeny of the class Colpodea; (2) examine the information contents of the colpodean 18S rRNA gene; (3) visualize the conflict between equally probable 18S rRNA gene phylogenies; and (4) reconstruct colpodean oral evolution accounting for phylogenetic uncertainties.

Material and Methods

Alignments, genetic distances and nucleotide homologies

All high quality 18S rRNA gene sequences of members of the class Colpodea were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) and aligned on the Guidance2 server (http://quidance.tau.ac.il/ver2/), using the MAFFT algorithm and 100 bootstrap repeats (Sela et al. 2015). Taxa cover all main colpodean lineages and their higher classification follows Foissner et al. (2011). Our rationale for selection the outgroup was based on the recent phylogenomic analyses that recognized the Nassophorea as a sister class of the Colpodea (e.g., Gentekaki et al. 2017; Lynn and Kolisko 2017; Lynn et al. 2018). The reliability of the base multisequence alignment was estimated to be 0.98 by comparing bootstrap trees to the alignment algorithm. Six datasets were generated from the base multisequence alignment: unmasked dataset and five datasets masked with cutoff values of 0.50, 0.75, 0.80, 0.90 and 0.93.

Average pairwise evolutionary divergences were estimated from the unmasked alignment in MEGA ver. 7.0 (Kumar et al. 2016), using the maximum composite likelihood model (Tamura et al. 2004) and 1000 bootstrap replicates. The rate variation among sites was assigned a gamma distribution having a shape parameter of 0.4, and evolutionary distances were calculated under the heterogenous substitution pattern among lineages (Tamura and Kumar 2002). Prior to analysis, all ambiguous positions and alignment gaps were removed for each sequence pair. The resulting pairwise genetic distances were assessed by multi-dimensional scaling, as implemented in the scikit-learn ver. 0.19.1 package in Python (Pedregosa et al. 2011). The SMACOF algorithm was run with 250 initializations, each run had 20 000 iterations, and ε was set to 1e–8 to declare convergence. Plotting of the ordination diagram was done with the Matplotlib module (Hunter 2007) and custom Python scripts.

The number of primary cluster-supporting nucleotide homologies was calculated for each dataset separately using the computer program SAMS (Wägele and Mayer 2007). Classification of types of nucleotide supporting positions follows Wägele and Rödding (1998). Briefly, symmetric, asymmetric and noisy positions were recognized. Symmetric (binary) positions have a different character state in each group of a split and hence support both groups equally. Asymmetric positions support only one group which possesses the same nucleotide at a particular position, while the other group contains different and more than one character state at this position. Noisy positions represent convergences or chance similarities between groups, as the same character state is present in all sequences of one group but also at least in one sequence of the other group.

Tree- and network-building methods

Prior to construction of phylogenetic trees, the best fitting evolutionary substitution models were found for each alignment under the Akaike Information Criterion in the jModelTest ver. 0.1.1 (Guindon and Gascuel 2003; Posada 2008). The best evolutionary models were summarized in Supplementary Table S1. Maximum likelihood (ML) analyses were performed under the GTR + Γ + I model in PhyML ver. 3.0 with SPR swapping algorithm and 1000 non-parametric

bootstrap replicates on the South of France bioinformatics platform (http://www.atgc-montpellier.fr/phyml/) (Guindon et al. 2010). Bayesian analyses were conducted on the CIPRES portal ver. 3.1 (http://www.phylo.org/), using the program MrBayes (Ronquist et al. 2012) on XSEDE ver. 3.2.6 (Miller et al. 2010), with two independent runs each having four chains 5 000 000 generation long. Prior parameters of the GTR + Γ + I models were specified with the 'lset' and 'prset' commands. Every 100th tree was sampled and the first 25% of trees were discarded as burn-in. Maximum clade credibility trees were constructed and calculation of posterior probabilities of their nodes was based on the remaining 75% of trees.

Conflicts between equally probable trees sampled during the stationary phase of the Bayesian analysis were visualized with Dendroscope ver. 2.7.4 as softwired galled networks (Huson and Scornavacca 2012). They were constructed from a set of 1000 randomly selected post burn-in trees for each dataset separately. A threshold of 20% was set as a minimum fraction of trees that must contain a cluster for that cluster to be included in a galled network. Alternative evolutionary trajectories, i.e., edges leading into reticulate nodes, were marked by curved lines. Apart from the galled networks, also phylogenetic networks were built in SplitsTree ver. 4 (Huson 1998; Huson and Bryant 2006), using the neighbornet algorithm with uncorrected distances (Bryant and Moulton 2004). The reliability of the phylogenetic networks was assessed by bootstrap analyses with 1000 replicates.

Statistical tree topology tests

The reliability of competing tree topologies was statistically tested, using the approximately unbiased test, the weighted Shimodaira–Hasegawa test and the weighted Kishino–Hasegawa test. Unconstrained and alternative trees were built under the best GTR + Γ + I evolutionary models, with a heuristic search, random sequence addition, and the SPR swapping algorithm in PAUP* ver. 4.0b8 (Swofford 2003). Consequently, per-site log likelihoods of the best scoring unconstrained and alternative trees were calculated and served for the estimation of *p*-values of the three topology tests. These were conducted using the programs makermt, consel and catpv from the package CONSEL (Shimodaira and Hasegawa 2001).

Reconstruction of ancestral oral traits

Evolution of the right and left side oral ciliature was performed in SIMMAP ver. 1.5.2 (Bollback 2006). Data were obtained mostly from Foissner (1993) and Dunthorn et al. (2012). Character coding was summarized in Supplementary Table S2. All character states were treated as unordered. Prior for the beta distribution of state frequencies and for the gamma distribution of the overall rate of character change were estimated on the 50%-majority rule consensus Bayesian trees inferred from the unmasked and 0.90 alignments, using the MCMC analysis implemented in SIMMAP. The best fitting distributions were found from the posterior distributions of the MCMC analyses with the R script included in the SIMMAP package. Phylogenetic uncertainty was incorporated by a set of 1000 randomly selected post burn-in trees from the Bayesian MCMC analyses. Branch lengths were rescaled so that the overall length of each tree is one. Ten samples were analyzed per tree with 20 priors drawn from the prior distribution. Results were plotted as pie charts and mapped onto the 50%-majority rule consensus Bayesian trees, with the help of the R script "PlotSimMap.R" (https://github.com/nylander/PlotSimMap).

Results

Phylogenetic relationships among main colpodean lineages

All phylogenetic techniques consistently recognized four main colpodean lineages with full or very strong statistical support in the six alignments analyzed: (1) Bursariomorphida including bryometopids, (2) Platyophryida including sorogenids, (3) Cyrtolophosidida and (4) Colpodida including bryophryids and grossglockneriids (Fig. 1, Supplementary Fig. S1). Phylogenetic relationships between these major clades were at first glance very well resolved and typically strongly supported statistically in Bayesian inferences while variably in maximum likelihood (ML) analyses (Fig. 1, Supplementary Fig. S1). The orders Colpodida and Cyrtolophosidida were classified as sister groups in all Bayesian and ML trees. This relationship was stable without any conflict also in the softwired galled networks constructed from thousands of phylogenetic trees sampled during the stationary phase of the Bayesian MCMC analyses (Fig. 2, Supplementary Fig. S2). Similarly, the Colpodida and Cyrtolophosidida formed a distinct split in NeighborNet analyses but its length and bootstrap support decreased from 95% to 69% along with the strength of the alignment masking (Table 1; Supplementary Fig. S3, arrowheads). The sister group relationship of the Colpodida and Cyrtolophosidida was consistently corroborated by four symmetric and five to seven asymmetric nucleotide positions (Table 1), which further underpined the robustness recognized in phylogenetic trees.

The phylogenetic position of the Bursariomorphida and Platyophryida was usually statistically corroborated but conflicted between the alignments analyzed. The Bursariomorphida branched off first in the unmasked, 0.50 and 0.75 alignments, while the Platyophryida branched off first in the 0.80–0.93 alignments (Fig. 1, Supplementary Fig. S1). The same pattern was observed in the galled networks while a conflicting position of the Platyophryida was revealed only in the 0.75 alignment (Supplementary Fig. S2). The placement of the Bursariomorphida was consistent in the phylogenetic networks, since they were separated together with the outgroup from the rest of colpodeans by a distinct set of parallel edges whose length and statistical support decreased from 85% to 59% with the strength of the alignment masking

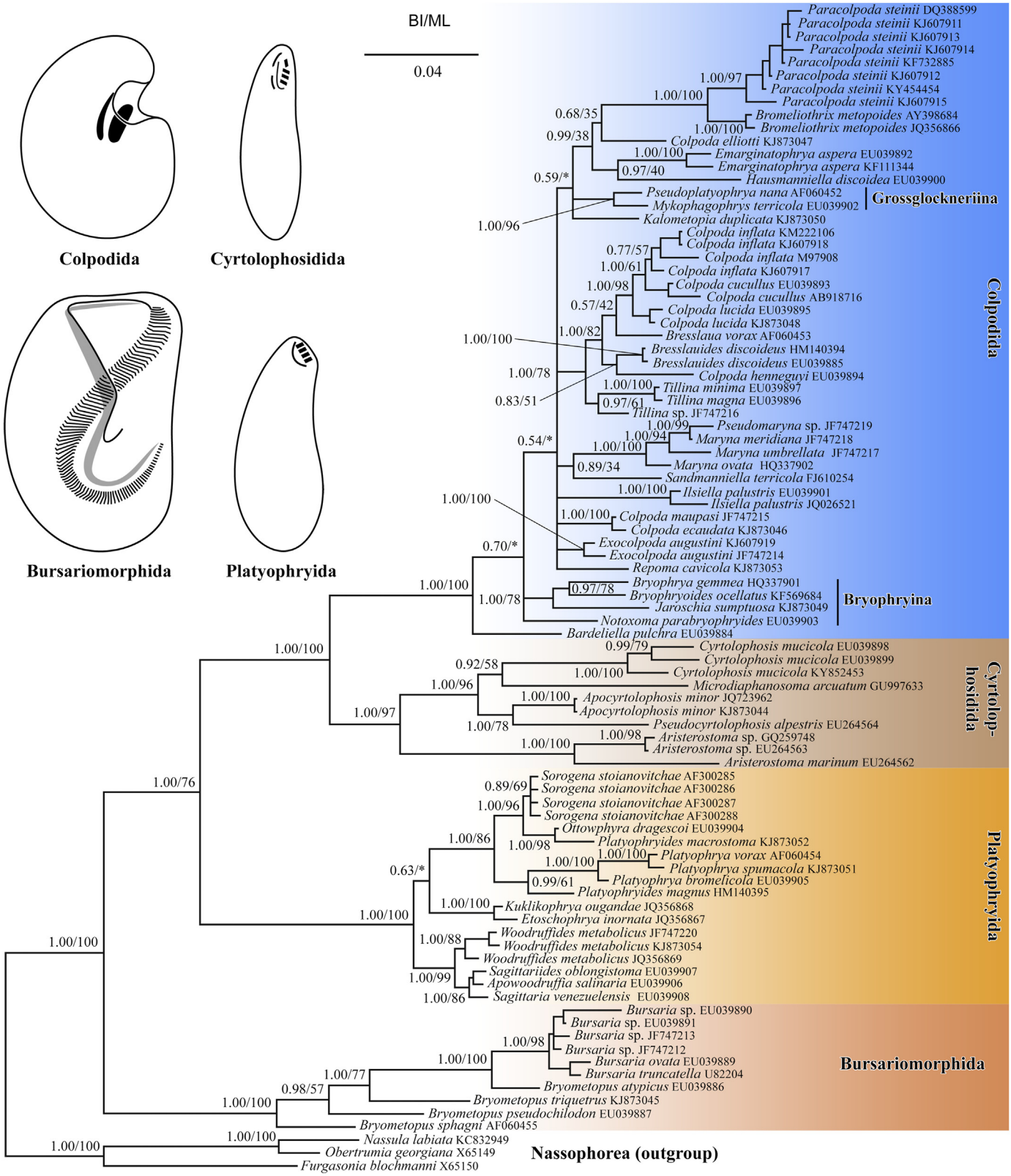

Fig. 1. 18S rRNA gene phylogeny of colpodean ciliates inferred from the unmasked alignment. There are four main lineages: Bursariomorphida, Platyophryida, Cyrtolophosidida and Colpodida including Bryophryina and Grossglockneriina. Bursariomorphida include Bryometopina and branch off first. Cyrtolophosidida and Colpodida are depicted as sister groups. Posterior probabilities for Bayesian Inference (BI) and bootstrap values for Maximum Likelihood (ML) are mapped onto the 50%-majority rule Bayesian consensus tree. Asterisks indicate mismatch in topology between Bayesian and ML tree. The scale bar denotes four substitutions per one hundred nucleotide positions.

