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ORIGINAL ARTICLE

Polyclad phylogeny persists to be problematic

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Abstract

Two conflicting morphological approaches to polyclad systematics highlight the relevance of molecular data for resolving the interrelationships of Polycladida. In the present study, phylogenetic trees were reconstructed based on a short alignment of the 28S rDNA marker gene with 118 polyclad terminals (24 new) including 100 different polyclad species from 44 genera and 22 families, as well as on a combined dataset using 18S and 28S rDNA genes with 27 polyclad terminals (19 new) covering 26 different polyclad species. In both approaches, Theamatidae and Cestoplanidae were included, two families that have previously been shown to switch from Acotylea to Cotylea. Three different alignment methods were used, both with and without alignment curation by Gblocks, and all alignments were subjected to Bayesian inference and maximum likelihood tree calculations. Over all trees of the combined dataset, an extended majority-rule consensus tree had weak support for Theamatidae and Cestoplanidae as acotyleans, and also the cotylean genera *Boninia*, *Chromyella* and *Pericelis* appeared as acotyleans. With the most inclusive short 28S dataset, on the other hand, there is good support for the aforementioned taxa as cotyleans. Especially with the short 28S matrix, taxon sampling, outgroup selection, alignment method and curation, as well as model choice were all decisive for tree topology. Well-supported parts of the phylogeny over all trees include Pseudocerotoidea, Prosthiostomoidea, Stylochoidea, Leptoplanoidea and Cryptoceloidea, the latter three with new definitions. Unstable positions in the tree were found not only for Theamatidae, Cestoplanidae, *Boninia*, *Chromyella* and *Pericelis*, but also for *Anonymus*, *Chromoplana* and *Cycloporus*.

Keywords Platyhelminthes · Polycladida · Cotylea · Acotylea · Molecular phylogenetics · Systematics

Introduction

Due to their colourful appearance, polyclad flatworms are among the most conspicuous members of the phylum Platyhelminthes, yet these animals are relatively poorly studied (Bahia et al. 2017). Usually, polyclads occur in diverse marine habitats, such as under coastal stones, on reefs and in interstitial spaces (Hyman 1951; Prudhoe 1985; Curini-Galletti et al. 2008). About 800 to 1000 species of polyclads are currently recognised (Rawlinson 2008; Martín-Durán and Egger 2012).

The phylogenetic position of Polycladida within Platyhelminthes used to be very controversial (Bahia et al. 2017). Only recently, Polycladida have been consistently recovered as sister group to Prorhynchida (a group harbouring only freshwater dwellers), forming the Amplimatricata, which is the sister group of all other Trepaxonemata (Egger et al. 2015; Laumer et al. 2015; Laumer and Giribet 2017).

Lang (1884) was the first to distinguish between two groups of 'marine planarians', the Tricladida and the Polycladida. He further grouped the Polycladida into forms with a ventral sucker behind the genital openings (Cotylea), and those without (Acotylea). This classification system persists after some modifications (e.g. Laidlaw 1903; Bock 1913; Hyman 1953; Marcus and Marcus 1966) until today, and in the 1980s, Faubel (1983, 1984) and Prudhoe (1985) separately published monographs attempting to further clarify the

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interrelationships of polyclads on morphological grounds, using genital organs, especially the organisation of the prostatic vesicle (Faubel 1983, 1984), the position of eyes and tentacles (Prudhoe 1985), or the pharynx organisation (Faubel 1983, 1984; Prudhoe 1985) as main systematic characters—however, the resulting classifications were largely incongruent. Interestingly, Faubel (1984) considered both Cotylea and Acotylea as not being monophyletic, but retained the names for taxonomic consistency. Prudhoe (1985) was also aware of problems with the classification and he cited several cases, where some families, such as Enantiidae and Boniniidae, have features fitting both to Cotylea and Acotylea.

For more than 30 years, these two conflicting systems have been in use by polycladologists (a term coined by J. Bahia, personal communication), stressing the need of a unifying system, based on morphology, on molecules, or both. The first molecular phylogenetic reconstruction of polyclad interrelationships was using a partial sequence of about 350 nucleotides of the marker molecule 28S (large nuclear ribosomal subunit) and was focussed on the family Pseudocerotidae, with *Pericelis* as the cotylean sister group of Pseudocerotidae (Litvaitis and Newman 2001). Another molecular phylogenetic analysis of Polycladida based on partial 28S sequences (about 900 nt long) included just eight cotylean and six acotyleans—Cotylea was not recovered as monophyletic, since the cotylean species *Pericelis cata* appeared outside the other Cotylea as sister group of Acotylea, while *Cestoplana rubrocincta* emerged as an acotylean as in Faubel's and Prudhoe's systems (Rawlinson et al. 2011). With a very similar dataset, Rawlinson and Stella (2012) recovered both, *Pericelis* and *Cestoplana*, as basally branching cotyleans, thereby stressing the problematic position of these taxa. In a flatworm-wide phylogenetic study based on four genes, the acotylean *Theama* was grouped with the remaining Cotylea, not with the Acotylea (Laumer and Giribet 2014), which was corrobarated in a transcriptomic study in the following year (Laumer et al. 2015).

In 2017, three large molecular phylogenies of polyclads were published, two with different stretches of the 28S marker gene (Bahia et al. 2017; Tsunashima et al. 2017), and one with mitochondrial genes (Aguado et al. 2017). Of these studies, only Bahia et al. dealt with the aforementioned problematic taxa, namely *Pericelis*, *Cestoplana* and *Theama*—all of them showing up as cotyleans in their tree (Bahia et al. 2017). However, this study only used a single alignment method and a single model with relatively low bootstrap support levels, so the reliability of the provided reconstruction remained unclear. During the review phase of this manuscript, another publication using the 28S marker gene was published (Litvaitis et al. 2019).

In the present study, we also have used partial 28S rDNA sequences, as well as a combined dataset of longer 18S and 28S sequences of a wide systematic range of polyclads. Most importantly, we have applied three different, widely used alignment algorithms and two different statistical approaches for tree reconstruction to test the stability and reliability of molecular phylogenies using one or two genes, and also, when possible, to infer relationships between groups based on a bigger data set.

Material and methods

Animal collection, identification of species and transcriptome data

An overview of newly generated and published sequences is provided in Table 1. For most collected material, tissue was stored in 99% ethanol, and histological sections were made as described by Aguado et al. (2017) and Dittmann et al. (2019). Several published polyclad transcriptomes (Egger et al. 2015, Laumer et al. 2015) were searched for 18S and 28S sequences (see Table 1) using BLAST (Altschul et al. 1990).

