

### ORIGINAL ARTICLE

### A Proposed Timescale for the Evolution of Armophorean Ciliates: Clevelandellids Diversify More Rapidly Than Metopids

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#### ABSTRACT

Members of the class Armophorea occur in microaerophilic and anaerobic habitats, including the digestive tract of invertebrates and vertebrates. Phylogenetic kinships of metopid and clevelandellid armophoreans conflict with traditional morphology-based classifications. To reconcile their relationships and understand their morphological evolution and diversification, we utilized the molecular clock theory as well as information contained in the estimated time trees and morphology of extant taxa. The radiation of the last common ancestor of metopids and clevelandellids very likely occurred during the Paleozoic and crown diversification of the endosymbiotic clevelandellids dates back to the Mesozoic. According to diversification analyses, endosymbiotic clevelandellids have higher net diversification rates than predominantly free-living metopids. Their cladogenic success was very likely associated with sharply isolated ecological niches constituted by their hosts. Conflicts between traditional classifications and molecular phylogenies of metopids and clevelandellids very likely come from processes, leading to further diversification without extinction of ancestral lineages as well as from morphological plesiomorphies incorrectly classified as apomorphies. Our study thus suggests that diversification processes and reconstruction of ancestral morphologies improve the understanding of paraphyly which occurs in groups of organisms with an apparently long evolutionary history and when speciation prevails over extinction.

THE class Armophorea unites microaerophilic or anaerobic, bacterivorous ciliates carrying an adoral zone of membranelles that is involved in motion and food acquisition. Lynn (2004) established this group solely on the gene coding for the small subunit ribosomal RNA and ranked it as a riboclass, because no morphological synapomorphies were detected. According to Lynn's (2008) textbook, the Armophorea are subdivided into the orders Armophorida and Clevelandellida. Armophorids are common in anoxic water habitats, but live also in oxygen-depleted soils (Esteban et al. 1995; Foissner 1998; Foissner et al. 1992, 2002; Hu 2014; Saccà 2012) and some are even endocommensals of sea urchins (Biggar and Wenrich 1932; Kattar 1982; da Silva-Neto et al. 2016). On the other hand, clevelandellids are exclusively inhabitants of

© 2018 International Society of Protistologists Journal of Eukaryotic Microbiology 2019, **66**, 167–181 the digestive tract of invertebrates and vertebrates (for a review, see Corliss 1979 and Earl 1972).

Molecular relationships among and within armophorids and clevelandellids conflict with traditional morphologybased classifications (Jankowski 2007; Lynn 2008). Specifically, the species rich genus *Metopus* is nonmonophyletic and the family Metopidae is consistently depicted as paraphyletic encompassing the monophyletic order Clevelandellida (Bourland et al. 2014, 2017a,b; Li et al. 2016, 2017a,b; Lynn and Wright 2013; da Silva-Neto et al. 2016). Ontogenetic data excluded metopids from the order Armophorida and are now considered as a distinct order, Metopida (Foissner and Agatha 1999; Vd'ačný and Foissner 2017a). The classification of the armophorid family Caenomorphidae is unstable in phylogenetic analyses. It could be a sister group of metopids and clevelandellids or could represent a distinct lineage at the base of the class Spirotrichea or the infraphylum Intramacronucleata (da Silva Paiva et al. 2013).

To reconstruct the evolutionary history of armophorean ciliates and to improve understanding of their phylogenetic relationships, diversification dynamics, and morphological evolution, we utilized the molecular clock theory as well as information contained in the estimated time trees and morphology of extant taxa. Diversification dynamics is only rarely examined in ciliates due to the lack of fossil records (Rajter and Vd'ačný 2016; Vd'ačný 2015; Vd'ačný et al. 2017; Wright and Lynn 1997). However, this problem could be overcome by the fossil appearance of hosts of the exclusively endosymbiotic clevelandellids, whose origin cannot precede that of their host groups. Similar assumptions were already proposed by Williams and Coleman (1992) and Wright and Lynn (1997), pioneers of divergence time estimates using molecular clock in ciliates.

In this study, we attempt to address outstanding questions about (i) the molecular clock rate of the 18S rRNA gene in ciliates; (ii) the diversification patterns of free-living and endosymbiotic ciliates; and (iii) the paraphyly problems in ciliates in the light of time scale and adaptive change in a new ecological situation.

#### **MATERIALS AND METHODS**

#### Sample collecting and processing

All newly sequenced metopids were collected from the upper 5 cm litter and soil layer of the floodplain of the Murray River at the Landside of Ryans road near to the town of Albury, Southeast Australia (S36°06' E146°54'). The material was sampled in February 2006, air-dried for 3 weeks, and stored in a plastic bag. Metopids were reactivated from resting cysts in summer 2006, using the non-flooded Petri dish method, as described in Vd'ačný and Foissner (2012). A more detailed description of the sample is available in Vd'ačný and Foissner (2017a,b).