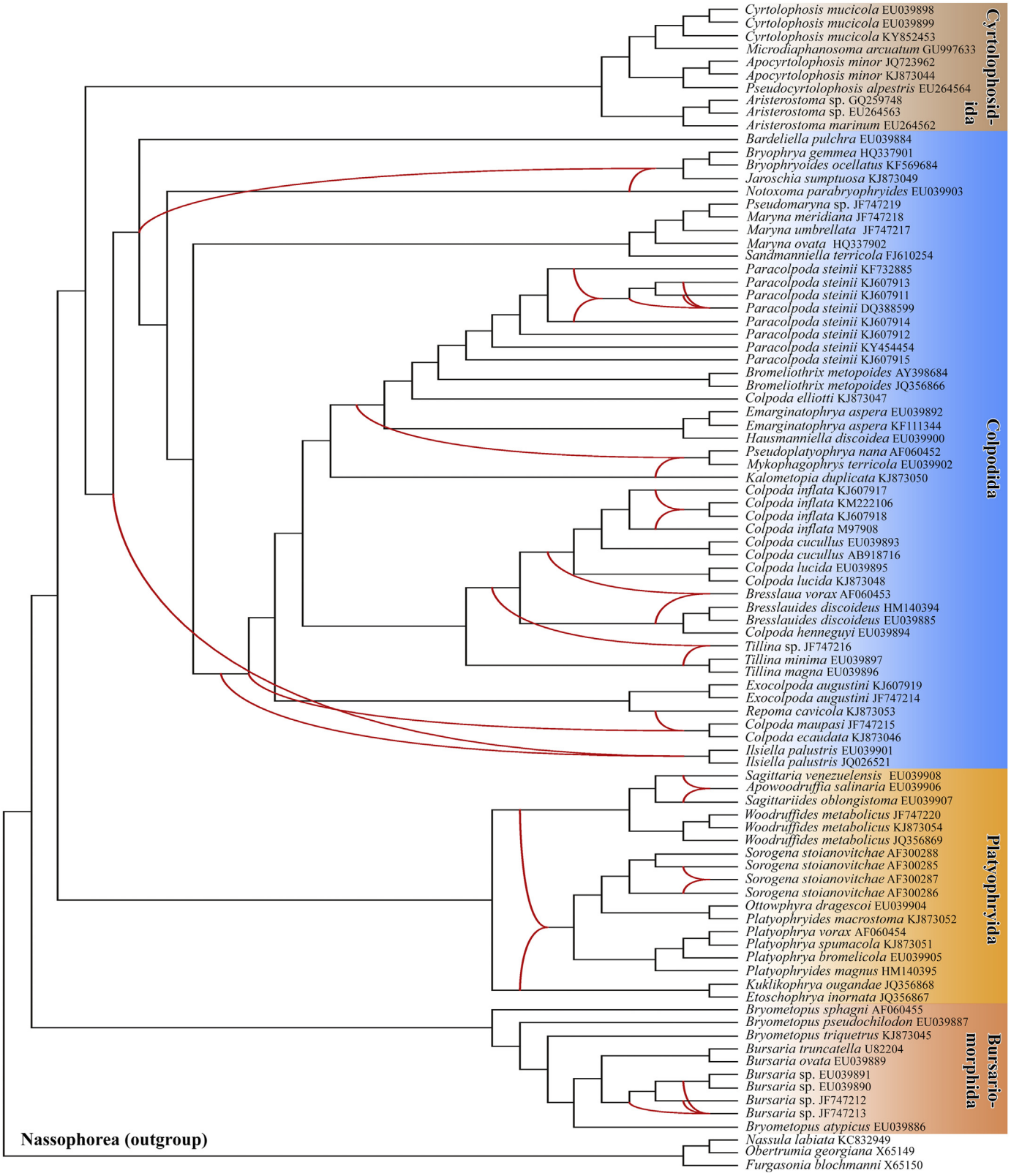

Fig. 2. Softwired galled network constructed from a set of thousand randomly selected trees obtained during the stationary phase of the Bayesian MCMC analyses of the unmasked alignment. A threshold of 20% was set as a minimum fraction of trees that must contain a cluster for that cluster to be included in the network. Alternative evolutionary trajectories, i.e., edges leading into reticular nodes, are marked by curved lines. Most conflicts concern the phylogenetic position of *Ilsiella*, bryophryids (*Bryophrya*, *Bryophryides*, *Jaroschia* and *Notoxoma*) and grossglockneriids (*Mycophagophrys* and *Pseudoplatyophrya*). *Ilsiella* and bryophryids are thus equally good candidates for being the deepest branches within the order Colpodida.

Table 1. Support for phylogenetic relationships among main colpodean lineages, as obtained with NeighborNet (NN), Bayesian Inference (BI), Maximum Likelihood (ML) and Split Spectrum (SS) analyses.

Taxa bipartition[a]	Alignment[b]	NN[c]	BI[d]	ML[e]	SS[f]
(Outgroup), (Colpodea)	Unmasked	99	1.00/1.00	100/100	6, 18, 0/6, 1, 15
	0.50	99	1.00/1.00	100/100	6, 18, 0/6, 1, 15
	0.75	99	1.00/1.00	100/100	6, 13, 0/6, 0, 13
	0.80	99	1.00/1.00	100/100	6, 12, 0/6, 0, 12
	0.90	99	1.00/1.00	100/100	6, 11, 0/6, 0, 12
	0.93	98	1.00/1.00	100/100	6, 11, 0/6, 0, 11
(Outgroup, Bursariomorphida),	Unmasked	85	1.00/1.00	100/76	4, 6, 38/4, 2, 35
(Platyophryida, Cyrtolophosidida, Colpodida)	0.50	78	1.00/1.00	100/72	4, 5, 19/4, 2, 35
	0.75	76	1.00/1.00	100/56	3, 3, 19/3, 2, 32
	0.80	69	–	100/51	3, 3, 12/3, 2, 30
	0.90	53	–	–	3, 1, 11/3, 2, 24
	0.93	59	–	–	3, 1, 9/3, 2, 22
(Outgroup, Platyophryida),	Unmasked	–	–	–	–
(Bursariomorphida, Cyrtolophosidida, Colpodida)	0.50	–	–	–	–
	0.75	–	–	–	–
	0.80	–	1.00/0.77	–	–
	0.90	–	1.00/1.00	100/65	–
	0.93	76	1.00/1.00	100/66	–
(Outgroup, Bursariomorphida, Platyophryida),	Unmasked	95	1.00/1.00	76/100	4, 2, 10/4, 7, 21
(Cyrtolophosidida, Colpodida)	0.50	97	1.00/1.00	72/99	4, 2, 10/4, 7, 20
	0.75	89	0.77/1.00	56/95	4, 1, 7/4, 7, 15
	0.80	87	0.77/1.00	51/94	4, 1, 7/4, 5, 15
	0.90	–	1.00/1.00	65/87	4, 0, 3/4, 5, 7
	0.93	69	1.00/1.00	66/86	4, 0, 3/4, 4, 4
(Outgroup, Bursariomorphida, Platyophryida, Cyrtolophosidida),	Unmasked	98	1.00/1.00	100/100	0, 6, 19/0, 5, 21
(Colpodida)	0.50	99	1.00/1.00	99/100	0, 6, 18/0, 5, 20
	0.75	98	1.00/1.00	95/96	0, 5, 13/0, 4, 18
	0.80	98	1.00/1.00	94/95	0, 5, 12/0, 4, 17
	0.90	98	1.00/1.00	87/93	0, 5, 6/0, 2, 6
	0.93	98	1.00/1.00	87/88	0, 4, 6/0, 2, 5

[a] Taxa bipartitions were represented in the Newick tree format.
[b] Six alignments were analyzed, unmasked and masked by Guidance2 with five different cut off values ranging from 0.50 to 0.93.
[c] Bootstrap support was calculated in SplitsTree with 1000 bootstrap replicates.
[d] Posterior probabilities were calculated in MrBayes. First values refer to the first group of a split while second values refer to the second group of a split.
[e] Bootstrap support was calculated in PhyML with 1000 bootstrap replicates. First values refer to the first group of a split while second values refer to the second group of a split.
[f] Numbers of split supporting nucleotide homologies were calculated in SAMS. Values refer to symmetric, asymmetric and noisy nucleotide positions supporting the first/second group of a split.

Table 2. Log likelihoods and *p*-values of the AU (approximately unbiased), WSH (weighted Shimodaira–Hasegawa), and WKH (weighted Kishino–Hasegawa) tests to compare different topological scenarios. Significant differences (*p*-value <0.05) between the best unconstrained and constrained topologies in bold face.

Topology	Alignment	Log likelihood (−ln L)	Δ (−ln L)[a]	AU	WSH	WKH	Conclusion
Best scoring maximum likelihood tree (unconstrained)	Unmasked	14,763.1686	–	0.831	0.993	0.635	–
	0.75	12,906.5545	–	0.679	0.995	0.550	–
	0.90	11,537.8243	–	0.598	0.967	0.502	–
Monophyly of *Bryometopus*	Unmasked	14,830.8533	67.68	**0.007**	**0.022**	**0.005**	**Rejected**
	0.75	12,968.0879	61.53	**1e–05**	**3e–04**	**1e–04**	**Rejected**
	0.90	11,598.6103	60.79	**0.000**	**1e–04**	**1e–04**	**Rejected**
Monophyly of the Bryometopida sensu Foissner (1993) (*Bryometopus* + *Jaroschia* + *Microdiaphanosoma*)	Unmasked	15,227.0795	463.91	**0.001**	**0.000**	**0.000**	**Rejected**
	0.75	13,289.0485	382.49	**0.000**	**0.000**	**0.000**	**Rejected**
	0.90	11,858.7891	320.96	**8e–07**	**0.000**	**0.000**	**Rejected**
Monophyly of Cyrtolophosidida and Platyophryida	Unmasked	14,781.8921	18.72	0.210	0.519	0.135	Not rejected
	0.75	12,917.8326	11.28	0.234	0.624	0.197	Not rejected
	0.90	11,550.2597	12.43	0.247	0.681	0.248	Not rejected
Monophyly of Colpodida sensu Foissner (1993)	Unmasked	14,792.8461	29.68	0.115	0.322	0.079	Not rejected
	0.75	12,930.4407	23.89	**0.034**	0.186	**0.039**	**Rejected**
	0.90	11,549.2711	11.45	0.232	0.760	0.232	Not rejected
Monophyly of Colpodida incl. Grossglockneriina (i.e., Bryophryina clustering outside Colpodida)	Unmasked	14,767.9056	4.74	0.268	0.162	0.625	Not rejected
	0.75	12,908.1197	1.56	0.537	0.949	0.450	Not rejected
	0.90	11,538.0757	0.25	0.582	0.947	0.498	Not rejected
Monophyly of *Colpoda* sensu Foissner (1993)	Unmasked	14,969.1681	206.00	**0.001**	**0.000**	**0.000**	**Rejected**
	0.75	13,082.1002	175.55	**0.000**	**0.000**	**0.000**	**Rejected**
	0.90	11,684.3465	146.52	**0.003**	**0.000**	**0.000**	**Rejected**
Monophyly of *Colpoda* incl. *Bromeliothrix*	Unmasked	14,853.5777	90.41	**4e–05**	**0.005**	**0.001**	**Rejected**
	0.75	12,982.0423	75.49	**2e–04**	**0.001**	**2e–04**	**Rejected**
	0.90	11,610.4014	72.58	**0.002**	**0.010**	**0.002**	**Rejected**
Monophyly of Hausmanniellidae (*Hausmanniella* + *Kalometopia* + *Bresslauides*)	Unmasked	14,820.5404	57.37	**0.007**	**0.033**	**0.007**	**Rejected**
	0.75	12,956.7026	50.15	**0.004**	**0.012**	**0.003**	**Rejected**
	0.90	11,589.6323	51.81	**0.002**	**0.022**	**0.004**	**Rejected**
Sister relationship of Marynidae and Ilsiellidae	Unmasked	14,765.5089	2.34	0.555	0.993	0.635	Not rejected
	0.75	12,908.0885	1.53	0.517	0.951	0.390	Not rejected
	0.90	11,539.1089	1.28	0.534	0.949	0.443	Not rejected
Monophyly of *Maryna*	Unmasked	14,784.0550	20.89	0.191	0.455	0.101	Not rejected
	0.75	12,927.6173	21.06	**0.024**	0.193	**0.041**	**Rejected**
	0.90	11,557.7743	19.95	0.086	0.505	0.124	Not rejected

[a] Difference between log likelihoods of constrained and best scoring (unconstrained) tree.

(Table 1; Supplementary Fig. S3, arrows). Nevertheless, this split was still corroborated by three or four symmetric and one to six asymmetric nucleotide positions present in the six alignments analyzed (Table 1). On the other hand, there were no splits separating the Platyophryida together with the outgroup from the rest of colpodeans in the phylogenetic networks. There were only some strongly statistically supported parallel edges that connected platyophryids with some but not all outgroup taxa (Supplementary Fig. S3). Interestingly, any primary nucleotide positions supporting the split of platyophryids from the rest of colpodeans were found neither in the unmasked nor in the masked alignments. Therefore, the platyophryids-early hypothesis inferred from the strongly masked alignments is very likely artifactual and based on noisy positions and evolutionary substitution models. On the other hand, the bursariomorphids-early hypothesis may be artifactual as well due to problems with homology assessment of some nucleotide positions in the unmasked alignment. Since a sister group relationship of platyophryids and cyrtolophosidids could not be rejected by any statistical tree topology test (Table 2), the phylogenetic position of platyophryids in the strongly masked alignments should be, indeed, taken with caution. Nevertheless, there are no primary nucleotide positions corroborating the sister relationship between platyophryids and cyrtolophosidids. Consequently, their close relatedness is unlikely although not rejected by statistical tree topology tests.