DNA extraction, PCR amplification and sequencing

For all specimens, DNA was extracted from a small piece of ethanol-preserved marginal tissue. DNA extraction was performed following phenol-chloroform protocols (Sambrook et al. 1989; Chen et al. 2010). Concentration and possible contamination of extracted DNA were checked using NanoDrop (NanoDrop Fluorospectrometer Thermo Fisher Scientific, USA). PCRs were performed in a total volume of 25 μl or 50 μl. 18S rDNA was amplified either in two overlapping fragments using the published primer combinations 4fb + 1806R (ca. 1200 bp) and 5fk + S30 (ca. 900 bp) (Larsson and Jondelius 2008) or in one approach using 18S-1F + 18S9R (ca. 1800 bp) (Álvarez-Presas et al. 2008). 28S rDNA was amplified with the primers 28_LSU5_fw + L1642R (ca.1450 bp) or 28S_1F + 28S_6R (ca. 1600 bp) (Larsson and Jondelius 2008). PCR was performed using a 'Touch Down' protocol using the following protocol: 5 min of initial denaturation at 94 °C; 30 s of denaturation at 94 °C, annealing at 68–45 °C for 30 s, extension at 72 °C for 2 min; 12 cycles; 30 s of denaturation at 94 °C, annealing at 45 °C for 30 s, extension at 72 °C for 2 min; 23 cycles; final extension at 72 °C for 10 min, hold at 4 °C. Successful products were purified using ExoSAP-IT (Affymetrix, USA), following manufacturer's protocol, or with the Wizard® SV gel and PCR clean-up system (Promega, USA) according to the manufacturer's quick protocol. PCR products were sequenced by CBMSO (Spain) or by Microsynth Austria GmbH, respectively. Sequences were assembled and edited by hand or using the software CLC Main Workbench 7 (Qiagen, Germany).

Table 1 List of all species used in this study, including authorities, sample locations and accession or SRA numbers. In trees using only a single sequence of the same species, the first listed sequence was included, with the number omitted

Species		Authority	Location	28Sshort6	18S28Slong	SRA
Ilyella	*gigas*	(Schmarda 1859)	Japan	LC100080.1		
Discocelis	*tigrina*	(Blanchard 1847)	Valencia, Spain	MN384690	MN334200, MN384690	
Adenoplana	*evelinae*	Marcus 1950	Brazil	KY263647.2		
Cestoplana	*rubrocincta* 1	(Grube 1840)	Naples, Italy	MN384689	MN334198, MN384689	
	rubrocincta 2		Australia	HQ659009.1		
	salar	Marcus 1949	Brazil	KY263653.2		
	techa	Du Bois-Reymond Marcus 1957	Brazil	KY263652.2		
Phaenocelis	*medvedica*	Marcus 1952	Brazil	KY263701.2		
Echinoplana	*celerrima* 1	Haswell 1907	Tunis, Tunisia	MN421930	MN421936, MN421930	SRS842092
	celerrima 2		Australia	HQ659020.1		
Hoploplana	*californica*	Hyman 1953	California	KC869850.1	KC869797.1, KC869850.1	
	divae	Marcus 1950	Brazil	KY263692.2		
	villosa	(Lang 1884)	Japan	LC100076.1		
Leptoplana	*tremellaris* 1	(Müller 1773)	Cornwall, UK	MN421931	MN421937, MN421931	SRS842637
	tremellaris 2		Spain	KY263695.2		
	sp.		Lizard Island (Australia)	MN384693		
Notoplana	*australis* 1	(Laidlaw 1904)	Australia	AY157153.1	AJ228786.1, AY157153.1	
	australis 2		Australia	HQ659015.1		
	delicata	(Jacubowa 1906)	Japan	LC100088.1		
	sp.		Brazil	KY263651.2		
Notocomplana	*humilis*	(Stimpson 1857)	Japan	LC100085.1		
	japonica	(Kato 1937a)	Japan	LC100087.1		
	koreana	(Kato 1937b)	Japan	LC100086.1		
	sp.		Japan	LC100089.1		
Melloplana	*ferruginea*	(Schmarda 1859)	Florida	HQ659014.1		
Comoplana	*agilis*	(Lang 1884)	Galicia, Spain	MN384685	MN334199, MN384685	
Armatoplana	*leptalea*	(Marcus 1947)	Brazil	KY263648.2		
Amemiyaia	*pacifica*	Kato 1944	Japan	LC100077.1		
Theama	*mediterranea*	Curini-Galletti et al. 2008	Rovinj, Croatia	MN384705	MN384707, MN384705	
	sp.		Panama	KC869845.1	KC869792.1, KC869845.1	
Callioplana	*marginata*	Stimpson1857	Japan	LC100082.1		
Planocera	*multitentaculata*	Kato 1944	Japan	LC100081.1		
	pellucida	(Mertens 1833)	Canary Island, Spain	MN384696	MN334203, MN384696	
Paraplanocera	*oligoglena*	(Schmarda 1859)	Hawaii	KC869849.1	KC869796.1, KC869849.1	
	sp.		Greece	KY263699.2		
Idioplana	*australiensis*	Woodworth 1898	Australia	HQ659008.1		
Pseudostylochus	*obscurus*	(Stimpson 1857)	Japan	LC100084.1		
	sp.		Japan	LC100083.1		
Stylochus	*ellipticus*	(Girard 1850)	Woods Hole, USA	Suppl. File 1	Suppl. File 1	SRS913554
	ijimai	Yeri and Kaburaki 1918	Japan	LC100079.1		
	oculiferus	(Girard 1853)	Florida	HQ659007.1		
	zebra	(Verrill 1882)	US Atlantic coast	AF342800.1	AF342801.1, AF342800.1	
	sp.		Peru	KY263743.2		
Imogine	*refertus*		Brazil	KY263694.2		

Table 1 (continued)