Isolated metopids were studied using a combination of detailed in vivo observation, silver impregnation, and scanning electron microscopy, as described by Foissner (2014). Briefly, living cells were observed at low (50–400×) and high (1000×, oil immersion) magnifications with bright field and differential interference contrast. The ciliary pattern of fixed cells was revealed with protargol and silver carbonate impregnation. Identification followed Kahl (1932), Foissner and Agatha (1999), Foissner et al. (2002), Bourland and Wendell (2014), and Vd'ačný and Foissner (2017b).

After identification, several specimens were picked from each species from enrichment cultures, washed several times to remove contaminants, and stored in ATL buffer. DNEasy Tissue Kit (Qiagen, Hildesheim, Germany) was used to extract the genomic DNA. Amplification of the 18S rRNA gene and quality check of the amplified DNA were performed as described by Vd'ačný et al. (2011a). PCR products were purified using the NucleoSpin Gel and PCR clean-up Kit (Macherey-Nagel, Düren, Germany) and cloned into a plasmid vector using the pGEM<sup>®</sup>-T and the pGEM<sup>®</sup>-T Easy Vector Systems (Promega, Fitchburg, Wisconsin, United States). Recombinant plasmids were introduced into the host organism *Escherichia coli* (strain JM109). After cultivation of transformed bacteria, plasmids were isolated using the PureYield<sup>™</sup>Plasmid Miniprep System (Promega, Fitchburg, Wisconsin, United States) and sequenced on an ABI 3730 automatic sequencer (Macrogen, Amsterdam, The Netherlands) with the M13 forward and reverse primers.

#### Alignment and tree-building methods

New sequences were checked and trimmed at the 5' and 3' ends in Chromas ver. 2.33 (Technelysium Pty Ltd.) and assembled into contigs using BioEdit ver. 7.2.5 (Hall 1999). An alignment of new and all available 18S rRNA gene sequences of free-living metopids and endosymbiotic clevelandellids was constructed on the GUIDANCE2 server (available at http://guidance.tau.ac.il/ver2/), using the MAFFT algorithm and 100 bootstrap repeats (Sela et al. 2015). Since the score of the resulting alignment was very high (>0.95), no special masking strategy was employed. Caeonomorphid taxa were selected as outgroup, because they are morphologically most similar to metopids and clevelandellids within the SAL super-cluster, and a sister group relationship of caenomorphids and metopids + clevelandellids cannot be excluded by statistical tree topology tests (da Silva Paiva et al. 2013).

 $GTR + I + \Gamma$  was selected as the best evolutionary substitution model for maximum likelihood and Bayesian analyses using jModelTest ver. 0.1.1 under the Akaike Information Criterion (AIC, Guindon and Gascuel 2003; Posada 2008). Maximum likelihood analyses were performed in PHYML ver. 3.0 with SPR tree-rearrangement and 1,000 nonparametric bootstrap replicates on the South of France bioinformatics platform (available at http:// www.atgc-montpellier.fr/phyml/) (Guindon et al. 2010). Bayesian analyses were run on the CIPRES portal ver. 3.1 (available at http://www.phylo.org/), using the program MrBayes (Ronquist et al. 2012) on XSEDE ver. 3.2.6 (Miller et al. 2010). Bayesian inference was performed with four chains running simultaneously for 5,000,000 generations. Prior parameters for stationary base frequencies, rate matrix for substitutions, gamma distribution shape and proportion of invariable sites, as estimated in jModelTest, were implemented into Bayesian analyses using the 'prset' command. Every 1000th tree was sampled and the first 25% of the sampled trees were considered as burnin and discarded prior to tree reconstruction and calculation of posterior probabilities.

#### **Molecular dating**

Divergence times were estimated in a Bayesian framework as implemented in the program BEAST ver. 2.4.5 (Bouckaert et al. 2014). The software BEAUti ver. 2.4.5 was used to generate a BEAST input XML file with the following settings: (i) GTR + I (=0.4430) +  $\Gamma$  (=0.3530) evolutionary model, as selected with jModelTest; (ii) four gamma categories for substitution rate heterogeneity; (iii) relaxed molecular clock; (iv) clock rate prior assuming a uniform distribution with an extremely large upper bound; (v) constant population function with gamma distribution; and (v) birth-death speciation model with uniform birth rate. Markov Chain Monte Carlo (MCMC) analyses started from a random seed, ran for 100 million generations, and trees as well as all other parameters were saved every 10,000th iteration. The convergence of all parameters to the stationary distribution, the effective sample size of parameters, and the burn-in fraction were inspected using the program Tracer ver. 1.6 (Rambaut and Drummond 2007). Two independent MCMC analyses were performed and tree files from different runs were merged in LogCombiner ver. 2.4.4. The final maximum credibility tree was then generated in TreeAnnotator ver. 1.8.1 (Rambaut and Drummond 2007) after discarding the first 20% of sampled trees.