Phylogenetic relationships within the order Colpodida

Tree- and network building methods consistently recognized with full or very strong statistical support the monophyletic origin of the order Colpodida, as defined by Foissner et al. (2011), i.e., including also members of the orders Bryophryida and Grossglockneriida (Fig. 1, Supplementary Figs. S1 and S3). This highly diverse cluster was supported also by asymmetric and noisy nucleotide positions, whose number ranged from five asymmetric and 21 noisy positions in the unmasked alignment to two asymmetric and five noisy positions in the 0.93 alignment (Table 1). Monophyly of the order Colpodida as defined by Foissner (1993), i.e., without bryophryids and grossglockneriids, could not be rejected by any statistical tree topology test performed on the unmasked and 0.90 alignments (Table 2). However, it could be rejected for the 0.75 alignment by the approximately unbiased ($p = 0.034$) and weighted Kishino–Hasegawa ($p = 0.039$) tests, but not by the weighted Shimodaira–Hasegawa test ($p = 0.186$). Interestingly, also clustering of bryophryids outside the Colpodida including grossglockneriids could not be refuted by any statistical tree topology test performed on all alignments (Table 2). None of these scenarios was, however, supported by primary nucleotide homologies, suggesting that the crown radiation of colpodids was very rapid and did not allow evolution and/or fixation of molecular synapomorphies in the comparatively conservative 18S rRNA gene.

Kinships within the order Colpodida were poorly resolved and usually weakly supported statistically. Many conflicts were recognized in the NeighborNet analyses (Supplementary Fig. S3) and in the softwired galled networks constructed from thousands of trees obtained during the stationary phase of the Bayesian MCMC analyses (Fig. 2, Supplementary Fig. S2). Network patterns thus revealed poor informativeness of the 18S rRNA gene to unambiguously resolve phylogenetic relationships among colpodids. Most conflicts concerned the phylogenetic position of *Bardeliella*, *Ilsiella*, *Sandmanniella*, marynids, bryophryids and grossglockneriids (Fig. 2). Most of these taxa were indicated equally good candidates for the deepest branches within the order Colpodida sensu Foissner et al. (2011).

Topology testing helped us to constrain some relationships within the core colpodids. Monophyly of the family Colpodidae sensu Foissner (1993) and its name-bearing genus *Colpoda* sensu Foissner (1993) could be rejected by all tree statistical topology tests conducted on the unmasked, 0.75 and 0.90 alignments. Likewise, a monophyletic origin of the family Hausmanniellidae sensu Foissner (1993) could be refuted. On the other hand, monophyly of the genus *Maryna* as well as of the families Marynidae and Ilsiellidae, both having a posteriorly displaced oral apparatus, could not be excluded by most topology tests given the three alignments analyzed and their best GTR + Γ + I evolutionary substitution models. The single exceptions were the approximately unbiased ($p = 0.024$) and weighted Kishino–Hasegawa ($p = 0.041$) tests conducted on the 0.75 alignment, which rejected monophyly of *Maryna* (Table 2).

Multi-dimensional scaling of taxa from the order Colpodida, based on genetic distances calculated under the heterogenous substitution pattern among lineages, revealed that (Fig. 3): (1) bryophryids, marynids and ilsiellids represent a distinct cluster each, (2) *Bardeliella* and *Sandmanniella* are orphan genera within the order Colpodida, (3) *Repoma*, *Kalometopia* and *Exocolpoda* are orphan genera within the core colpodids, and (4) traditional *Colpoda* species either form small isolated clusters (e.g., *C. maupasi* and *C. ecaudata*) or group with morphologically more derived genera (e.g., *Bresslaua*, *Bresslauides*, *Bromeliothrix* or *Hausmanniella*). Multi-dimensional scaling analyses thus indirectly corroborated non-monophyly of the family Colpodidae as well as of its type genus *Colpoda*. Pairwise genetic distances of colpodids were summarized in Supplementary Table S3.

Evolution of the oral ciliature

The morphological evolution of the oral apparatus was reconstructed from thousands of phylogenetic trees sampled during the stationary phase of the Bayesian MCMC analyses of the unmasked (Figs. 4 and 5) and the 0.90 alignment (data not shown). Using this strategy, we also accounted for uncertainty in the phylogenetic position of bursariomorphids and platyophryids as well as of some key colpodid taxa, such as *Bardeliella*, *Sandmanniella*, *Ilsiella*,

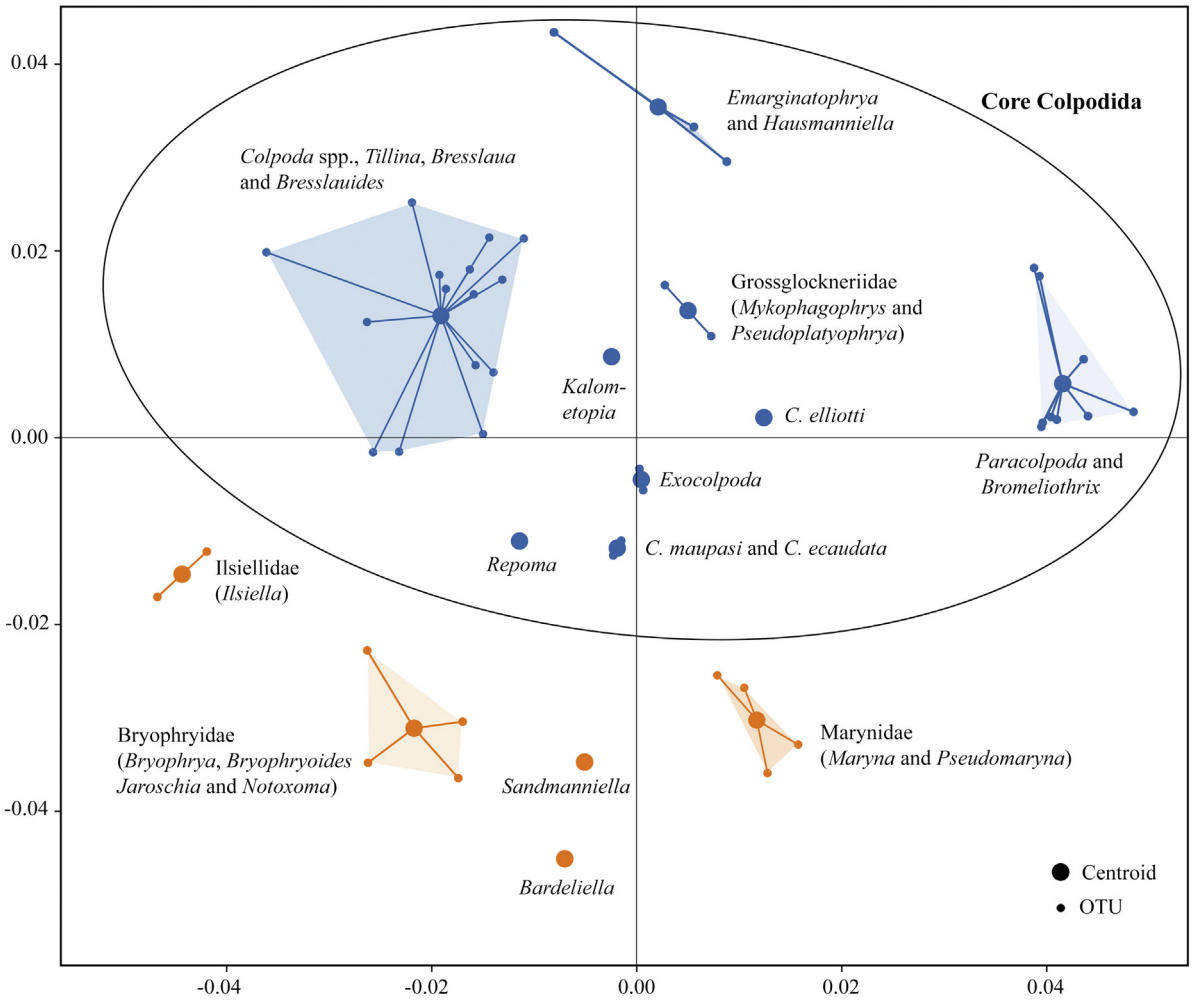

Fig. 3. Multidimensional scaling of members of the order Colpodida based on pairwise evolutionary divergences estimated from the unmasked alignment in MEGA. Groups are delimited when corresponding clusters are recovered also in phylogenetic analyses. Note that traditional *Colpoda* species form clusters with morphologically more derived genera (e.g., *Bresslaua*, *Bresslauides*, *Bromeliothrix*, *Hausmanniella*) and distances between colpodid groups are much larger than between individual genera of bryophryids. OTU = operational taxonomic unit.

marynids, bryophryids and grossglockneriids. Although the 50%-majority rule consensus Bayesian trees inferred from the unmasked and 0.90 alignments differed in the position of bursariomorphids and platyophryids (Fig. 1, Supplementary Fig. S1), the reconstruction of ancestral oral morphologies of the main four colpodean lineages was unequivocal and therefore only results from the unmasked alignment are presented (Figs. 3 and 4). The right oral field was composed of dikinetids and the left oral field consisted of brick-shaped adoral organelles already in the last common ancestor (LCA) of the class Colpodea, supporting the view of Foissner et al. (2011). These plesiomorphic conditions were maintained also in the LCAs of the Bursariomorphida, Platyophryida and Cyrtolophosidida. However, the oral ciliature overcame a dramatic transformation in the bryophryid-grossglockneriid-colpodid (BGC) cluster. The LCA of this assemblage very likely had a right oral ciliature composed of polykinetids and a complex left oral ciliature built from brick-shaped organelles in the anterior part and from a polykinetidal field in the posterior part. The probabilistic approach of the stochastic mapping also indicated that the right oral ciliature became polykinetidal independently in the LCA of *Bursaria* and in the LCA of the BGC cluster. Stochastic mapping also suggested that regardless of the phylogenetic position of *Ilsiella*, plesiomorphic features of its oral ciliature are homoplasies, i.e., they were not inherited from the LCA of the BGC cluster whose oral ciliature was complex (Figs. 4 and 5).

Discussion

Phylogeny of the class Colpodea

The recent 18S rRNA gene phylogenies suggested several changes in the traditional morphology-based framework of the class Colpodea (for reviews, see Foissner 1993 and Foissner et al. 2011). However, the present evaluation of the quality of phylogenetic information in the colpodean 18S rRNA gene with multiple statistical approaches showed