Species		Authority	Location	28Sshort6	18S28Slong	SRA
		Du Bois-Reymond Marcus 1965				
	stellae	Marquina et al. 2014	Valencia, Spain	MN384692	MN334201, MN384692	
Leptostylochus	*gracilis*	Kato 1934	Japan	LC100078.1		
Cycloporus	*gabriellae* 1	Marcus 1950	Brazil	KY263656.2		
	gabriellae 2			KY263658.2		
	variegatus 1	Kato 1934	Brazil	KY263657.2		
	variegatus 2		Spain	KY263659.2		
	variegatus 3		Brazil	KY263660.2		
	variegatus 4		Brazil	KY263661.2		
	japonicus	Kato 1944	Japan	LC100092.1		
Maritigrella	*crozieri* 1	(Hyman 1939)	Florida Keys, USA	MN421933	MN421939, MN421933	SRS844631
	crozieri 2		Aquaria in Virginia, USA	HQ659013.1		
	crozieri 3		Florida	KY263686.2		
	fuscopunctata	(Prudhoe 1978)	Maltese coast	KU674837.1		
	newmanae	Bolaños et al. 2007	Belize	EF514798.1		
Prostheceraeus	*roseus*	Lang 1884	Tenerifa	KY263688.2		
	vittatus	(Montagu 1815)	unknown	Suppl. File 1	Suppl. File 1	SRS913668
Stylostomum	*ellipse*	(Dalyell 1853)	Punat, Croatia	MN384704	MN334208, MN384704	
Eurylepto des	*galikias*	Noreña et al. 2014	Galicia, Spain	MN384691		
Prosthiostomum	*grande*	Stimpson 1857	Japan	LC100090.1		
	siphunculus 1	(Delle Chiaje 1822)	Barcelona, Spain	MN421934	MN421940, MN421934	SRS842699
	siphunculus 2		Asturias, Spain	MN384697	MN334204, MN384697	
	siphunculus 3		Spain	HQ659012.1		
	vulgaris	Kato 1938	Japan	LC100091.1		
Amakusaplana	*acroporae*	Rawlinson et al. 2011	Aquaria US East Coast	HQ659010.1		
Lurymare	*katoi*	Poulter 1975	Lizard Island (Australia)	MN384694		
Enchiridium	*evelinae*	Marcus 1949	Brazil	KY263662.2		
	sp. 1		Lizard Island (Australia)	MN384686		
	sp. 2		Santa Helena Island	KY263665.2		
Chromyella	sp.		Panama	KC869848.1	KC869795.1, KC869848.1	
Anonymus	*ruber*	Cuadrado et al. 2017	Canary Island, Spain	MN384687	MN334197, MN384687	
	virilis	Lang 1884	Canary Island, Spain	MN384688		
Boninia	*divae*	Marcus and Marcus 1968	Panama	KC869846.1	KC869793.1, KC869846.1	
Chromoplana	sp.		Panama	KC869847.1	KC869794.1, KC869847.1	
Pericelis	*byerleyana*	(Collingwood 1876)	Red Sea	MH047291.1		
	cata 1	Marcus and Marcus 1968	unknown	EU679114.1		
	cata 2		Brazil	KY263700.2		
	orbicularis	(Schmarda 1859)	unknown	EU679116.1		
	tectivorum	Dittmann et al. 2019	Aquaria Innbruck, Austria	MK181524	MN334202, MK181524	
Pseudoceros	*astorum*	Bulnes and Torres 2014	Brazil	KY263737.2		
	bicolor 1	Verrill 1902	Belize	GQ398095.1		
	bicolor 2		Brazil	KY263732.2		
	bicolor marcusorum	Litvaitis et al. 2010	Belize	GQ398098.1		

Table 1 (continued)

Species		Authority	Location	28Sshort6	18S28Slong	SRA
	cf *bicolor*		Brazil	KY263729.2		
	bimarginatus	Meixner 1907	Lizard Island (Australia)	MN384700	MN334207, MN384700	
	contrarius	Newman and Cannon 1995	Papua New Guinea	KY263728.2		
	harrisi	Bolaños et al. 2007	Panama	EF514802.1		
	jebborum	Newman and Cannon 1994	Lizard Island (Australia)	MN384701		
	cf *maximus*	Lang 1884	Spain	KY263708.2		
	nipponicus	Kato 1944	Japan	LC100096.1		
	periaurantius	Newman and Cannon 1994	Lizard Island (Australia)	MN384702		
	rawlinsonae 1	Bolaños et al. 2007	Bahamas	GQ398101.1		
	rawlinsonae 2		Brazil	KY263733.2		
	stimpsoni	Newman and Cannon 1998	Lizard Island (Australia)	MN384703		
	velutinus 1	(Blanchard 1847)	Spain	KY263726.2		
	velutinus 2		Japan	LC100095.1		
Pseudobiceros	*bedfordi*	(Laidlaw 1903)	Papua New Guinea	KY263715.2		
	caribbensis	Bolaños et al. 2007	Curaçao	EF514804.1		
	evelinae	(Marcus 1950)	Brazil	KY263716.2		
	flowersi	Newman and Cannon 1997	Lizard Island (Australia)	MN384698	MN334205, MN384698	
	hancockanus	(Collingwood 1876)	Lizard Island (Australia)	MN384699	MN384706, MN384699	
	nigromarginatus	(Yeri & Kaburaki 1918)	Japan	LC100097.1		
	pardalis 1	(Verrill 1900)	Panama	EF514807.1		
	pardalis 2		Brazil	KY263723.2		
	splendidus	(Lang 1884)	Florida	HQ659016.1		
	wirtzi	Bahia and Schroedl 2016	Senegal	KY263725.2		
	sp.		Santa Helena Island	KY263724.2		
Maiazoon	*orsaki*	Newman and Cannon 1996	Papau New Guinea	KY263697.2		
Thysanozoon	*alagoensis*	Bahia et al. 2015	Brazil	KY263747.2		
	brocchii 1	(Risso 1818)	Philip Island, Australia	HQ659017.1		
	brocchii 2		Brazil	KY263744.2		
	raphaeli	Bolaños et al. 2007	Panama	EF514809.1		
Yungia	sp.		Florida	HQ659018.1		
Phrikoceros	*mopsus*	(Marcus 1952)	Brazil	KY263707.2		
Monobiceros	*langi*	Faubel 1984	Spain	KY263710.2		
Macrostomum	*lignano*	Ladurner et al. 2005		MN421932	MN421938, MN421932	SRS842645
Xenoprorhynchus	sp.			KC869852.1	KC869813.1, KC869852.1	

Datasets for phylogenetic analyses

We made eight different single gene sequence collections of 'short' 28S sequences (see Table 1 for accession numbers of all newly generated and used published data). In general, we only used one sequence per species from the same authors.