As there is no fossil record available for the class Armophorea, calibration points were based on the fossil appearance of hosts of endosymbiotic clevelandellids. Occurrence of endosymbiotic ciliates is usually not restricted to a single host species, but it is generally restricted to higher taxa of their hosts (Corliss et al. 1965; Moon-van der Staay et al. 2014; Rataj and Vd'ačný 2018; Vďačný 2018; Williams and Coleman 1992). According to phylogenetic analyses, there are two distinct clevelandellid lineages (Lynn and Wright 2013; present study): one occurring in cockroaches of the families Blaberidae and Blattidae, while the other one is from amphibians of the families Ranidae and Bufonidae. As clevelandellids are exclusively endosymbiotic, their occurrence cannot precede that of their host groups, a fact that can be incorporated into dating analyses with minimum bounds. Since these are secondary calibration points, we used uniform distributions with extremely large upper bounds. The node uniting clevelandellids occurring in blaberiid and blattid cockroaches, was assigned a lower bound of 61 Ma and an upper bound of 407 Ma. The minimum calibration date was based on the oldest known, blaberiid cockroach Gyna obesa (Evangelista et al. 2017) and the upper bound on the earliest insect fossils (Bourguignon et al. 2018). The node uniting clevelandellids from anurans, was assigned a lower bound of 65 Ma and an upper bound of 275 Ma. The minimum calibration date represents the Neobatrachia-Pelobatoidea split and the upper bound is the origin of frogs (Zhang et al. 2013).

#### **Diversification analyses**

Diversification rates were estimated in a Bayesian framework using BayesRate ver. 1.3.41 which accommodates uncertainty in the inferred phylogeny and incomplete taxon sampling (Silvestro et al. 2011). Thus, prior to computation, the proportion of the taxa sampled was summarized (Table S1) and incorporated into diversification analyses. Phylogeny of Metopids and Clevelandellids

Fit of the models with and without extinction was assessed by BayesFactors, for which log marginal likelihoods were estimated over 100 trees from the posterior distribution of the BEAST analysis, using the thermodynamic integration option. Since the birth-death model was favored over the pure-birth model, clade specific rates were calculated with assumption of extinction during evolutionary history. MCMC simulations included 100,000 iterations, with a sampling frequency of 100 and discarding the first 1,000 samples per tree as burn-in.

# Reconstruction of ancestral traits and correlation analyses

Ancestral state reconstruction was performed for one ecological and two morphological traits with SIMMAP ver. 1.5.2 (Bollback 2006). Characters and their states are summarized in Table S2. Terminology follows Lynn (2008) and Foissner and Agatha (1999). Specifically, the perizonal stripe is a ciliary structure made of up to five somatic, densely ciliated kineties extending over the dorsal side of the preoral dome. The paroral membrane is a ciliary structure lying along the right border of the oral region. We recognize two types of paroral membranes in armophoreans. The first type is made of a single row of cilia and we call it single-rowed. The second type is made of two distinctly separated rows of cilia and we call it double-rowed. de Puytorac and Grain (1976) termed it also diplostichomonad.

A set of 100 randomly selected trees from the posterior distribution of the BEAST analysis served to incorporate phylogenetic uncertainty. Since all characters are binary, analyses were run using default settings: (i)  $\alpha = 1.00$  and k = 31 for the beta distribution of state frequencies; (ii) equal bias prior 1/k for the beta distribution; and (iii)  $\alpha = 1.25$ ,  $\beta = 0.25$  and k = 60 for the gamma distribution of the overall rate of character change. Ten samples were analyzed per each tree and branch lengths were re-scaled that the overall length of trees is one. Results were plotted as pie charts using the R script "PlotSimMap.R" (available at https://github.com/nylander/PlotSimMap) and mapped onto the BEAST maximum credibility tree.

Character correlation was evaluated on the BEAST maximum credibility tree using Pagel's test (1994) implemented in MESQUITE ver. 2.73 (Maddison and Maddison 2007). This test estimates log-likelihood scores for a fourparameter model without correlation and an eight-parameter model with correlation (Pagel 1994). Fit of the correlated and uncorrelated models was evaluated through the likelihood ratio test.

#### RESULTS

#### **Phylogenetic analyses**

We obtained 12 new 18S rRNA gene sequences from Australian free-living metopids. Their length, GC content, and GenBank accession numbers are provided in Table 1. To determine their phylogenetic positions and to

 Table 1. Characterization of new 18S rRNA gene sequences obtained

 from Australian metopids (arranged alphabetically)