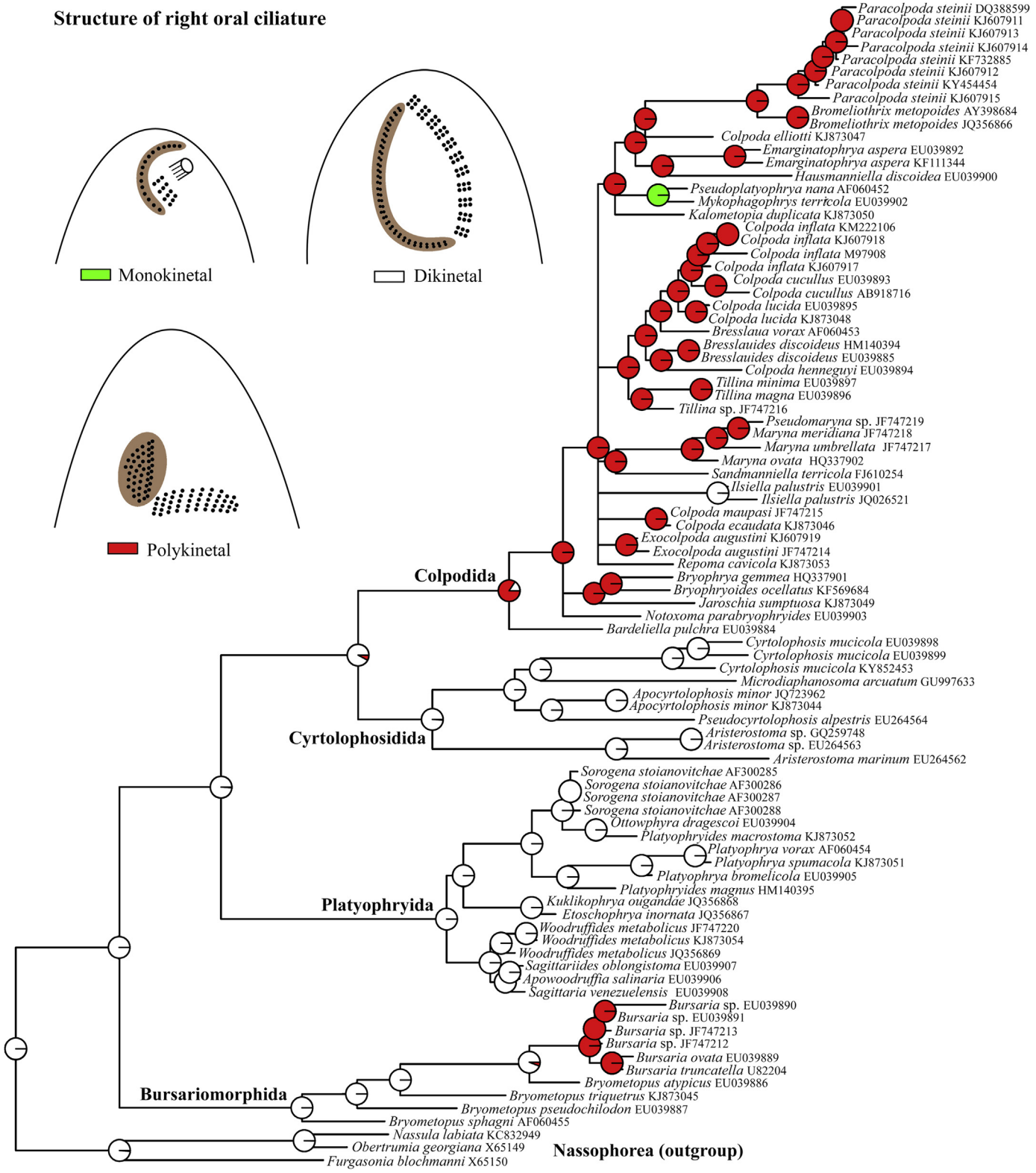

Fig. 4. Reconstruction of ancestral states of the right oral ciliature based on a set of thousand randomly selected trees from the stationary phase of the Bayesian MCMC analyses of the unmasked alignment. Relative proportions of character states were mapped as pie charts onto the nodes of the 50%-majority rule Bayesian consensus tree shown in Fig. 1. The right oral field was composed of dikinetids already in the last common ancestor of the class Colpodea and this plesiomorphic condition was maintained also in the last common ancestors of the orders Bursariomorphida, Platyophryida and Cyrtolophosidida. The probabilistic approach of the stochastic mapping also indicated that the right oral ciliature became polykinetal independently in the last common ancestor of the genus *Bursaria* and in that of the order Colpodida including bryophryids and grossglockneriids.

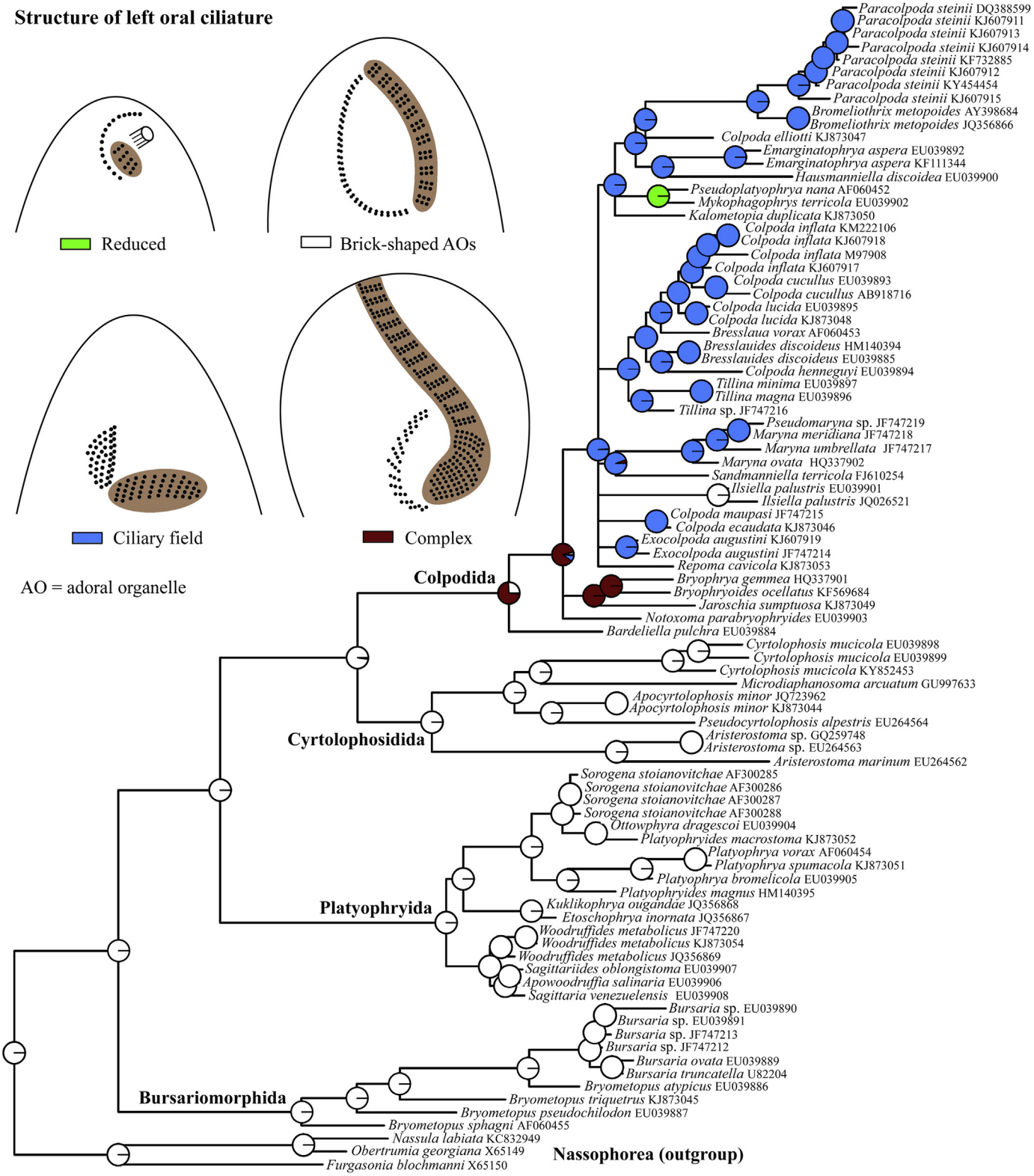

Fig. 5. Reconstruction of ancestral states of the left oral ciliature based on a set of thousand randomly selected trees from the stationary phase of the Bayesian MCMC analyses of the unmasked alignment. Relative proportions of character states were mapped as pie charts onto the nodes of the 50%-majority rule Bayesian consensus tree shown in Fig. 1. The left oral field was composed of brick-shaped adoral organelles (AOs) already in the last common ancestor of the class Colpodea, and this plesiomorphic condition was maintained also in the last common ancestors of the orders Bursariomorphida, Platyophryida and Cyrtolophosidida. However, the left oral ciliature overcame a dramatic transformation in the last common ancestor of the order Colpodida, where it became composed from brick-shaped organelles in the anterior part and a polykinetal field in the posterior part.

that the evolutionary relationships and molecular delimitation of the main colpodean lineages are not so clear, as it may appear at first glance. Specifically, we revealed three issues in the molecular phylogenies of the class Colpodea: (1) the phylogenetic positions of bursariomorphids and platyophryids are unstable and depend on the alignment masking (Fig. 1, Supplementary Fig. S1), (2) the sister relationship of platyophryids and cyrtolophosidids cannot be excluded by statistical tree topology tests (Table 2) and (3) clustering of bryophryids and grossglockneriids outside colpodids are also statistically valid possibilities (Table 2). Interestingly, phylogenetic inferences using mitochondrial SSU rDNA do not support the bursariomorphids-early hypothesis, but with variable statistical support corroborate the platyophryids-early hypothesis (Dunthorn et al. 2011). Considering the phylogenetic uncertainty in 18S rRNA gene inferences, the concept of the order Cyrtolophosidida including platyophryids and sorogenids as well as the distinctness of the orders Bryophryida and Grossglockneriida still remain reliable taxonomic hypotheses open for further testing, when sequences from other phylogenetically informative genes become available. However, bryophryids, grossglockneriids and colpodids are indisputably very closely related and belong to the crown radiation of the class Colpodea, as already recognized in all previous analyses (Bourland et al. 2011; Dunthorn et al. 2012; Foissner et al. 2014; Quintela-Alonso et al. 2011). This highly diverse cluster is supported also by primary nucleotide homologies (Table 1), corroborating the concept of the order Colpodida revised by Foissner et al. (2011). On the other hand, the monophyly and validity of the former order Bryometopida are unambiguously refuted and bryometopids represent an old stem lineage that is at best to be submerged within the order Bursariomorphida, as already proposed by Foissner et al. (2011). Although our present analyses indicated that there are still some problems in the higher classification of the Colpodea, we prefer not to perform further changes until more data become available.

Evolutionary taxonomy of the order Colpodida

Natural classification of the highly diverse order Colpodida remains the hardest riddle within the class Colpodea (Dunthorn et al. 2008, 2009, 2012; Foissner and Stoeck 2009; Foissner et al. 2011, 2014). Possible reasons are uninformativeness of the 18S rRNA gene and an uncoupling of morphological and molecular evolution. The latter phenomenon might have caused inconsistencies between morphological and molecular classifications. In this respect, we have recognized four types of taxa groups (Fig. 6):

(1) The first type is characterized by small morphological and molecular differences (genetic distance <0.015), when two species are compared (Fig. 6A). A good example is the type species *Colpoda cucullus* and *C. inflata*. Both are morphologically rather similar, sharing the same oral pattern and general body bauplan (Foissner 1993). Likewise, their genetic distance is comparatively small (0.011 ± 0.003; Supplementary Table S3) and both are placed close to each other in phylogenetic trees (Figs. 1 and 2). In this case, there is no problem between morphological and molecular classifications and both species can be unambiguously assigned to the same genus.

(2) The second type includes taxa pairs with small morphological but comparatively deep molecular divergence (genetic distance >0.030) (Fig. 6B). For instance, *C. cucullus* is morphologically rather similar to *C. henneguyi* in the general body organization but their genetic distance is three times greater (0.032 ± 0.005) than between *C. cucullus* and *C. inflata* (0.011 ± 0.003) (Supplementary Table S3). This problem is most likely caused by morphostasis, a phenomenon when morphological evolution slows down but molecular evolution proceeds. Morphostasis typically causes paraphyly problems in the colpodean tree of life. This phenomenon is, however, highly important in reconstruction and understanding morphological evolution, as already recognized in the alveolate evolution by Leander and Keeling (2003).

(3) The third type is, for instance, represented by *C. cucullus* and *Bresslaua vorax* (Fig. 6C). *Bresslaua*, a predator, clearly differs morphologically from *Colpoda*, a bacterivor, by the large, funnel-shaped buccal cavity (Foissner, 1993). Interestingly, the genetic distance of *C. cucullus* and *B. vorax* (0.024 ± 0.005) is even smaller than between *C. cucullus* and *C. henneguyi* (0.032 ± 0.005) (Supplementary Table S3), indicating that *C. henneguyi* diverged earlier from *C. cucullus* than did *B. vorax*. Classification of *C. cucullus* and *B. vorax* into distinct genera appears justified in a morphological but not in a molecular point of view. This problem can be overcome, when evolutionary classification (Mayr and Bock 2002) is anticipated, as already suggested by Foissner et al. (2011) and Dunthorn et al. (2012).

(4) The last group is characterized by large morphological and comparatively deep molecular divergence (genetic distance >0.030), when two species are compared. A good example is *C. cucullus* and the grossglockneriid *Pseudoplatyophrya nana* (Fig. 6D). The latter species is morphologically highly derived, its oral ciliary structures are reduced and a special feeding tube is developed (Foissner 1993). Genetic distance is also comparatively high (0.032 ± 0.005; Supplementary Table S3) and they even belong to different clusters in the ordination diagram (Fig. 3). In this case, there is no problem between morphological and molecular classification and both species can be placed in different genera and also in different higher taxa. Interestingly, the genetic distance of *C. cucullus* from morphologically highly dissimilar bryophryids, marynids and ilsiellids ranges between 0.041 and 0.059 (Supplementary Table S3), indicating that these taxa diverged from *C. cucullus* even before