The first sequence collection used 108 polyclad terminals (including the first, gappy version of sequences published by Bahia et al. 2017 on NCBI, which was corrected and reuploaded by Bahia et al. in 2019 with a non-gappy version), 20 of which were generated by us, and *Macrostomum lignano* as an outgroup ('28Sshort1'), while all subsequent 'short' 28S sequence collections worked with the updated second sequence versions of Bahia et al. (2017): '28Sshort2' added *Cycloporus japonicus*, two *Pericelis* and four pseudocerotoid sequences, while '28Sshort3' only included all (updated) sequences of '28Sshort1'.

Variations of '28Sshort2' included only *Xenoprorhynchus* sp. ('28Sshort2X') or both *Xenoprorhynchus* sp. and

Macrostomum lignano ('28Sshort2XM') as outgroups. '28Sshort4' is identical to '28Sshort3', except the removal of *Chromoplana* sp., whereas in '28Sshort5', we also removed *Cycloporus variegatus*. Finally, for '28Sshort6', we used '28Sshort2' sequences and included all available sequences of *Cycloporus variegatus* (four sequences) and *Cycloporus gabriellae* (two sequences). Most of the shown trees deal with the last sequence collection, which includes 118 polyclad terminals (24 sequences provided by us), covering 100 polyclad species.

Additionally, we made a combined dataset of 'long' 18S and 28S sequences ('18S28Slong'), including 27 polyclad terminals (19 of which were newly generated) and *Macrostomum lignano* as an outgroup.

Sequences for each gene were separately aligned using three methods: MUSCLE v3.8.31 (Edgar 2004), MAFFT Q-INS-i and MAFFT E-INS-i v7.310 (Katoh and Standley 2013). They were manually trimmed, and in the case of the combined dataset, concatenated. For several alignments, we also used Gblocks with the least stringent settings (Castresana 2000). Conversion of fasta alignments to Nexus and Phylip formats was done using ALTER (Glez-Peña et al. 2010).

Two different approaches for phylogenetic reconstructions were pursued: maximum likelihood (ML) reconstructions using RAxML (Stamatakis 2014), and Bayesian inference (BI) with MrBayes (Ronquist et al. 2012). The best models (GTR + I + G) were determined with jModelTest v2.1.10 using the Akaike Information Criterion AIC(c) (Posada 2008).

For ML trees, between 500 and 10,000 tree searches were performed, and between 500 and 1000 separate bootstrap replicates. At least 5–10 million generations were calculated for BI trees, or more until convergence (average standard deviation of split frequences < 0.01) was reached. For extended majority-rule consensus trees, we used RAxML with the concatenated trees of BI and ML analyses of the 28Sshort6 dataset (see Table 2). Phylogenetic trees were visualised in Figtree (http://tree.bio.ed.ac.uk/software/figtree/) and adapted in Inkscape (https://inkscape.org/) and Adobe Illustrator CS4.

The sequences generated during and/or analysed during the current study are available in the GenBank repository, under the accession numbers listed in Table 1. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Effects of model choice, alignment, outgroup selection and taxon sampling on tree topology

We recovered varied results with our combined 18S28Slong matrix (see Table 2, Suppl. Figs. S1–12): without using Gblocks for alignment curation, three of the six phylogenetic reconstructions supported Cestoplanoidea, Chromoplanoidea, Periceloidea, Anonymidae and *Chromoplana* as cotyleans (Suppl. Figs. S5, S10, S11), while three trees rendered most of these taxa as acotyleans or polytomic (Suppl. Figs. S4, S6, S12). We have visualised these changes caused by model choice in Fig. 1. Using Gblocks, only the two trees based on a Q-INS-i alignment recovered Cestoplanoidea, Chromoplanoidea, Periceloidea, Anonymidae and *Chromoplana* as cotyleans (Suppl. Figs. S2,8). In both E-INS-i trees and the ML MUSCLE tree, Anonymidae and *Chromoplana* are sister group of all other Polycladida, while in the BI MUSCLE tree, they are polytomic with Cotylea and Acotylea (see Table 2).

We continued our analyses with the first 28S-only dataset (28Sshort1) with many more taxa than available in the 18S28Slong dataset, including the first version of sequences published by Bahia et al. (2017). With this dataset, we calculated BI and ML trees based on three different alignments, and consistently (100%) recovered Cestoplanoidea, Chromoplanoidea, Periceloidea, Anonymidae and *Chromoplana* as Cotylea. The corresponding MRE tree exhibited an identical topology as the BI MUSCLE tree shown in Fig. 2a. After obtaining the new sequence versions of Bahia et al. (2017) in January 2019, we recalculated all trees with the new sequences (and adding additional sequences, see 28Sshort2) and consistently (100%) recovered the aforementioned groups as Acotylea, regardless of outgroup selection or alignment curation (Fig. 3).

We tested different parameters, always using a short 28S dataset with BI MUSCLE with and without Gblocks for tree reconstruction. Using Gblocks, outgroup selection markedly changed other parts of the topology, such as Prosthiostomoidea alternating between Acotylea and Cotylea (Fig. 3). With only *Xenoprorhynchus* as outgroup, *Chromoplana* is the sister group of all other Polycladida (Fig. 3b), while with only *Macrostomum* as outgroup, *Cycloporus variegatus* takes the place of sister group of all other Polycladida (Fig. 3c). Using the same ingroup and outgroup taxa as in Fig. 3c, but without Gblocks, we recovered a topology with many basal polytomies (Fig. 3d). With both non-polyclad outgroups, a basal polytomy between *Cycloporus variegatus*, Euryleptidae and all other Polycladida was recovered (Fig. 3a). Prosthiostomidae are basally branching Acotylea with *Macrostomum* + *Xenoprorhynchus*, and only *Macrostomum* as outgroups. *Xenoprorhynchus* alone as outgroup provides a basal polytomy of *Anonymus*, Cotylea and Acotylea, except *Chromoplana* (Fig. 3b).