Taxon	Clone <sup>c</sup>	Length (nt)	GC (%)	GenBank entry
Atopospira galeata (Kahl, 1927) Bourland and Wendell (2014)	Clone 1 Ag	1706	44.31	MH086814
Atopospira galeata (Kahl, 1927) Bourland and Wendell (2014)	Clone 2 Ag	1706	44.67	MH086815
<i>Metopus hasei</i> Sondheim, 1929	Clone 1 Mh	1706	44.78	MH086816
<i>Metopus hasei</i> Sondheim, 1929	Clone 2 Mh	1706	44.43	MH086817
<i>Metopus hasei</i> Sondheim, 1929	Clone 3 Mh	1706	44.43	MH086818
Metopus hasei Sondheim, 1929	Clone 4 Mh	1706	44.43	MH086819
<i>Metopus hasei</i> Sondheim, 1929	Clone 5 Mh	1706	44.37	MH086820
Metopus laminarius Kahl, 1927	Clone 1 MI	1702	44.24	MH086821
Metopus minor Kahl, 1927	Clone 1 Mm	1706	44.49	MH086822
<i>Metopus setosus</i> Kahl, 1927 <sup>a</sup>	Clone 1 Ms	1709	44.24	MH086823
<i>Metopus setosus</i> Kahl, 1927 <sup>a</sup>	Clone 2 Ms	1710	44.21	MH086824
<i>Metopus</i> sp. <sup>b</sup>	Clone 1 Msp	1712	44.16	MH086825

<sup>a</sup>This population was morphologically described in detail by Vd'ačný and Foissner (2017b).

<sup>b</sup>Very likely a new species.

<sup>c</sup>A single sequence was obtained from each clone.

reconstruct the evolutionary history of metopids and clevelandellids, Bayesian and maximum likelihood (ML) analyses were carried out. They resulted in almost identical topologies. The order Metopida was depicted as paraphyletic, encompassing the order Clevelandellida and their common node obtained full statistical support in both analyses (Fig. 1). The genus Metopus was nonmonophyletic: (i) M. es, the type species of Metopus, grouped with Brachonella contorta but with very poor support (posterior probability 0.64/ML bootstrap 45%); (ii) American populations of *M. fuscus* and *M. setosus* clustered together with Palmarella lata with poor to strong support (0.99/62%); and (iii) M. hasei and M. yantaiensis formed a very weakly supported and poorly structured clade along with Parametopidium circumlabens (Fig. 1). Monophyly of the genus Urostomides was strongly statistically supported (1.00/98%) and relationships among its species were comparatively well-resolved. A common origin of Atopospira species obtained strong statistical support in Bayesian analyses (0.98), while only poor support in ML analyses (52%). American and Australian populations of A. galeata clustered together with high support (1.00/99%). Likewise, American and Australian populations of *M. laminarius* formed a well supported clade (1.00/82%). On the other hand, American and Australian isolates of *M. setosus* did not group together and were separated by several strongly to fully statistically supported nodes, indicating that they represent morphologically cryptic species. Validity and distinctness of *M. minor*, which was considered as a subspecies of *M. setosus* by Kahl (1932), is strongly corroborated in that *M. minor* did not cluster with any of the *M. setosus* populations studied. Similarly, *B. contorta* was revealed to contain two morphologically cryptic and genetically fairly distant groups (Fig. 1).

Monophyly of the order Clevelandellida was statistically fully supported in Bayesian and ML analyses. Clevelandellids formed a robust clade (1.00/91%) along with the following metopids: Atopospira spp., M. hasei, M. laminarius, M. minor, M. setosus (Australian population), Metopus sp., M. yantaiensis, and P. circumlabens. Two distinct and statistically well-supported lineages were recognized within the order Clevelandellida: (i) one clade contained species isolated from invertebrates (N. ovalis, Nyctotherus velox, Nyctotheroides sp. AF147882.1, Nyctotherus sp. KC139721.1, and Clevelandella spp.) and (ii) the other clade included species found in anurans (Nyctotheroides cordiformis, N. deslierresae, N. hubeiensis, N. parvus, N. pyriformis, and Nyctotheroides sp. AF147882.1). The genus Nyctotherus was shown paraphyletic and included the monophyletic genus Clevelandella (Fig. 1).

#### **Estimation of divergence times**

The time tree obtained with Bayesian relaxed molecular clock is shown in Fig. 2. The molecular clock rate for the armophorean 18S rRNA gene was estimated to be on average  $3.96 \times 10^{-4}$  nucleotide substitutions per site per one million years, with the 95% credibility interval spanning a range from  $1.63 \times 10^{-4}$  to  $6.25 \times 10^{-4}$ . The rate of evolution of different lineages in the analyses varied by 68.1% of the clock rate, documenting that a strict clock would be inappropriate for dating the armophorean phylogeny.

The crown radiation of the last common ancestor of metopids and clevelandellids very likely occurred during the Paleozoic period and its posterior mean was estimated to be 440 Ma ago. The genus *Urostomides* originated in the early history of the order Metopida and has begun to diversify about 178 Ma ago. On the other hand, the genus *Atopospira* emerged comparatively recently and split into *A. violacea* and *A. galeata* about 45 Ma ago (Fig. 2). *Parametopidium circumlabens*, a metopid obligate endosymbiont of sea urchins, branched off from its nearest free-living relatives about 51 Ma ago.