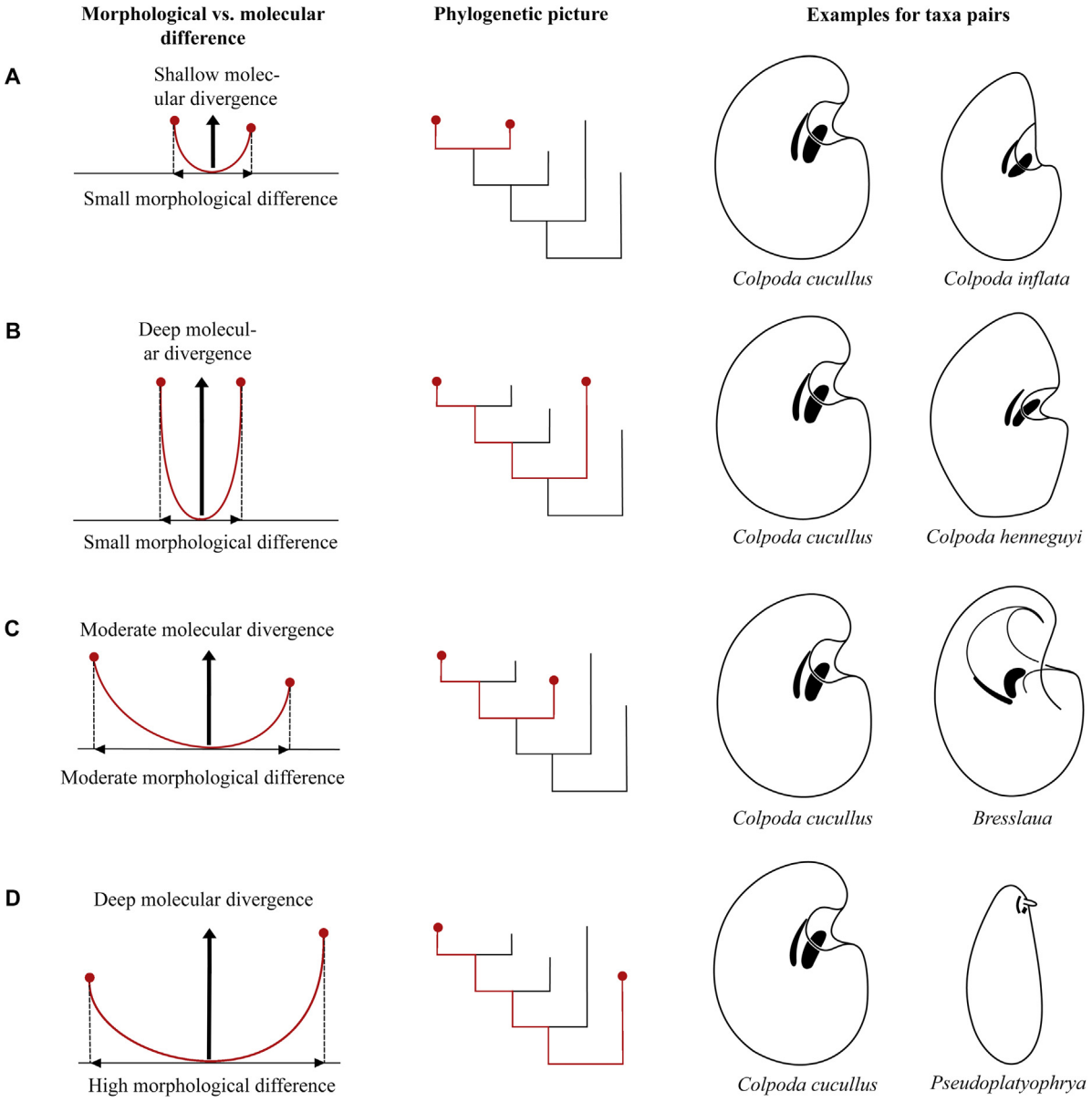

Fig. 6. Morphological and molecular differences between *Colpoda*-like and morphologically derived colpodids branched off from the Ur-*Colpoda* stem lineage. Molecular divergence is a function of time which runs upward and is denoted by thick arrows. **A**: Small morphological and molecular difference shown in *C. cucullus* and *C. inflata*. **B**: Small morphological and high molecular difference shown in *C. cucullus* and *C. henneguyi*. **C**: Moderate morphological and molecular difference shown in *C. cucullus* and *B. vorax*. **D**: High morphological and molecular difference shown in *C. cucullus* and *P. nana*.

grossglockneriids. Bryophryids, marynids and ilsiellids represent a distinct cluster each in the ordination diagram similar as grossglockneriids (Fig. 3), corroborating their genetic and taxonomic distinctness within the order Colpodida.

Taking into account that morphological and molecular evolution do not necessarily run in tandem, might significantly help to reconcile morphological and molecular phylogenies of ciliates. Already Foissner et al. (2011) and Dunthorn et al. (2012) advocated the "Ur-*Colpoda*" concept, in which *Colpoda* represents the stem lineage. Both, *Colpoda*-like taxa and morphologically more derived taxa, such as *Bresslauides*, *Hausmanniella* or *Pseudoplatyophrya*, might have branched off from the Ur-*Colpoda* stem lineage. In this light, the validity of the paraphyletic *Colpoda* as well as of the derived genera appears justified, since this taxonomic concept preserves information not only on morphology but also on ecology and evolutionary processes (Hörandl 2014).

Splitting of the traditional genus *Colpoda*

The traditional genus *Colpoda* has been always depicted in molecular phylogenies as non-monophyletic (Bourland et al.

2011, 2012, 2014; Dunthorn et al. 2009, 2012; Foissner et al. 2011, 2014; Quintela-Alonso et al. 2011). Also statistical tree topology tests have consistently rejected monophyly of this species-rich genus (Table 2). To solve this taxonomic problem, several genera were resurrected and some other were established: *Tillina*, *Repoma*, *Paracolpoda*, *Exocolpoda* and *Emarginatophrya*. Foissner et al. (2011) redefined *Tillina* by having a large body carrying a distinct postoral groove, a contractile vacuole with collecting canals, a large oral cavity, several to many roof kineties and oblong extrusomes. *Repoma* also possesses a diagonal groove and a contractile vacuole with collecting canals but differs from *Tillina* by having multiple micronuclei and a comparatively small oral cavity (Foissner 1993; Novotny 1970; Novotny et al. 1977). Foissner (1985) restricted *Paracolpoda* to *C. steinii* and, at the present state of knowledge, this resurrected genus could be characterized by two long caudal cilia, left oral cilia forming a conspicuous beard, and at least some left somatic kineties shortened posteriorly (Dragesco and Dragesco-Kernéis 1986; Foissner 1993, 2016). *Exocolpoda* is outstanding for division in freely motile (non-encysted) condition, a small, conical vestibulum and a right oral polykinetid composed of few to many short, more or less disordered kineties (Foissner et al. 2002). *Emarginatophrya* is distinguished by a distally emarginated left oral polykinetid (Foissner 2016). However, also these resurrected and new colpodid genera have not helped to completely erase the paraphyly problem of *Colpoda* because generic apomorphies which could define several problematic molecular lineages (e.g., *C. maupasi*, *C. ecaudata*, *C. elliotti*, *C. henneguyi*) have been as yet not recognized.

Visualization of the colpodid pair-wise genetic distances with multidimensional scaling also revealed that grouping of *Colpoda*-like species and morphologically divergent taxa does not mirror traditional taxonomic concepts (Fig. 3). Although distances between some traditional *Colpoda* species are much larger than between individual genera of bryophryids, no morphological apomorphies sustaining the colpodid groups delimited by multidimensional scaling have been recognized so far. Moreover, the softwired galled network unraveled that (1) *Repoma* might be a sister group of *C. maupasi* and *C. ecaudata*, indicating that these taxa with a comparatively small oral cavity could represent a distinct lineage within the Colpodidae and (2) *Tillina* might be paraphyletic, which would on the other hand question its distinctness in the already paraphyletic genus *Colpoda* (Fig. 2). The phylogenetic positions of *Exocolpoda*, *Paracolpoda* and *Emarginatophrya* are so far without conflicts, which would corroborate their validity. Obviously only an integrative morphological-molecular approach, along with critical examination of morphological and molecular data quality by multiple tests, can help to decipher the intricate evolutionary history of colpodids. The importance of integrative approach in phylogenetic studies of ciliates was reviewed by Vďačný (2017).

Application of evolutionary taxonomy concepts in ciliates

Taxonomy comprises the theory and practice of describing, naming, and ordering groups of organisms (Wägele 2005; Wiley and Lieberman 2011). It is vitally important that taxonomy is logically consistent with phylogeny and integrated into the field of evolutionary theory (Wiley et al. 1991). Unfortunately, the existing morphology-based taxonomy of ciliates is often discordant with molecular frameworks, especially at genus and family levels (e.g., Foissner et al. 2004, 2011; Liu et al. 2016; Lynn and Wright 2013; Omar et al. 2017; Pan and Stoeck 2017; Shao et al. 2014; Sun et al. 2012; Vďačný et al. 2018). Recognizing generic and family characters is not straightforward until a sound phylogeny is available. The biological rationale of this problem was already discussed by Wiley et al. (1991). Briefly, there are no processes that allow for a genus or a family to give rise to other taxa and each monophyletic group begins as a single species in an array of processes termed speciation. Since there are no processes such as “genusation” and “familization”, apomorphies defining monophyletic groups arisen from the ancestral species need to be *a posteriori* recognized when a sound phylogeny become available. However, due to morphostasis of some lineages this can be a very difficult task in ciliates, causing traditional ciliate genera to become paraphyletic in molecular analyses. Although application of evolutionary taxonomy has been criticized (for a review, see Wägele 2005 and Wiley and Lieberman 2011), the paraphyly problems are sometimes so pronounced that evolutionary systematics appear to be a more practical solution than phylogenetic systematics (Hörandl 2006, 2007, 2014; Hörandl and Stuessy 2010; Mayr and Bock 2002; Stuessy and Hörandl 2014). These authors argued that holophyletic (=monophyletic *s. s.*) groups mostly reflect extinction gaps, while paraphyletic groups appear in clades having ecological shifts, as also recognized in the ciliate classes Armophorea and Litostomatea (Vďačný and Rajter 2015; Vďačný et al. 2014, 2018). According to Hörandl (2014), recognizing of paraphyletic and monotypic groups preserves information on morphology, ecology, and evolutionary processes. Hörandl (2014) also stated that this pluralistic approach facilitates practicability and stability of taxonomic work, as the broadening of criteria restricts the number of equally valid options for classification. Moreover, preserving information content on phenotypes ensures connection to traditional literature and to modern information systems.

In the traditional ciliate systematics, new taxa are established on the basis of derived, shared morphological characters, i.e., synapomorphies. However, when morphological characteristics used as diagnostic features of taxa turn out to be plesiomorphies or homoplasies, this situation has to be solved. The best practice is to search for new morphological synapomorphic characters that will help to delimit the natural units of evolution and will erase the para- or poly-

phyly problems. If such characters cannot be found or may even not exist, the evolutionary taxonomy concept could be applied (e.g., Foissner et al. 2011; Vďačný and Rajter 2015; Vďačný et al. 2018), as discussed above. Or molecular characters could be used to resolve taxonomic incongruities. For instance, Sun et al. (2012, 2013) used molecular characters and secondary structure features of the 18S rRNA and the ITS2 molecule to solve the paraphyly problem and to redefine the genus *Vorticella* and the families Vorticellidae and Astylozoidae. This strategy could be broadened in the context of ecological niches and physiological properties, as already documented on the example of peritrichs (Sun et al. 2016) and euplotids (Petroni et al. 2002; Zhao et al. 2018). However, one should be aware that molecular, ecological and physiological characters are influenced by the same problems of variations as morphological traits. Moreover, molecular characters can be also homoplastic due to various selection pressures (Rajter and Vďačný 2018) or molecular synapomorphies might be not fixed or might have been erased by multiple substitutions that took place during the very long evolutionary history of ciliates (Rataj and Vďačný 2018). Since the ciliate taxonomy has now become a very complex matter, an integrative approach is an absolute necessity to establish a natural, phylogeny-based system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ejop.2018.11.003.

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Appendix A. Supplementary data

Re-analysis of the 18S rRNA gene phylogeny of the ciliate class Colpodea

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Supplementary Table S1. Best fitting evolutionary models for the six datasets analyzed.

Dataset	Model	A	C	G	T	[AC]	[AG]	[AT]	[CG]	[CT]	[GT]	p-inv	Γ
Unamasked	GTR+I+Γ	0.2731	0.1978	0.2577	0.2715	0.8873	2.7747	1.2032	0.6912	5.2668	1.000	0.4450	0.4160
0.50	GTR+I+Γ	0.2741	0.1952	0.2574	0.2733	0.8496	2.7317	1.1996	0.6040	5.2186	1.000	0.4540	0.4260
0.75	GTR+I+Γ	0.2772	0.1926	0.2582	0.2720	0.9890	2.9675	1.3107	0.7042	6.0639	1.000	0.4770	0.4190
0.80	GTR+I+Γ	0.2784	0.1898	0.2543	0.2774	1.0088	3.0500	1.2763	0.7187	5.8083	1.000	0.4810	0.4180
0.90	GTR+I+Γ	0.2794	0.1881	0.2557	0.2767	1.0723	3.2632	1.3802	0.8278	6.1321	1.000	0.4940	0.4280
0.93	GTR+I+Γ	0.2797	0.1897	0.2525	0.2782	1.0657	3.4295	1.3845	0.8039	5.9589	1.000	0.5030	0.4300

A, C, G, T = base frequencies; [AC], [AG], [AT], [CG], [CT], [GT] = rate substitution matrices; p-inv = proportion of invariable sites; Γ = gamma distribution shape parameter.