Consequently, we tested if the newly added sequences were responsible for the change in tree topology, especially of Cestoplanoidea, Chromoplanoidea, Periceloidea, Anonymidae and *Chromoplana*. We therefore removed all additional sequences compared to our first dataset leading to the 28Sshort3 alignment, and with the same alignment and

Table 2 Summary and overview of all trees calculated with the 18S28Slong and the 28Sshort6 datasets. *ML* maximum likelihood, *BI* Bayesian inference, *M* MUSCLE, *Q* Q-INS-i, *E* E-INS-i alignment, *GB* with Gblocks, *No GB* without Gblocks alignment curation, *x* yes, – no, *p* polytomic, *?* no data

Suppl. Fig. S	18S28Slong												28Sshort6												Support
	ML						BI						ML						BI						
	GB			No GB			GB			No GB			GB			No GB			GB			No GB			
	M 1	Q 2	E 3	M 4	Q 5	E 6	M 7	Q 8	E 9	M 10	Q 11	E 12	M 13	Q 14	E 15	M 16	Q 17	E 18	M 19	Q 20	E 21	M 22	Q 23	E 24	
1. Cotylea and Acotylea *sensu* Bahia et al. 2017 are monophyletic	–	x	–	–	x	–	–	x	–	x	x	–	x	x	x	x	–	x	x	x	x	x	–	x	15/24
2. Cestoplanidae appear monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	x	x	x	x	x	x	x	x	x	x	x	x	12/12
3. Cestoplanidae appear within Cotylea	–	x	–	–	x	–	p	x	–	x	x	–	x	x	x	x	–	x	x	x	x	x	–	x	15/24
4. Cestoplanoidea is sister group to all other Cotylea	–	x	–	–	x	–	–	x	–	x	x	–	x	x	x	x	–	x	x	x	x	x	–	x	15/24
5. Pericelidae is monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	x	x	x	x	x	x	x	x	x	x	x	x	12/12
6. Pericelidae is sister group to all Cotylea except Cestoplanoidea	–	–	–	–	x	–	–	–	–	x	x	–	–	x	–	x	–	–	–	–	–	x	–	–	6/24
7. *Chromoplana* and *Anonymus* recover as clade 1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	p	x	x	x	23/24
8. Clade 1 appears as sister group to a clade including Prosthiostomoidea and Pseudocerotoidea	–	x	–	–	x	–	p	x	–	x	x	x	x	x	x	x	–	x	x	–	–	x	–	x	14/24
9. Pseudocerotoidea and Pseudocerotidae *sensu* Bahia et al. 2017 are monophyletic	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	–	x	x	x	x	x	–	x	22/24
10. The species *Pseudoceros*, *Pseudobiceros* and *Thysanozoon* are not monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	x	x	x	x	x	x	x	x	x	x	x	x	12/12
12. Euryleptidae *sensu* Faubel 1984 is split into two clades	x	x	x	x	x	x	x	x	x	x	x	x	x	–	x	x	–	x	x	x	x	x	–	x	21/24
12. Clade 2 is monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	x	x	x	x	x	x	x	x	x	x	x	x	12/12
13. The clade still called Euryleptidae is recovered as paraphyletic	–	–	–	–	–	–	–	–	–	–	–	–	–	x	–	–	x	–	–	–	–	–	x	–	21/24
14. The genera *Cycloporus* and *Prostheceraeus* are recovered as monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	–	–	–	–	–	–	–	–	–	–	p	–	0/12
15. *Maritigrella* is recovered as monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	–	x	–	–	x	–	–	x	–	–	x	–	4/12
16. Prosthiostomoidea appears as monophyletic	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	24/24
17. Prosthiostomoidea is sister group to Pseudocerotoidea	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	–	x	x	x	x	x	–	x	22/24
18. Within Prosthiostomidae, *Enchiridium* appears as sister group to a clade consisting of *Prosthiostomum, Lurymare* and *Amakusaplana*	?	?	?	?	?	?	?	?	?	?	?	?	x	x	x	x	x	x	x	x	x	x	x	x	12/12
19. *Prosthiostomum* appears paraphyletic, as *Amakusaplana* and *Lurymare* cluster within	?	?	?	?	?	?	?	?	?	?	?	?	x	x	x	x	–	x	x	x	x	x	–	x	10/12
20. Chromoplanoidea (including *Theama, Chromyella* and *Boninia*) clusters within Cotylea	–	x	–	–	x	–	–	x	–	x	x	–	x	x	x	x	–	x	x	x	x	x	–	x	15/24
	–	–	–	–	–	–	–	–	–	–	–	–	x	–	x	–	–	x	x	x	x	–	–	x	7/24

Table 2 (continued)

Suppl. Fig. S	18S28Slong												28Sshort6												Support
	ML						BI						ML						BI						
	GB			No GB			GB			No GB			GB			No GB			GB			No GB			
	M 1	Q 2	E 3	M 4	Q 5	E 6	M 7	Q 8	E 9	M 10	Q 11	E 12	M 13	Q 14	E 15	M 16	Q 17	E 18	M 19	Q 20	E 21	M 22	Q 23	E 24	
21. Chromoplanoidea as sister group to all other Cotylea, except Cestoplanoidea																									
22. Theamatidae is sister group to a clade consisting of *Boninia* and *Chromyella*	x	x	x	x	x	x	x	x	x	x	x	x	–	x	–	x	x	x	x	x	–	x	x	x	21/24
23. Leptoplanoidea *sensu* Faubel 1983 (in whose definition *Hoploplana* and *Theama* are included) is supported	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/24
24. Clade 3 can be subdivided into two clades (clades 5 and 6)	x	x	x	x	x	x	x	x	x	x	x	x	x	–	x	x	–	x	x	–	x	x	p	x	21/24
25. Clade 5 is synonymous with Leptoplanoidea *sensu* Bahia et al. 2017	x	x	x	x	x	x	x	x	x	x	x	x	–	–	–	–	–	–	–	–	–	–	–	–	12/24
26. *Pseudostylochus* is part of clade 5	?	?	?	?	?	?	?	?	?	?	?	?	x	x	x	x	x	x	x	x	x	x	x	x	12/12
27. *Leptoplana* is monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	x	x	x	x	x	x	x	x	x	x	x	x	12/12
28. Notoplanidae as a whole, as well as *Notoplana* are monophyletic, while *Notocomplana* is not monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	–	–	–	–	–	–	–	–	–	–	–	–	0/12
29. Clade 6 appears monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	x	–	x	x	–	x	x	–	x	x	p	x	8/12
30. Clade 6 appears as sister group to clade 5	x	x	x	x	x	x	x	x	x	x	x	x	x	–	x	x	–	x	x	–	x	x	p	x	20/24
31. Clade 4 can be subdivided into two clades, clades 7, 8 and *Callioplana*, where the latter is sister group to clades 7 + 8	?	?	?	?	?	?	?	?	?	?	?	?	–	–	–	x	–	x	p	–	–	p	–	p	2/12
32. A polytomy exists between *Callioplana*, clade 3 and clade 4	?	?	?	?	?	?	?	?	?	?	?	?	–	–	–	–	–	–	x	–	x	x	x	x	5/12
33. Clade 7 appears not monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	–	x	x	–	x	–	–	x	x	–	x	–	6/12
34. *Hoploplana* clustering as sister group to *Idioplana,* as clade 7	?	?	?	?	?	?	?	?	?	?	?	?	x	–	–	x	–	x	x	–	–	x	–	x	6/12
35. *Hoploplana* is sister group to Planoceridae/*Planocera pellucida*	x	x	x	x	x	–	x	x	x	x	p	–	–	x	–	–	x	–	–	x	p	–	x	–	14/24
36. Clade 8 is monophyletic	–	–	–	–	–	x	–	–	–	–	p	x	–	–	–	x	–	x	p	p	p	p	p	p	4/24
37. *Planocera* is monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	–	x	–	–	x	–	–	x	–	–	x	–	4/12
38. *Paraplanocera* is monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	–	–	–	–	–	–	–	–	–	–	–	–	0/12
39. Planoceridae *sensu* Faubel 1983 are recovered as monophyletic	–	–	–	–	–	–	–	–	–	–	p	–	–	–	–	–	–	–	–	–	–	–	–	–	0/24
40. *Stylochus* is monophyletic	–	x	–	–	x	–	–	x	–	x	–	–	–	–	–	–	x	–	–	–	–	–	x	–	6/24
41. *Imogine* is monophyletic	?	?	?	?	?	?	?	?	?	?	?	?	–	–	–	–	–	–	–	–	–	–	–	–	0/24
Total score	17	21	17	17	20	15	17	21	17	20	18	16	34	32	34	33	23	33	32	29	30	32	21	32	
Total number of points possible	22 (all lines except lines with ?)												38 (all lines except lines #14, 33, and 34)												