The origin of the order Clevelandellida dates back to the Mesozoic and its segregation into two main lineages occurred about 156 Ma ago. The divergence of the lineage inhabiting invertebrates started in the Upper Cretaceous about 129 Ma ago, and radiation of the lineage living in ranid and bufonid anurans began approximately 97 Ma ago



Figure 1 Phylogeny of the class Armophorea based on the 18S rRNA gene. Posterior probabilities for Bayesian Inference (BI) and bootstrap values for Maximum Likelihood (ML) are mapped onto the 50%-majority rule Bayesian consensus tree. Dashes indicate mismatch in topology between Bayesian and ML tree. Sequences in bold face were obtained during this study. The scale bar denotes seven substitutions per one hundred nucleotide positions.



Figure 2 Maximum credibility tree showing posterior means of divergence times of the class Armophorea obtained with the Bayesian relaxed molecular dating in BEAST. Crown divergence times of the orders Metopida and Clevelandellida are in bold face. The 95% credibility intervals are shown for all nodes as bars. Horizontal axis represents the time scale in million years. Group-specific net diversification rates estimated under the birth-death model implemented in BayesRate are shown in left indent.

(Fig. 2). The radiation of the ciliate genus *Clevelandella*, which is restricted to the blaberiid cockroach genus *Panesthia*, was estimated here to be about 50 Ma ago.

#### **Diversification dynamics**

According to the Bayesian diversification analyses taking into account incomplete taxon sampling, a birth-death model was strongly favored over a pure-birth model (BayesFactor BF = 22.66), indicating that some lineages of the metopid-clevelandellid clade have gone extinct during the armophorean phylogeny. Analyses of group-specific rates under the birth-death model indicated that endosymbiotic clevelandellids have diversified at a much higher rate than metopids (inset, Fig. 2). Specifically, metopids have a net diversification rate of an average of 0.008 lineages per one million years (Myr), while that of clevelandellids is almost four times higher, being 0.028 lineages/Myr (Table S3).

# Reconstruction of ancestral states and correlation analyses

All analyzed key intrinsic traits are significantly associated with phylogenetic clades, as illustrated by SIMMAP reconstructions mapped onto the BEAST maximum credibility tree (Fig. 3, 4). The ancestor of the metopid-clevelandellid clade was very likely free-living and shifted to endosymbiosis two times independently: in Parametopidium circumlabens, which became an inhabitant of sea urchins, and in the progenitor of the clevelandellid clade. Ancestrally, the paroral membrane was single-rowed and a perizonal stripe was present. Loss of the perizonal stripe is confined to the progenitor of the clevelandellid clade and very likely is associated with the switch to endosymbiosis. Indeed, according to Pagel's correlation tests (Table S4), there is a strong association between lifestyle and type of paroral membrane ( $\chi^2$  = 13.46, df = 4, p < 0.001) as well as between lifestyle and presence/absence of a perizonal stripe ( $\chi^2$  = 13.02, df = 4, p < 0.001) during the metopidclevelandellid phylogeny. There is also a strong correlation between type of paroral membrane and presence/ absence of a perizonal stripe ( $\chi^2 = 8.52$ , df = 4, p = 0.01) (Table S4).

The endosymbiotic lifestyle is thus typically associated with a double-rowed paroral membrane and with the loss of the perizonal stripe. The single exception is the endosymbiotic *Parametopidium* which displays a double-rowed (diplostichomonad) paroral membrane and still possesses a perizonal stripe. In turn, this indicates that the endosymbiotic lifestyle of *Parametopidium* evolved independently from clevelandellids.

On the other hand, the free-living way of life is mostly connected with a single-rowed paroral and a welldeveloped perizonal stripe. The single exception is the genus *Atopospira* which is free-living but exhibits a double-rowed paroral. According to SIMMAP reconstructions, the double-rowed paroral most likely evolved three times independently from the single-rowed paroral, that is, in Atopospira, Parametopidium and the progenitor of the whole clevelandellid clade (Fig. 4).

#### DISCUSSION

#### Molecular clock rate of the 18S rRNA gene in ciliates

The concept of a molecular clock is based on the assumption that processes such as DNA replication, transcription, and translation are similar in all organisms and the proteins and RNAs carrying out all these housekeeping functions should be highly conserved. In spite of this fact, nucleotide substitution rates can vary considerably between species and molecular clock may not "tick" at a steady rate (Thomas et al. 2006). To overcome this problem, Bayesian relaxed clock methods, allowing for rate variation, have been developed (for a review, see Yang 2014).