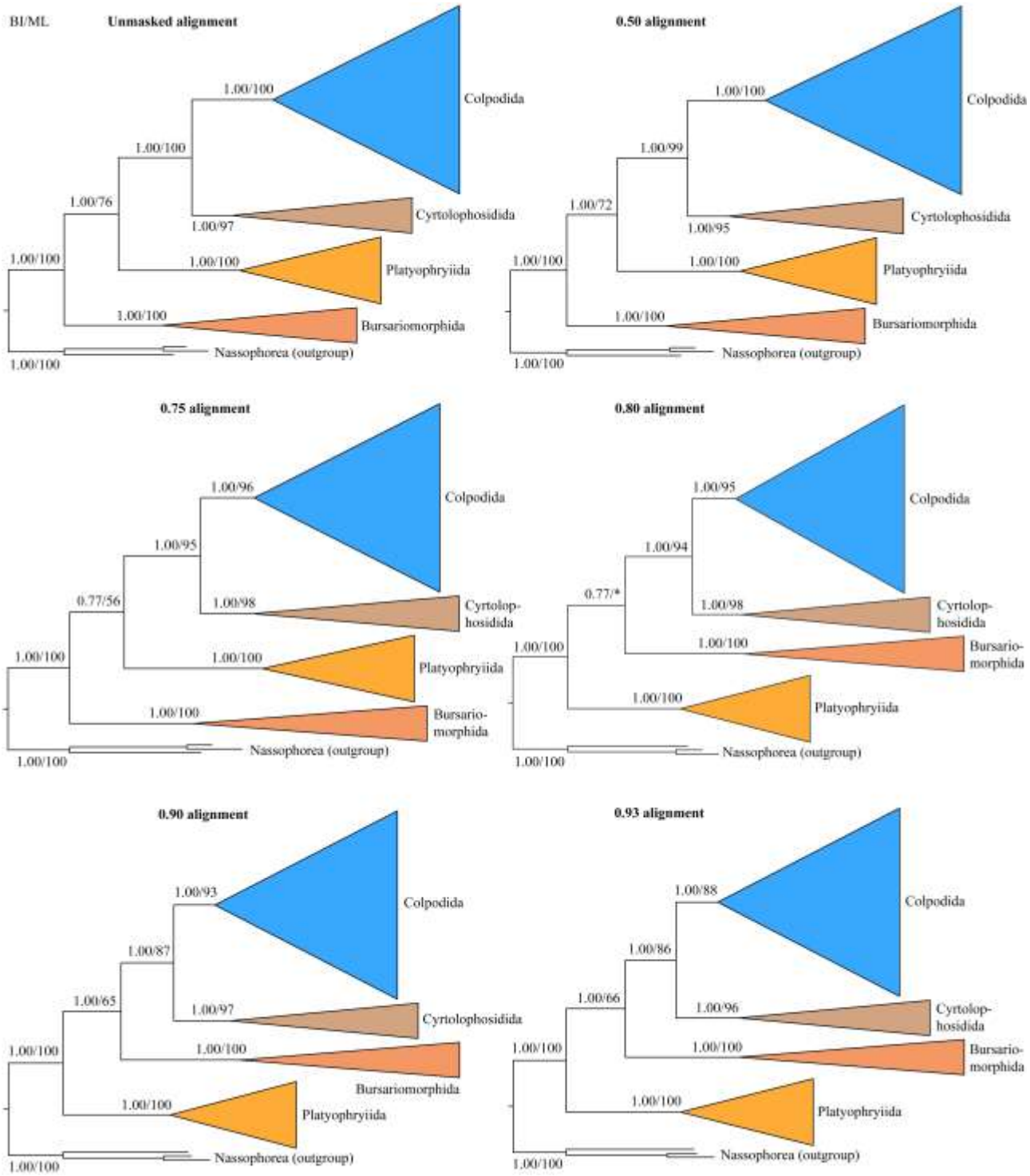
Supplementary Table S2. Distribution of oral ciliature characters in the colpodean taxa analyzed with the computer program SIMMAP.

Taxon	Right oral ciliature	Left oral ciliature	Taxon	Right oral ciliature	Left oral ciliature
Bursariomorphida			**Colpodida – Grossglockneriina**		
Bryometopus atypicus	Dikinetal	Brick-shaped AOs	*Mykophagophrys terricola*	Monokinetal	Reduced
Bryometopus pseudochilodon	Dikinetal	Brick-shaped AOs	*Pseudoplatyophrya nana*	Monokinetal	Reduced
Bryometopus sphagni	Dikinetal	Brick-shaped AOs	**Colpodida – Colpodina**		
Bryometopus triquetrus	Dikinetal	Brick-shaped AOs	*Bardeliella pulchra*	Polykinetal	Complex
Bursaria ovata	Polykinetal	Brick-shaped AOs	*Bresslaua vorax*	Polykinetal	Ciliary field
Bursaria truncatella	Polykinetal	Brick-shaped AOs	*Bresslauides discoideus*	Polykinetal	Ciliary field
Bursaria spp.	Polykinetal	Brick-shaped AOs	*Bromeliothrix metopoides*	Polykinetal	Ciliary field
Platyophryida			*Colpoda aspera*	Polykinetal	Ciliary field
Apowoodruffia salinaria	Dikinetal	Brick-shaped AOs	*Colpoda cucullus*	Polykinetal	Ciliary field
Etoschophrya inornata	Dikinetal	Brick-shaped AOs	*Colpoda ecaudata*	Polykinetal	Ciliary field
Kuklikophrya ougandae	Dikinetal	Brick-shaped AOs	*Colpoda elliotti*	Polykinetal	Ciliary field
Ottowphyra dragescoi	Dikinetal	Brick-shaped AOs	*Colpoda henneguyi*	Polykinetal	Ciliary field
Platyophrya bromelicola	Dikinetal	Brick-shaped AOs	*Colpoda inflata*	Polykinetal	Ciliary field
Platyophrya spumacola	Dikinetal	Brick-shaped AOs	*Colpoda lucida*	Polykinetal	Ciliary field
Platyophrya vorax	Dikinetal	Brick-shaped AOs	*Colpoda maupasi*	Polykinetal	Ciliary field
Platyophryides macrostoma	Dikinetal	Brick-shaped AOs	*Exocolpoda augustini*	Polykinetal	Ciliary field
Platyophryides magnus	Dikinetal	Brick-shaped AOs	*Hausmanniella discoidea*	Polykinetal	Ciliary field
Sagittaria venezuelensis	Dikinetal	Brick-shaped AOs	*Ilsiella palustris*	Dikinetal	Brick-shaped AOs
Sagittariides oblongistoma	Dikinetal	Brick-shaped AOs	*Kalometopia duplicata*	Polykinetal	Ciliary field
Sorogena stoianovitchae	Dikinetal	Brick-shaped AOs	*Maryna meridiana*	Polykinetal	Ciliary field
Woodruffides metabolicus	Dikinetal	Brick-shaped AOs	*Maryna ovata*	Polykinetal	Ciliary field
Cyrtolophosidida			*Maryna umbrellata*	Polykinetal	Ciliary field
Apocyrtolophosis minor	Dikinetal	Brick-shaped AOs	*Paracolpoda steinii*	Polykinetal	Ciliary field
Aristerostoma marinum	Dikinetal	Brick-shaped AOs	*Pseudomaryna sp.*	Polykinetal	Ciliary field
Aristerostoma sp.	Dikinetal	Brick-shaped AOs	*Repoma cavicola*	Polykinetal	Ciliary field
Cyrtolophosis mucicola	Dikinetal	Brick-shaped AOs	*Sandmanniella terricola*	Polykinetal	Complex
Microdiaphanosoma arcuatum	Dikinetal	Brick-shaped AOs	*Tillina magna*	Polykinetal	Ciliary field
Pseudocyrtolophosis alpestris	Dikinetal	Brick-shaped AOs	*Tillina minima*	Polykinetal	Ciliary field
Colpodida – Bryophryina			*Tillina* sp.	Polykinetal	Ciliary field
Bryophrya gemmea	Polykinetal	Complex	**Outgroup**		
Bryophryoides ocellatus	Polykinetal	Complex	*Furgasonia blochmanni*	Dikinetal	Brick-shaped AOs
Jaroschia sumptuosa	Polykinetal	Complex	*Nassula labiata*	Dikinetal	Brick-shaped AOs
Notoxoma parabryophryides	Polykinetal	Reduced	*Obertrumia georgiana*	Dikinetal	Brick-shaped AOs

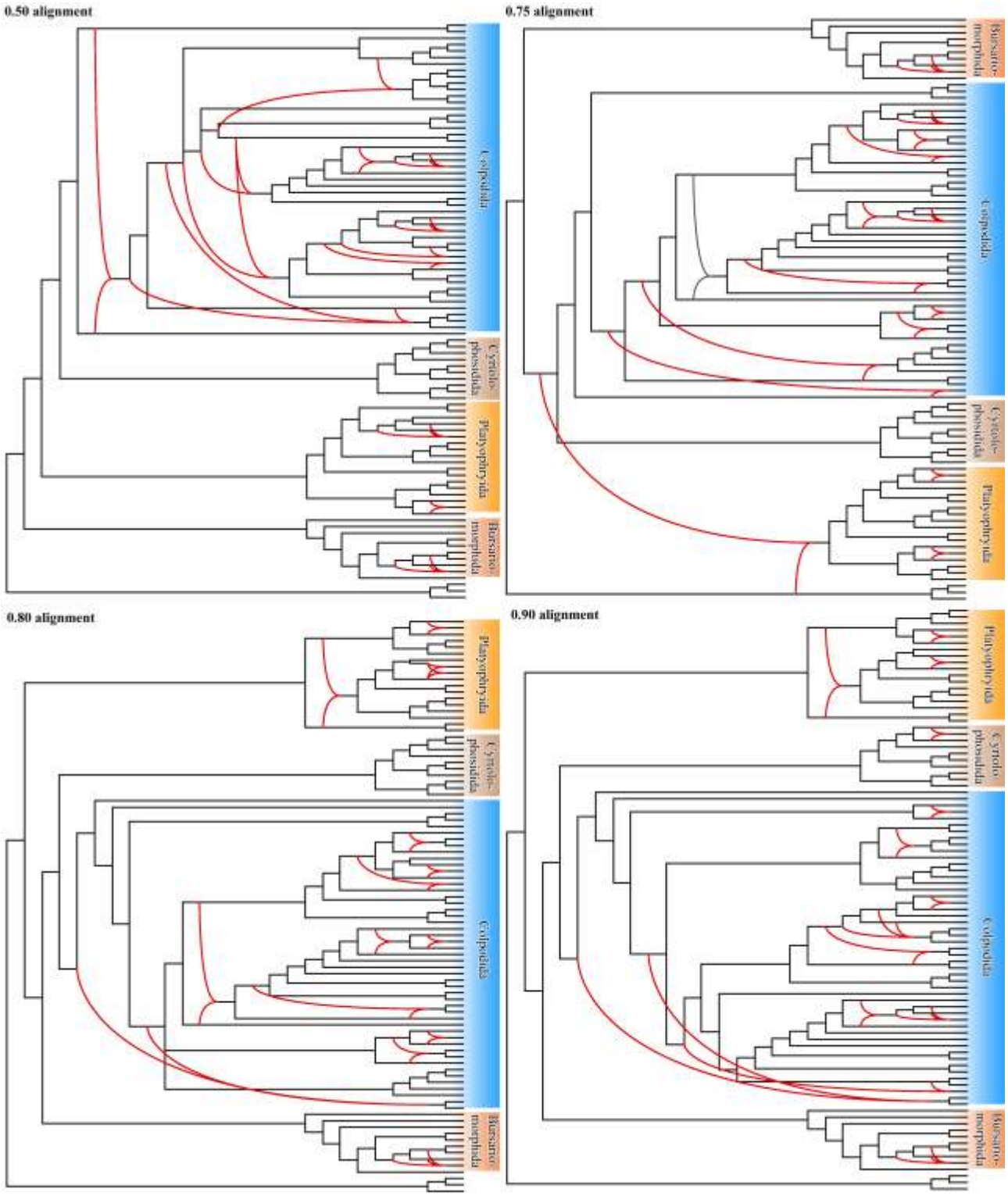
[a] AOs = adoral organelles.

Supplementary Table S3. Average pairwise genetic distances (below diagonal) of colpodid taxa and their standard errors (above diagonal) calculated from the unmasked 18S rRNA gene alignment under the heterogenous substitution pattern among lineages.