model choice, recovered a tree topology very different (Fig. 2b) from the one obtained with the 28Sshort1 alignment (Fig. 2a)—again with *Cycloporus variegatus* as sister group to the remaining Polycladida (Fig. 2b).

Now we also removed *Chromoplana* from the dataset (28Sshort4) and once more had *C. variegatus* as sister group to all other Polycladida. Also, Cestoplanoidea, Chromoplanoidea, Pericelloidea and Anonymidae emerged as Acotylea (Fig. 4a). With the additional removal of *C. variegatus* from the sequence collection (28Sshort5), we recovered Cestoplanoidea, Chromoplanoidea, Pericelloidea and Anonymidae as Cotylea once more—but only after alignment curation with Gblocks (Fig. 4b).

In a last change, we returned to the full dataset with updated sequences, but also used all available sequence variations for *Cycloporus variegatus* and *Cycloporus gabriellae*, instead of only using one sequence per species from the same authors (28Sshort6, Suppl. Figs. S13–24). We now recovered *Cycloporus* again within Cotylea, and present the detailed results using this dataset in the following section.

Comparative tree topology using 28Sshort6 and 18S28Slong matrices

All 12 trees using the 28Sshort6 matrix (Suppl. Figs. S13–24), and most of the 12 trees using the 18S28Slong matrix (Suppl. Figs. S1–12) are different from each other. We have analysed the tree topologies to identify stable and unstable taxa (Table 2). This table also gives an overview of which tree supports which topology. Additionally, we computed extended majority-rule consensus (MRE) trees from all 12 trees of the 18S28Slong matrix (Fig. 5), and all 12 trees of the 28Sshort6 matrix (Fig. 6). We also calculated separate 28Sshort6 and 18S28Slong matrix-based MREs for all alignments treated with or without Gblocks, respectively (Suppl. Figs. S25–28). If not otherwise stated, the MRE tree always refers to the MRE calculated from all twelve trees of each matrix. 'Trees' refers to both BI and ML trees, unless it is preceded by 'MRE'. Instead of citing all trees supporting a particular placement of a taxonomic group, we provide this information in Table 2 for better accessibility and overview.

In the following, we focus our comparisons on already defined families and superfamilies, mainly of the systems established by Faubel (1983, 1984) and Bahia et al. (2017).

The majority (63%) of our trees, and the 28Sshort6 MRE tree support Cotylea and Acotylea *sensu* Bahia et al. (2017) and in the following we use these terms according to their definition: in brief, *Theama* and *Cestoplana* are cotyleans instead of acotyleans.

Cestoplanoidea (Bahia et al. 2017) and thereby its only family, Cestoplanidae, appear monophyletic in all our trees, even if its position within the trees differs widely. The majority (63%) of our trees, and the 28Sshort6 MRE tree, support Cestoplanoidea within (and as sister group to all other) Cotylea, but in the remaining trees, it is sister group to Acotylea (33%) or, in one case, polytomic.