As concerns the ciliate 18S rRNA gene, Wright and Lynn (1997) estimated the rate of nucleotide substitution on the basis of genetic distance between the obligate freshwater fish ectoparasite, Ichthyophthirius, and its closest free-living relative, Ophryoglena, using the origin of freshwater fishes in the fossil record. They have found that the 18S rRNA gene of Ichthyophthirius has diverged approximately 1.8 to 2.0% over 145 Myr or 1% per 72 to 80 Myr. This is equivalent to a rate of 1.25–1.40  $\times$   $10^{-4}$ nucleotide substitutions per site per one million years. Vďačný (2015) estimated the rate for litostomatean ciliates to be  $1.75 \times 10^{-4}$  on average with a 95% credibility interval to be  $1.18 \times 10^{-4}$  and  $2.36 \times 10^{-4}$  substitutions per site per one million years (calculated from the original data). An independently timed event in our analysis came from the endosymbiotic genus Entodinium which lives both in cammelids and ruminants (Williams and Coleman 1992; Wright and Lynn 1997). In the present study, we used relaxed Bayesian molecular clock and external information about fossil appearance of hosts of the exclusively endosymbiotic clevelandellids. The mean posterior estimate of the clock rate of the armophorean 18S rRNA gene was  $3.96 \pm 0.05 \times 10^{-4}$  substitutions per site per one million years.

A comparison of these three independent clock-rate estimates indicates that the 18S rRNA gene evolves at the same order of magnitude in three divergent groups of ciliates (oligohymenophoreans, litostomateans, and armophoreans). This information can be particularly useful for Bayesian dating analyses that cannot be calibrated with independently derived fossil data. The uncertainty in the clock rate prior can be incorporated by a uniform distribution with reasonably defined lower and upper bounds or by a diffuse prior for the gamma distribution, as recommended by Yang (2014) and Drummond and Bouckaert (2015).

#### Coevolution of ciliates with their hosts

Phylogenetic studies have suggested that endosymbiotic ciliates group according to associations with higher taxa of their hosts (e.g., Lynn and Wright 2013; Moon-van der



Figure 3 SIMMAP reconstruction of ancestral lifestyle and presence/absence of a perizonal stripe based on a set of 100 randomly selected trees from the posterior distribution of the BEAST analysis. Relative proportions of characters states were mapped onto the maximum credibility tree. Circles at tips of branches show the character state of a respective taxon. Coding of characters is summarized in Table S2. AZ, adoral zone; PS, perizonal stripe.



Figure 4 SIMMAP reconstruction of ancestral types of paroral membrane based on a set of 100 randomly selected trees from the posterior distribution of the BEAST analysis. Relative proportions of characters states were mapped onto the maximum credibility tree. Circles at tips of branches show the character state of a respective taxon. Coding of characters is summarized in Table S2. AZ, adoral zone; PM, paroral membrane; PS, perizonal stripe.

Staay et al. 2014; Rataj and Vd'ačný 2018; Sauvadet et al. 2017; Vďačný 2018). The present time-calibrated phylogeny indicates that evolution of metopid and clevelandellid ciliates is also coupled with that of their hosts. Specifically, Parametopidium circumlabens, a metopid obligate endosymbiont of sea urchins, branched off from its nearest free-living relatives about 51 Ma ago (Fig. 2). According to Kattar (1982), most records of P. circumlabens are from globular sea urchins of the order Camarodonta, namely from the families Temnopleuridae, Toxopneustidae, and Echinometridae. Interestingly, the oldest camarodont fossils are from the Upper Eocene about 56 Ma ago (Kroh and Smith 2010). Similarly, the radiation of the ciliate genus Clevelandella, which is restricted to the blaberiid cockroach genus Panesthia (Kidder 1937, 1938; Lynn and Wright 2013; Mandal and Nair 1974; Yamasaki 1939), was estimated here to be about 50 Ma ago (Fig. 2). Interestingly, divergence of the host genus Panesthia was estimated to be on average about 45 Ma ago by Wang et al. (2017). This indicates that *Clevelandella* might have been already present in the last common ancestor of the genus *Panesthia*.

#### **Diversification dynamics in ciliates**

Time-trees based on extant species include also information about the past diversification events that can be inferred by complex mathematical models (Stadler 2013). The present diversification analyses suggest that the establishment of symbiotic associations between clevelandellids and their invertebrate as well as vertebrate hosts has increased their net diversification rates. It is well-known that different host species constitute sharply isolated ecological niches and adaptation to a new host might permit rapid speciation of endosymbionts or parasites (for a review, see Coyne and Orr 2004). Various host species thus act as barriers preventing gene flow between populations of endosymbionts, which in turn enhances their speciation processes. We suppose that evolution of isolating barriers requires comparatively more time in freeliving metopids due to more homogenous properties of the aquatic environment. This, in turn, might have caused a slower speciation rate of metopids, which is indirectly corroborated in that speciation and net diversification rates of clevelandellids are several times higher than those of metopids (Fig. 2, Table S3). Indeed, there are only about 80 recognized metopid taxa, while almost 200 clevelandellid forms have been described so far from a variety of hosts (Table S1).