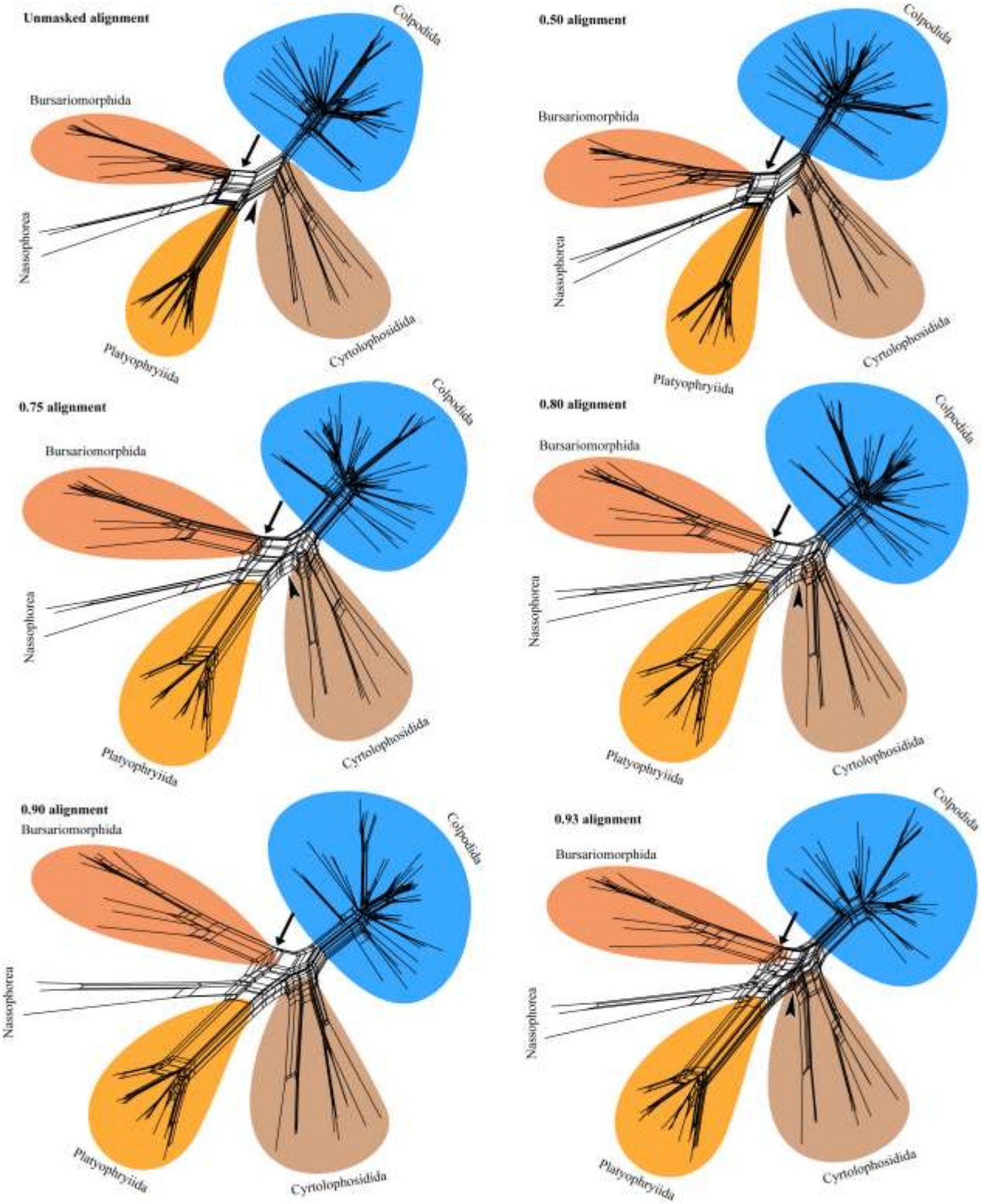
Taxon		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
Bryophryoides ocellatus KF569684	1		0.004	0.006	0.006	0.006	0.007	0.006	0.007	0.006	0.006	0.006	0.006	0.006	0.006	0.005	0.005	0.005	0.007	0.006	0.006	0.005	0.005	0.005	0.005	0.005	0.006	0.007	0.007	0.005	0.006	0.007	0.006	0.005	0.006	0.005	0.006	0.006	0.006	0.008	0.008	0.007	0.007	0.007	0.007	0.007	0.007	0.008	0.008
Bryophrya gemmea HQ337901	2	0.025		0.005	0.006	0.006	0.007	0.006	0.006	0.006	0.006	0.005	0.006	0.005	0.006	0.006	0.006	0.006	0.006	0.006	0.005	0.005	0.005	0.005	0.005	0.006	0.006	0.006	0.006	0.005	0.005	0.007	0.006	0.006	0.006	0.006	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.008	0.008
Kalometopia duplicata KJ873050	3	0.047	0.043		0.005	0.005	0.005	0.005	0.005	0.004	0.004	0.004	0.004	0.004	0.004	0.005	0.005	0.005	0.004	0.004	0.005	0.004	0.004	0.005	0.004	0.006	0.005	0.005	0.005	0.006	0.006	0.005	0.006	0.005	0.006	0.005	0.006	0.005	0.007	0.007	0.006	0.006	0.006	0.006	0.007	0.006	0.007	0.007	0.007
Bresslauides discoideus EU039885	4	0.050	0.046	0.031		0.001	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.003	0.004	0.004	0.005	0.005	0.005	0.005	0.005	0.006	0.005	0.006	0.006	0.006	0.006	0.006	0.006	0.005	0.006	0.005	0.006	0.005	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Bresslauides discoideus HM140394	5	0.049	0.044	0.030	0.001		0.004	0.003	0.004	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.003	0.004	0.004	0.004	0.005	0.005	0.005	0.005	0.005	0.005	0.006	0.005	0.006	0.006	0.007	0.006	0.005	0.006	0.005	0.006	0.005	0.006	0.005	0.006	0.006	0.006	0.007	0.007	0.006	0.007	0.007	0.007
Colpoda henneguyi EU039894	6	0.065	0.058	0.039	0.019	0.022		0.004	0.005	0.004	0.004	0.005	0.005	0.004	0.005	0.005	0.005	0.005	0.005	0.006	0.006	0.006	0.005	0.006	0.006	0.007	0.006	0.006	0.006	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.006	0.007	0.006	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.009	0.008
Bresslaua vorax AF060453	7	0.057	0.052	0.035	0.018	0.017	0.026		0.004	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.005	0.004	0.005	0.005	0.005	0.005	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.006	0.005	0.006	0.006	0.007	0.005	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.008	0.007
Colpoda cucullus AB918716	8	0.056	0.053	0.031	0.026	0.026	0.032	0.027		0.002	0.002	0.002	0.003	0.002	0.003	0.005	0.004	0.005	0.004	0.005	0.005	0.005	0.005	0.005	0.005	0.007	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.006	0.006	0.006	0.006	0.008	0.007	0.007	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.007
Colpoda lucida KJ873048	9	0.050	0.050	0.029	0.019	0.019	0.025	0.018	0.008		0.001	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.003	0.005	0.005	0.005	0.004	0.005	0.004	0.006	0.005	0.005	0.006	0.006	0.006	0.006	0.007	0.006	0.006	0.006	0.006	0.005	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Colpoda lucida EU039895	10	0.054	0.054	0.029	0.022	0.021	0.028	0.021	0.008	0.003		0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.003	0.005	0.005	0.005	0.005	0.005	0.005	0.006	0.006	0.005	0.005	0.006	0.006	0.007	0.006	0.007	0.006	0.007	0.005	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Colpoda cucullus EU039893	11	0.050	0.046	0.026	0.022	0.021	0.033	0.022	0.006	0.011	0.011		0.002	0.002	0.003	0.004	0.004	0.004	0.003	0.004	0.005	0.005	0.004	0.005	0.005	0.006	0.005	0.005	0.005	0.006	0.006	0.006	0.007	0.005	0.006	0.005	0.006	0.005	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.006	0.007	0.007	0.007
Colpoda inflata KJ607918	12	0.051	0.045	0.028	0.022	0.021	0.033	0.022	0.011	0.012	0.014	0.011		0.002	0.000	0.004	0.004	0.004	0.003	0.005	0.004	0.004	0.005	0.005	0.005	0.006	0.006	0.006	0.005	0.006	0.006	0.006	0.006	0.006	0.005	0.006	0.005	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Colpoda inflata KJ607917	13	0.051	0.044	0.028	0.019	0.018	0.027	0.020	0.010	0.011	0.013	0.008	0.007		0.002	0.004	0.004	0.004	0.003	0.004	0.004	0.004	0.004	0.005	0.005	0.006	0.006	0.006	0.006	0.005	0.006	0.006	0.006	0.007	0.006	0.006	0.006	0.006	0.005	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Colpoda inflata KM222106	14	0.052	0.045	0.030	0.023	0.023	0.035	0.023	0.011	0.013	0.015	0.011	0.000	0.007		0.004	0.005	0.004	0.003	0.005	0.005	0.004	0.005	0.005	0.005	0.007	0.006	0.006	0.005	0.007	0.006	0.006	0.007	0.006	0.006	0.006	0.007	0.005	0.008	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Tillina magna EU039896	15	0.038	0.045	0.034	0.026	0.025	0.037	0.032	0.032	0.028	0.031	0.028	0.027	0.027	0.029		0.001	0.003	0.005	0.006	0.006	0.005	0.005	0.004	0.004	0.005	0.006	0.006	0.005	0.007	0.005	0.006	0.006	0.005	0.006	0.005	0.006	0.006	0.006	0.008	0.008	0.007	0.007	0.007	0.007	0.007	0.007	0.008	0.007	0.007	0.007
Tillina minima EU039897	16	0.036	0.046	0.034	0.026	0.025	0.034	0.032	0.028	0.027	0.030	0.025	0.027	0.026	0.028	0.002		0.003	0.005	0.006	0.006	0.005	0.005	0.004	0.004	0.005	0.006	0.006	0.005	0.007	0.004	0.006	0.006	0.005	0.006	0.005	0.006	0.006	0.007	0.006	0.008	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Tillina sp. JF747216	17	0.040	0.042	0.031	0.018	0.017	0.030	0.026	0.027	0.021	0.023	0.022	0.022	0.022	0.023	0.020	0.020		0.004	0.005	0.005	0.004	0.004	0.004	0.004	0.005	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.007	0.005	0.005	0.005	0.006	0.005	0.007	0.007	0.007	0.006	0.006	0.006	0.006	0.006	0.007	0.007
Colpoda inflata M97908	18	0.060	0.051	0.030	0.025	0.025	0.038	0.025	0.018	0.017	0.017	0.014	0.012	0.013	0.012	0.035	0.035	0.028		0.005	0.005	0.005	0.005	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.007	0.007	0.006	0.006	0.006	0.007	0.006	0.008	0.007	0.007	0.007	0.007	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008
Mykophagophrys terricola EU039902	19	0.047	0.042	0.029	0.029	0.028	0.042	0.038	0.032	0.032	0.032	0.030	0.032	0.031	0.034	0.038	0.036	0.034	0.036		0.003	0.004	0.004	0.005	0.005	0.006	0.006	0.005	0.005	0.006	0.006	0.006	0.007	0.005	0.006	0.005	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.006
Pseudoplatyophrya nana AF060452	20	0.045	0.041	0.030	0.031	0.029	0.046	0.034	0.034	0.032	0.032	0.031	0.035	0.031	0.037	0.039	0.040	0.033	0.036	0.014		0.004	0.004	0.005	0.004	0.006	0.005	0.005	0.005	0.006	0.006	0.006	0.007	0.005	0.006	0.005	0.006	0.005	0.006	0.006	0.005	0.005	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.006
Exocolpoda augustini JF747214	21	0.038	0.036	0.031	0.033	0.032	0.046	0.037	0.035	0.032	0.035	0.033	0.029	0.030	0.030	0.033	0.034	0.026	0.034	0.029	0.025		0.002	0.004	0.004	0.005	0.005	0.005	0.005	0.005	0.006	0.005	0.006	0.004	0.005	0.005	0.006	0.004	0.006	0.005	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.007
Exocolpoda augustini KJ607919	22	0.037	0.035	0.028	0.031	0.031	0.041	0.037	0.029	0.030	0.033	0.030	0.029	0.028	0.031	0.033	0.033	0.025	0.036	0.026	0.025	0.007		0.004	0.004	0.005	0.005	0.005	0.005	0.005	0.005	0.004	0.006	0.004	0.005	0.005	0.005	0.004	0.006	0.005	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.007
Colpoda ecaudata KJ873046	23	0.037	0.040	0.031	0.036	0.035	0.047	0.038	0.038	0.035	0.038	0.037	0.036	0.037	0.038	0.030	0.030	0.030	0.044	0.035	0.032	0.024	0.022		0.001	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.006	0.005	0.006	0.004	0.006	0.005	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.006
Colpoda maupasi JF747215	24	0.036	0.040	0.029	0.033	0.031	0.049	0.034	0.036	0.032	0.035	0.035	0.033	0.035	0.035	0.029	0.028	0.028	0.041	0.034	0.031	0.023	0.022	0.003		0.005	0.005	0.005	0.005	0.005	0.005	0.006	0.006	0.005	0.005	0.005	0.006	0.005	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006
Sandmanniella terricola FJ610254	25	0.041	0.046	0.045	0.045	0.043	0.058	0.045	0.055	0.048	0.052	0.050	0.050	0.050	0.051	0.036	0.036	0.037	0.055	0.050	0.047	0.039	0.039	0.040	0.037		0.006	0.006	0.006	0.006	0.005	0.007	0.007	0.005	0.006	0.005	0.006	0.006	0.007	0.006	0.008	0.008	0.007	0.008	0.008	0.007	0.008	0.008	0.008	0.008
Emarginatophrya aspera KF111344	26	0.058	0.049	0.034	0.037	0.036	0.053	0.042	0.044	0.040	0.041	0.040	0.043	0.040	0.046	0.045	0.046	0.042	0.041	0.038	0.037	0.039	0.037	0.040	0.039	0.052		0.003	0.005	0.007	0.006	0.006	0.006	0.006	0.005	0.006	0.006	0.006	0.006	0.006	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Emarginatophrya aspera EU039892	27	0.054	0.050	0.035	0.040	0.041	0.053	0.047	0.044	0.039	0.039	0.040	0.044	0.040	0.047	0.044	0.045	0.042	0.044	0.039	0.042	0.037	0.034	0.041	0.042	0.050	0.010		0.005	0.007	0.007	0.006	0.007	0.006	0.006	0.006	0.006	0.007	0.006	0.007	0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Colpoda elliotti KJ873047	28	0.048	0.045	0.033	0.038	0.037	0.048	0.046	0.040	0.041	0.043	0.035	0.041	0.039	0.040	0.038	0.036	0.035	0.046	0.033	0.036	0.033	0.030	0.039	0.037	0.046	0.035	0.035		0.007	0.005	0.005	0.007	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006
Jaroschia sumptuosa KJ873049	29	0.037	0.037	0.050	0.046	0.045	0.055	0.052	0.052	0.048	0.052	0.050	0.052	0.051	0.055	0.052	0.051	0.047	0.059	0.047	0.047	0.038	0.041	0.039	0.040	0.053	0.059	0.058	0.052		0.006	0.007	0.006	0.006	0.006	0.006	0.007	0.006	0.007	0.006	0.007	0.008	0.007	0.007	0.007	0.007	0.007	0.008	0.008
Notoxoma parabryophryides EU039903	30	0.035	0.035	0.046	0.041	0.041	0.050	0.048	0.045	0.046	0.043	0.045	0.045	0.042	0.042	0.038	0.034	0.038	0.055	0.042	0.047	0.042	0.039	0.038	0.038	0.036	0.054	0.054	0.038	0.046		0.006	0.006	0.006	0.006	0.005	0.006	0.006	0.007	0.006	0.008	0.007	0.008	0.008	0.007	0.007	0.008	0.008	0.008
Hausmanniella discoidea EU039900	31	0.059	0.059	0.040	0.045	0.049	0.056	0.054	0.045	0.045	0.044	0.048	0.044	0.041	0.045	0.046	0.040	0.049	0.055	0.044	0.05	0.044	0.032	0.040	0.043	0.061	0.044	0.043	0.039	0.058	0.054		0.006	0.006	0.007	0.007	0.007	0.006	0.007	0.007	0.008	0.007	0.008	0.008	0.008	0.008	0.007	0.008	0.008	0.007
Bardeliella pulchra EU039884	32	0.046	0.046	0.043	0.051	0.049	0.060	0.052	0.056	0.054	0.055	0.052	0.052	0.052	0.053	0.053	0.051	0.056	0.052	0.052	0.045	0.046	0.047	0.044	0.054	0.054	0.055	0.049	0.052	0.044	0.059		0.006	0.007	0.006	0.007	0.007	0.006	0.006	0.008	0.008	0.007	0.007	0.007	0.007	0.007	0.008	0.008	0.008
Maryna ovata HQ337902	33	0.043	0.045	0.039	0.045	0.043	0.059	0.042	0.046	0.046	0.049	0.041	0.043	0.043	0.045	0.041	0.040	0.037	0.049	0.044	0.044	0.031	0.030	0.035	0.033	0.039	0.043	0.048	0.043	0.049	0.046	0.051	0.053		0.003	0.003	0.004	0.005	0.007	0.006	0.007	0.007	0.006	0.006	0.006	0.006	0.007	0.006	0.007
Maryna umbrellata JF747217	34	0.048	0.050	0.044	0.051	0.049	0.067	0.053	0.050	0.051	0.053	0.047	0.048	0.049	0.051	0.049	0.048	0.042	0.053	0.049	0.049	0.039	0.037	0.043	0.041	0.045	0.049	0.051	0.046	0.056	0.051	0.064	0.056	0.018		0.003	0.003	0.006	0.007	0.007	0.007	0.007	0.006	0.006	0.006	0.006	0.007	0.007	0.007
Maryna meridiana JF747218	35	0.045	0.045	0.042	0.043	0.042	0.059	0.045	0.044	0.045	0.047	0.041	0.043	0.043	0.045	0.042	0.041	0.038	0.048	0.045	0.043	0.035	0.033	0.036	0.034	0.040	0.041	0.047	0.046	0.052	0.044	0.058	0.050	0.013	0.013		0.002	0.006	0.007	0.007	0.007	0.006	0.006	0.006	0.006	0.006	0.006	0.007	0.007
Pseudomaryna sp. JF747219	36	0.052	0.053	0.049	0.050	0.049	0.060	0.052	0.049	0.051	0.054	0.048	0.049	0.049	0.052	0.048	0.047	0.045	0.055	0.051	0.05	0.042	0.039	0.042	0.041	0.047	0.047	0.052	0.052	0.058	0.051	0.060	0.056	0.019	0.019	0.005		0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Repoma cavicola KJ873053	37	0.046	0.045	0.034	0.033	0.032	0.043	0.036	0.038	0.032	0.035	0.037	0.035	0.034	0.037	0.040	0.040	0.032	0.040	0.042	0.039	0.025	0.022	0.029	0.029	0.047	0.043	0.040	0.040	0.047	0.050	0.040	0.051	0.037	0.041	0.039	0.045		0.006	0.006	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.008	0.007
Ilsiella palustris JQ026521	38	0.054	0.054	0.051	0.047	0.046	0.053	0.051	0.064	0.056	0.059	0.059	0.057	0.056	0.059	0.051	0.053	0.050	0.065	0.054	0.053	0.044	0.045	0.049	0.048	0.056	0.056	0.057	0.052	0.063	0.056	0.061	0.052	0.052	0.061	0.054	0.061	0.045		0.002	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.009	0.008
Ilsiella palustris EU039901	39	0.052	0.055	0.043	0.038	0.040	0.051	0.046	0.056	0.047	0.051	0.051	0.051	0.047	0.053	0.042	0.044	0.044	0.059	0.049	0.052	0.042	0.039	0.039	0.041	0.051	0.049	0.051	0.047	0.056	0.050	0.058	0.047	0.050	0.061	0.053	0.057	0.039	0.010		0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.009	0.008
Bromeliothrix metopoides JQ356866	40	0.068	0.062	0.050	0.053	0.051	0.068	0.059	0.053	0.057	0.057	0.055	0.061	0.058	0.062	0.064	0.063	0.057	0.063	0.047	0.041	0.049	0.048	0.054	0.051	0.061	0.048	0.053	0.046	0.067	0.066	0.068	0.071	0.058	0.059	0.055	0.063	0.057	0.072	0.070		0.001	0.004	0.004	0.004	0.004	0.004	0.004	0.004
Bromeliothrix metopoides AY398684	41	0.069	0.063	0.051	0.053	0.052	0.069	0.060	0.051	0.058	0.057	0.054	0.062	0.057	0.062	0.065	0.063	0.057	0.063	0.046	0.041	0.049	0.048	0.054	0.052	0.063	0.049	0.054	0.046	0.070	0.060	0.065	0.069	0.057	0.060	0.056	0.063	0.057	0.073	0.071	0.002		0.004	0.004	0.004	0.004	0.004	0.004	0.004
Paracolpoda steinii KY454454	42	0.063	0.060	0.053	0.050	0.049	0.066	0.056	0.051	0.056	0.056	0.055	0.057	0.057	0.057	0.060	0.059	0.051	0.061	0.053	0.044	0.045	0.047	0.049	0.046	0.061	0.051	0.056	0.046	0.062	0.065	0.068	0.063	0.052	0.056	0.052	0.060	0.054	0.068	0.066	0.020	0.021		0.001	0.001	0.001	0.001	0.002	0.002	0.002
Paracolpoda steinii KJ607913	43	0.065	0.062	0.054	0.051	0.050	0.067	0.058	0.052	0.057	0.055	0.056	0.057	0.056	0.057	0.061	0.058	0.053	0.065	0.053	0.046	0.047	0.047	0.050	0.048	0.063	0.054	0.059	0.046	0.063	0.066	0.066	0.064	0.055	0.058	0.054	0.062	0.055	0.070	0.068	0.021	0.020	0.002		0.000	0.001	0.000	0.001	0.001	0.002
Paracolpoda steinii KJ607911	44	0.065	0.062	0.054	0.051	0.050	0.067	0.058	0.052	0.057	0.055	0.056	0.057	0.056	0.057	0.061	0.058	0.053	0.065	0.053	0.046	0.047	0.047	0.050	0.048	0.063	0.054	0.059	0.046	0.063	0.066	0.066	0.064	0.055	0.058	0.054	0.062	0.055	0.070	0.068	0.021	0.020	0.002	0.000		0.001	0.000	0.001	0.001	0.002
Paracolpoda steinii KJ607912	45	0.063	0.060	0.052	0.050	0.048	0.066	0.057	0.051	0.055	0.055	0.054	0.056	0.056	0.057	0.059	0.058	0.051	0.063	0.052	0.045	0.046	0.046	0.048	0.046	0.061	0.052	0.057	0.046	0.062	0.064	0.067	0.063	0.053	0.056	0.053	0.060	0.054	0.068	0.066	0.020	0.020	0.001	0.001	0.001		0.000	0.001	0.001	0.002
Paracolpoda steinii KF732885	46	0.065	0.062	0.053	0.051	0.050	0.062	0.059	0.051	0.056	0.056	0.056	0.058	0.056	0.059	0.061	0.058	0.053	0.065	0.053	0.046	0.047	0.047	0.049	0.048	0.063	0.054	0.056	0.046	0.063	0.060	0.061	0.065	0.055	0.058	0.054	0.061	0.055	0.069	0.064	0.020	0.021	0.001	0.000	0.000	0.000		0.001	0.001	0.002
Paracolpoda steinii DQ388599	47	0.068	0.065	0.057	0.055	0.053	0.071	0.061	0.056	0.061	0.055	0.060	0.059	0.055	0.056	0.065	0.057	0.056	0.067	0.057	0.049	0.050	0.046	0.054	0.051	0.069	0.058	0.062	0.046	0.067	0.071	0.069	0.067	0.058	0.063	0.059	0.067	0.059	0.074	0.071	0.027	0.022	0.007	0.002	0.002	0.004	0.002		0.002	0.002
Paracolpoda steinii KJ607914	48	0.074	0.072	0.062	0.059	0.060	0.076	0.068	0.061	0.065	0.056	0.064	0.061	0.057	0.060	0.069	0.058	0.062	0.075	0.060	0.056	0.057	0.047	0.058	0.057	0.073	0.063	0.068	0.046	0.072	0.072	0.074	0.070	0.065	0.067	0.064	0.070	0.064	0.078	0.077	0.030	0.023	0.008	0.002	0.002	0.002	0.001	0.007		0.002
Paracolpoda steinii KJ607915	49	0.074	0.069	0.057	0.054	0.054	0.068	0.060	0.052	0.057	0.057	0.059	0.059	0.058	0.058	0.065	0.062	0.061	0.066	0.054	0.049	0.052	0.053	0.050	0.049	0.070	0.053	0.055	0.051	0.069	0.064	0.061	0.069	0.059	0.063	0.058	0.064	0.056	0.074	0.070	0.023	0.022	0.009	0.010	0.010	0.010	0.009	0.010	0.010	