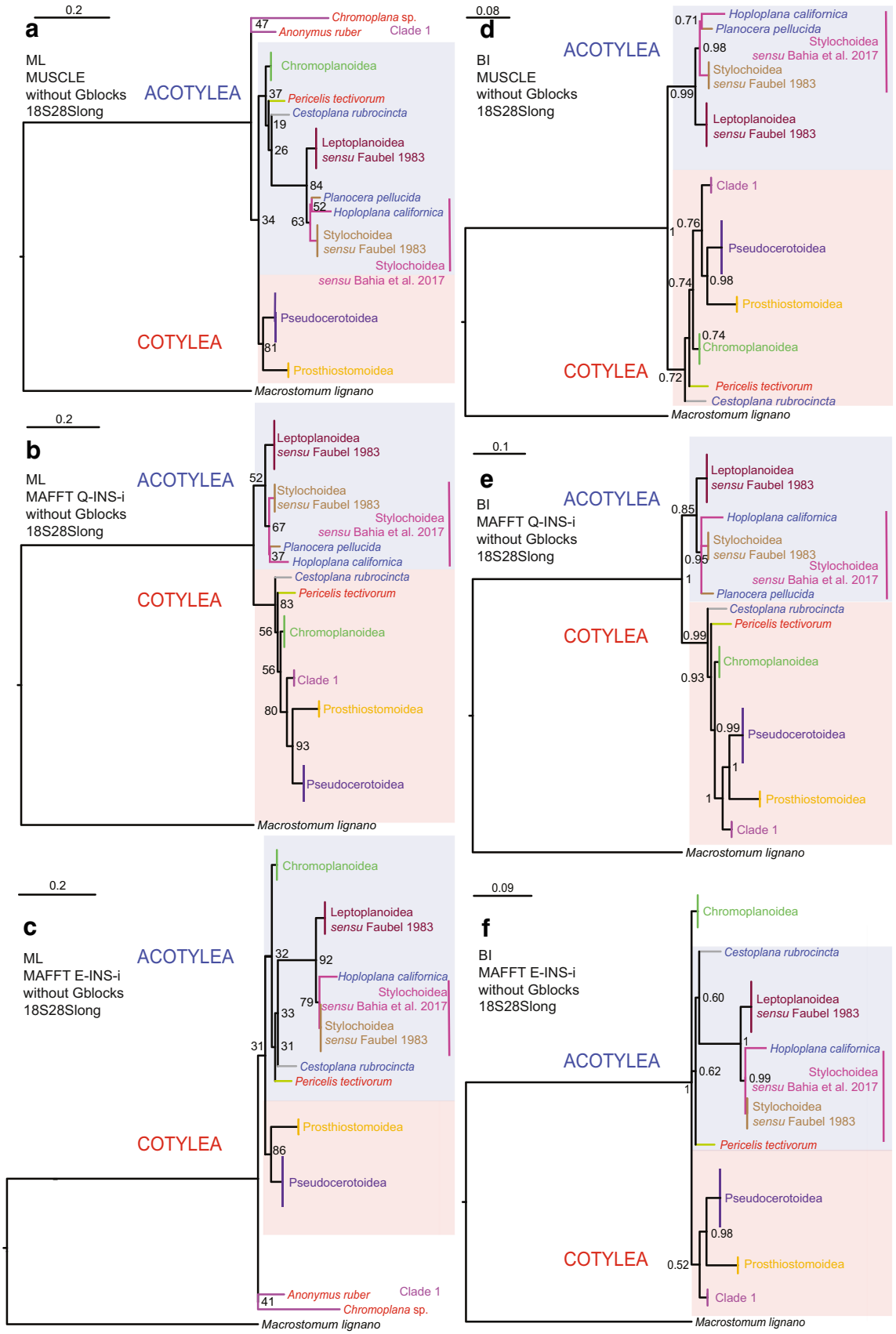
Also 63% of our trees, and the 28Sshort6 MRE tree, support the phylogenetic position of Chromoplanoidea (Bahia et al. 2017, including *Theama*, *Chromyella* and *Boninia*) within Cotylea. Only 29% of our trees (all of them 28Sshort6 trees), as well as the 28Sshort6 MRE tree, place Chromoplanoidea as sister group to all other Cotylea, except Cestoplanoidea. In 88% of our trees, and in both MRE trees, Theamatidae is sister group to a clade consisting of *Boninia* and *Chromyella*.

Pericelloidea (Bahia et al. 2017) and thereby its only family, Pericelidae, is also monophyletic in all of our phylogenetic reconstructions and both MRE trees. They are most often either sister group to all Cotylea except Cestoplanoidea and Chromoplanoidea (25% and the 28Sshort6 MRE tree), or sister group to Chromoplanoidea within Acotylea (25% of all trees, but 100% of the 18S28Slong Gblocks trees, and the 18S28Slong MRE tree). However, in 21% of the trees, Pericelloidea is placed as sister group to all Cotylea except Cestoplanoidea, or, also in 21% of the trees, Pericelloidea is sister group to all Acotylea and Cestoplanoidea.

All but one of our trees, and both MRE trees recover *Chromoplana* and *Anonymus* as clade 1 and this clade mostly (58% and both MRE trees) appears as sister group to a clade including Prosthiostomoidea (with the single family Prosthiostomidae) and Pseudocerotoidea (consisting of Pseudocerotidae, Euryleptidae and clade 2, see paragraph below).

Pseudocerotoidea and Pseudocerotidae *sensu* Bahia et al. 2017 are monophyletic in all but two trees, and in both MRE trees. Within Pseudocerotidae, all of our 28Sshort6 trees show that neither *Pseudoceros*, nor *Pseudobiceros*, nor *Thysanozoon* are monophyletic. The traditional family Euryleptidae *sensu* Faubel 1984 does not appear monophyletic in any of our trees, including the MRE trees. It is split into two clades (21 trees) or three clades (3 trees). In this work, we termed one of these clades 'clade 2' (while retaining the name Euryleptidae for the larger clade). The larger clade includes *Cycloporus japonicus*, *Cycloporus variegatus*, *Prostheceraeus* and *Maritigrella* in the 28Sshort6 trees, while *Cycloporus* is lacking in the 18S28Slong trees. Clade 2 consists of *Euryleptodes galikias, Cycloporus gabriellae* and *Stylostomum ellipse* in the 28Sshort6 trees, and only *Stylostomum ellipse* in the 18S28Slong trees. In the three trees, where the Euryleptidae *sensu* Faubel 1984 are split into three clades, even the clade still called Euryleptidae is recovered as paraphyletic. The genera *Cycloporus* and *Prostheceraeus* are never recovered as monophyletic in any of the 28Sshort6 trees, and also *Maritigrella* is only recovered as monophyletic in one third of the 28Sshort6 trees.

Prosthiostomoidea (Bahia et al. 2017) appears monophyletic in all trees, and in all but two trees as sister group to Pseudocerotoidea. Within Prosthiostomoidea, *Enchiridium* appears as sister group to a clade consisting of *Prosthiostomum, Lurymare* and *Amakusaplana* in all


a
ML
MUSCLE
without Gblocks
18S28Slong
0.2
ACOTYLEA
COTYLEA
Chromoplana sp.
Anonymus ruber
Clade 1
Chromoplanoidea
Pericelis tectivorum
Cestoplana rubrocincta
Leptoplanoidea
sensu Faubel 1983
Planocera pellucida
Hoploplana californica
Stylochoidea
sensu Faubel 1983
Stylochoidea
sensu Bahia et al. 2017
Pseudocerotoidea
Prosthiostomoidea
Macrostomum lignano
b
ML
MAFFT Q-INS-i
without Gblocks
18S28Slong
0.2
c
ML
MAFFT E-INS-i
without Gblocks
18S28Slong
0.2
d
BI
MUSCLE
without Gblocks
18S28Slong
0.08
e
BI
MAFFT Q-INS-i
without Gblocks
18S28Slong
0.1
f
BI
MAFFT E-INS-i
without Gblocks
18S28Slong
0.09