Like in metopids and clevelandellids, topologies of phylogenetic trees of rhynchostomatian and pleurostome ciliates indicate a comparatively homogeneous diversification without mass or gradual extinctions (Rajter and Vd'ačný 2016). Nevertheless, Bayesian diversification analyses favors a birth-death model over a pure-birth model both in the metopid-clevelandellid group (present study) and in rhynchostomatians (Vd'ačný et al. 2017), suggesting attrition of some lineages via extinction. Diversification patterns of spathidiid ciliates might be, however, comparatively different from those of metopids, clevelandellids, pleurostomes and rhynchostomatians. Rajter and Vd'ačný (2016) speculated that the long branches connected by short internodes in the spathidiid tree of life might reflect very rapid initial radiation followed by gradual extinction. Our supposition was based on lineage-through-time (LTT) plots constructed from spathidiid phylogenies, which exhibited a gradual decline in accumulation of lineages towards present times. Such a diversification pattern seems to be rather atypical in ciliates, although extinction is very likely an integral part of their evolutionary history.

#### Paraphyly problems in ciliates

Paraphyletic genera and suprageneric groups are comparatively common and scattered throughout the ciliate tree of life. Well-known examples are the time-honored and species-rich genera Blepharisma (Pan and Stoeck 2017), Colpoda (Dunthorn et al. 2011), Dileptus (Vd'ačný and Foissner 2012; Vďačný and Rajter 2015), Oxytricha (Foissner et al. 2014; Shao et al. 2014), Spathidium (Rajter and Vd'ačný 2016), Strombidium (Liu et al. 2016), and Vorticella (Sun et al. 2012). Paraphyly was detected also in Metopus and Nyctotherus (Bourland et al. 2014, 2017a,b; da Silva-Neto et al. 2016; Li et al. 2016, 2017a,b; Lynn and Wright 2013; Omar et al. 2017; present study). Paraphyletic suprageneric groups are, for instance, the order Metopida which encompasses the order Clevelandellida (Li et al. 2016, 2017a,b; Lynn and Wright 2013), the subclass Haptoria which contains the sublass Trichostomatia (Vd'ačný et al. 2011a,b), the subclass Scuticociliatia which includes the subclass Apostomatia (Gao et al. 2013), and the class Prostomatea which contains the class Plagiopylea (Zhang et al. 2014).

According to Hörandl (2006) and Hörandl and Stuessy (2010), main natural sources for paraphyly are diversification processes leading to speciation without extinction of an ancestral species, causing co-existence of ancestralderivative taxa. On the other hand, artificial sources for creating paraphyletic groups are plesiomorphies incorrectly used as apomorphies (for reviews, see Wägele 2005 and Vd'ačný 2017). The paraphyly problems encountered in the metopid-clevelandellid group are obviously a combination of both natural and artificial sources. Nevertheless, paraphyly within the metopid-clevelandellid clade might be attributed to evolutionary budding, a process typically occurring along with adaptive changes in a new ecological situation (Foissner et al. 2011; Mayr and Bock 2002). During budding, origin of a new taxon is typically associated with a high amount of phenotypic change, i.e., evolution of distinct morphological apomorphies in derivative taxa, while ancestral lineages survive phenotypically almost unchanged (Foissner et al. 2011).

Metopus species maintained key morphological attributes of the putative parental taxon of the whole metopidclevelandellid group, causing the genus Metopus to be defined by plesiomorphies and not by apomorphies (Fig. 3-5). Nonetheless, it would be artificial to split Metopus into several small monophyletic groups defined solely by molecular apomorphies, because this would suppress our knowledge about morphological evolution of the metopid-clevelandellid ciliates and would ignore that Metopus represents a stem lineage from which multiple derivative taxa have branched off (Fig. 1, 5). A stem genus is a paraphyletic assemblage that is defined by the same evolutionary novelties as the last species of the stem lineage. It is not surprising that a genus with an apparently long evolutionary history is paraphyletic. Indeed, several Metopus species are extremely old (Fig. 2) and diversification in the metopid-clevelandellid group prevails over extinction (Table S3). A very similar phylogenetic picture has been revealed not only in various ciliate genera (Przyboś et al. 2015; Rajter and Vd'ačný 2016; Vd'ačný and Rajter 2015), but also in some invertebrate groups. A good example is the paraphyletic genus Holothuria which includes four morphologically distinct genera (Borrero-Pérez et al. 2010) and whose time-scale is similar to that of Metopus.