Supplementary Fig. S1. Comparison of phylogenies of colpodean ciliates based on the 18S rRNA gene inferred from the unmasked, 0.50, 0.75, 0.80, 0.90 and 0.93 alignments. Trees were constructed under the respective GTR + I + Γ substitution evolutionary models selected by jModelTest (Supplementary Table S1). Only relationships between main colpodean lineages were shown. Posterior probabilities for Bayesian Inference (BI) and bootstrap values for Maximum Likelihood (ML) were mapped onto the 50%-majority rule Bayesian consensus trees. Note that the phylogenetic position of the orders Bursariomorphida and Platyophryida was usually statistically strongly supported but conflicts between the alignments analyzed. The order Bursariomorphida branched off first in the unmasked, 0.50 and 0.75 alignments, while the order Platyophryida in the 0.80–0.93 alignments. On the other hand, the orders Colpodida and Cyrtolophosidida were consistently classified as sister groups.



Supplementary Fig. S2. Softwired galled networks constructed from a set of thousand randomly selected trees obtained during the stationary phase of the Bayesian MCMC analyses of the 0.50, 0.75, 0.80 and 0.90 alignments. A threshold of 20% was set as a minimum fraction of trees that must contain a cluster for that cluster to be included in the network. Alternative evolutionary trajectories, i.e., edges leading into reticulate nodes, were marked by curved red lines. Most of the conflicts were on the base of the order Colpodida and included the phylogenetic position of *Bardeliella*, *Ilsiella*, *Sandmanniella*, bryophryids and grossglockneriids. The order Bursariomorphida branched off first in the 0.50 alignment, while the order Platyophryida in the 0.80 and 0.90 alignments. The position of bursariomorphids and platyophryids was conflicting in the 0.75 alignment.



Supplementary Fig. S3. Phylogenetic networks constructed from six alignments. With the strength of alignment masking, networks loose the tree-like structure becoming star-like in the central part due to the loss of phylogenetic signals. The set of splits uniting the orders Colpodida and Cyrtolophosidida (arrowheads) became shorter and more weakly statistically supported along with the strength of alignment masking. Likewise, the set of splits separating the order Bursariomorphida from the rest of colpodeans (arrows) became weaker in more strongly masked alignments. Note that there were no splits separating the order Platyophryida from the rest of colpodeans, although this relationship was shown with strong statistical support in phylogenetic trees (see Supplementary Fig. S1).