## Evolutionary taxonomy and morphological evolution of metopids and clevelandellids

Metopus Claparède and Lachmann, 1858 and Nyctotherus Leidy, 1849 are among the earliest genus-group names for microaerophilic and anerobic free-living and endosymbiotic "heterotrichs". However, several studies have suggested that both Metopus and Nyctotherus are paraphyletic assemblages (see above). In the cladistic point of view, their paraphyly invalidates the taxonomic concepts in the order Metopida Jankowski, 1980 and the family Metopidae Kahl, 1927 as well as in the order Clevelandellida Puytorac and Grain, 1976 and its family Nyctotheridae Amaro, 1972. Indeed, diagnostic characters of the genus *Metopus*, i.e., the twisted body carrying a perizonal stripe composed of five rows and a single-rowed paroral membrane, are ancient plesiomorphies present already in the last common progenitor of the whole metopid-clevelandellid group (Fig. 3-5). Similarly, the loss of the perizonal stripe and the formation of a deep vestibulum



Figure 5 Hypothesis for the morphological evolution of some armophorean genera based on 18S rRNA gene phylogenies and SIMMAP reconstructions. Diagnostic features of the genus *Metopus* are ancient plesiomorphies present already in the last common progenitor of the whole metopid-clevelandellid group. The genus *Metopus* thus represents a stem lineage that has independently given raise to multiple derivative taxa that are considered as distinct genera.

and a double-rowed paroral membrane, are features of the last common ancestor of the order Clevelandellida (Lynn 2008; Fig. 3–5). These features have, however, no power to reveal phylogenetic relationships among clevelandellid families and genera, because they are plesiomorphies at these taxonomic levels.

There are several conspicuous metopid morphospecies that were classified as distinct genera and for which molecular data are available (Fig. 5). Specifically, Atopospira Jankowski 1964a can be clearly defined by the bipartition of the adoral zone (Bourland and Wendell 2014), Brachonella Jankowski 1964a by the spiralization of the adoral zone around the entire body and posteriorization of the cytostome (Bourland and Wendell 2014; Bourland et al. 2017a), Urostomides Jankowski 1964a by a fourrowed perizonal stripe (Bourland et al. 2017b; Foissner 2016), and Parametopidium Aescht, 2001 by a doublerowed paroral membrane (da Silva-Neto et al. 2016). However, the double-rowed paroral is a homoplastic trait that evolved convergently also in Atopospira and clevelandellids (Fig. 4). This is not surprising, since oral structures are under strong selective pressure in many ciliates and hence similar oral morphologies might have formed independently in distantly related taxa (Lynn 2008).

There are several morphologically distinct metopids which need to be sequenced and/or whose morphology needs to be studied with modern taxonomic methods. A good example is the genus *Palmarella* Jankowski, 1975 which is well recognized by the strongly flattened anterior body portion (Jankowski 1964a,b). At the present state of knowledge, we cannot exclude that this genus is also paraphyletic and encompasses the genera *Tesnospira* Jankowski 1964a which, in addition, displays a suture in the anterior pole area and lacks a side stripe (Jankowski 1964a,b), *Tropidoatractus* Levander, 1894 which possesses a ribbed armor (Foissner et al. 1992; Jankowski 1964a,b; Kahl 1932), and *Lepidometopus* Vd'ačný and Foissner 2017a which is covered with epicortical scales (Vd'ačný and Foissner 2017a).

As advocated by Hörandl (2007), in such a complex taxonomic situation, we do not prefer cladistic but evolutionary classification in the sense of Mayr and Bock (2002). Specifically, we suggest to maintain the order Metopida, the family Metopidae and the genus *Metopus* which represents the stem lineage of the whole metopid-clevelandellid clade. Likewise, we find the order Clevelendellida as valid in the light of adaptive changes in a new ecological situation (colonization of the hindgut), which was very likely associated with a high amount of phenotypic change (Fig. 5). Adaptive radiation of the clevelandellids culminated in the name-bearing genus *Clevelandella* Kidder 1938 whose body has even reversed polarity and has evolved an elongated posterior end bearing the peristome (Fig. 5; Kidder 1937, 1938).

#### **CONCLUSIONS**

• The 18S rRNA gene evolves at the same order of magnitude in genetically fairly distant groups of ciliates. On average, the clock rate spans a range of

1.24–3.96  $\times$  10<sup>-4</sup> substitutions per site per one million years.

• Although particular ciliate groups might have undergone rapid radiations and are still flourishing, fitting of diversification models also indicates attrition of some lineages via extinction during evolution.

• Clevelandellids are cladogenically much more successful than metopids, which is very likely associated with sharply isolated ecological niches provided by hosts of clevelandellids. On the other hand, evolution of isolating barriers very likely requires comparatively more time in free-living metopids due to more homogenous properties of the aquatic environment.

• Main sources for paraphyly in the metopid-clevelandellid clade are processes leading to further diversification without extinction of ancestral lineages as well as plesiomorphies incorrectly classified as apomorphies.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article:

 Table S1. Number of species described in the class Armophorea and proportion of the taxa sampled.

 Table S2.
 Characters, character states, and their coding used for ancestral state reconstruction.

**Table S3.** Parametrization and fitting of constant-rate diversification models to the phylogeny of metopids and clevelandellids.

 Table S4.
 Pagel's test of correlated character evolution

 performed on the BEAST maximum credibility tree.