◀ **Fig. 1** Effect of model choice and alignment on tree topology. Tree reconstructions based on the 18S28Slong dataset, without using Gblocks. **a**–**c** Maximum likelihood. **d**–**f** Bayesian inference used for phylogenetic reconstructions. **a**, **d** MUSCLE alignments. **b**, **e** MAFFT Q-INS-i alignments. **c**, **f** MAFFT E-INS-i alignments. Node numbers indicate bootstrap support values (**a**–**c**) or posterior probabilities (**d**–**f**). Acotylea and Cotylea *sensu* Faubel 1983 and 1984 are written in blue and red fonts, respectively. Species recovered as Acotylea or Cotylea in our trees are displayed with blue and red background, respectively. Branches and nodes are given the same colour as their respective taxon

28Sshort6 trees. *Prosthiostomum* appears polyphyletic in 83% of our 28Sshort trees and also the 28Sshort MRE tree, as *Amakusaplana* and *Lurymare* cluster within.

Leptoplanoidea *sensu* Faubel 1983 (in whose definition *Hoploplana* and *Theama* are included) is not supported in any of our trees. We have termed Leptoplanoidea *sensu* Faubel 1983, but without *Hoploplana* and *Theama*, clade 3 (supported by all trees), which we further subdivided into two clades (clades 5 and 6).

In all 18S28Slong trees, clade 5 is synonymous with Leptoplanoidea *sensu* Bahia et al. (2017), as the genus *Pseudostylochus* is not available in these datasets. In all 28Sshort6 trees, *Pseudostylochus* is part of clade 5, but *Pseudostylochus* is not included in the superfamily's definition given by Bahia et al. (2017). All of our 28Sshort6 trees show that the genus *Leptoplana* is monophyletic, and that Notoplanidae as a whole and also its genera *Notoplana* and *Notocomplana* are not monophyletic.

In 58% of the 28Sshort6 trees and also the corresponding MRE tree, clade 6 is monophyletic, appears as sister group to clade 5 and includes *Discocelis, Adenoplana, Ilyella, Phaenocelis* and *Amemiyaia*. In our 18S28Slong trees and the corresponding MRE tree, clade 6 is represented only by *Discocelis* and always recovered as the sister group of clade 5.

Clade 4 can be subdivided into two clades, clades 7 + 8 and their sister group *Callioplana*. This topology is supported by two of twelve 28Sshort6 trees, as well as the respective MRE tree, while in ten 28Sshort6 trees, either a polytomy exists

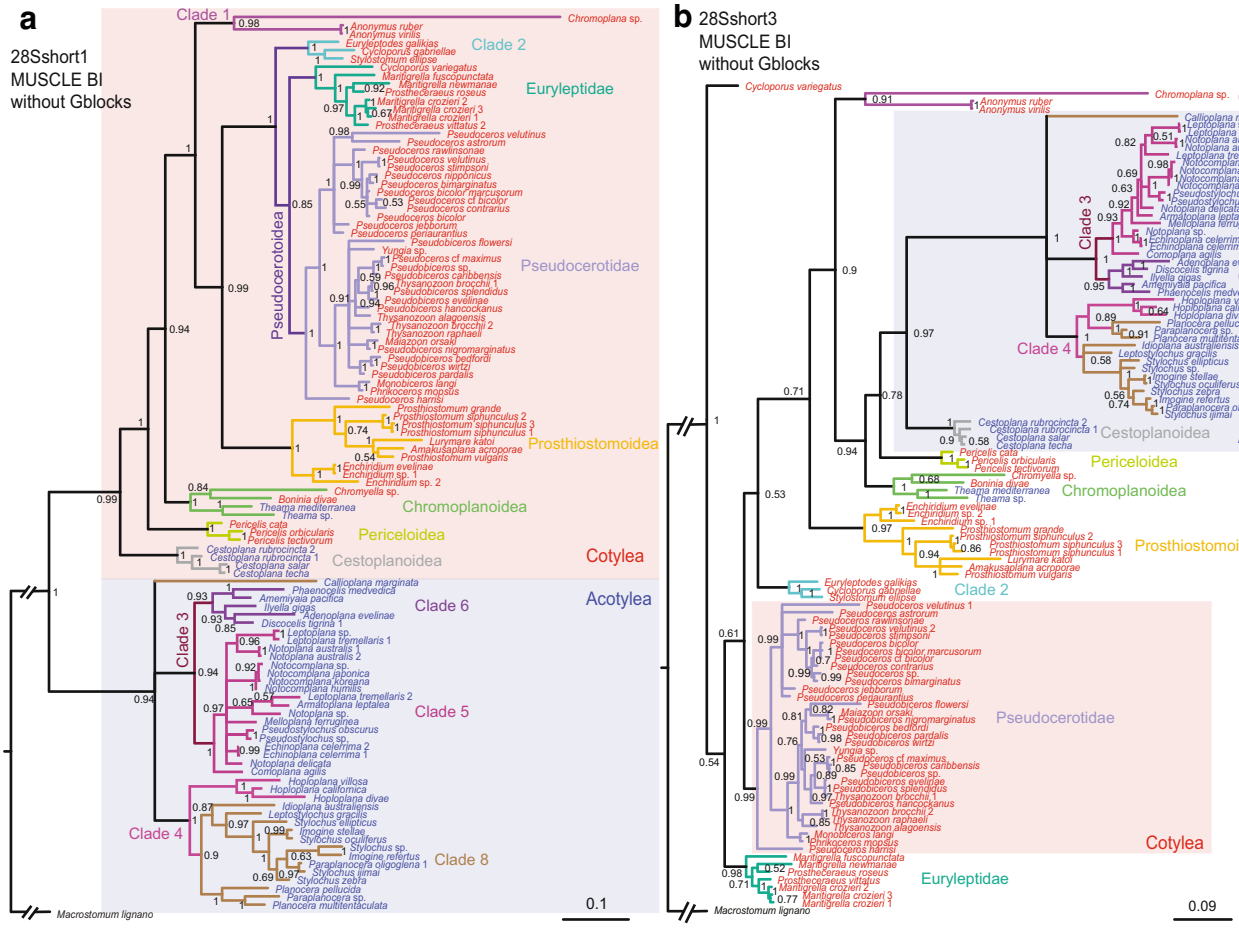
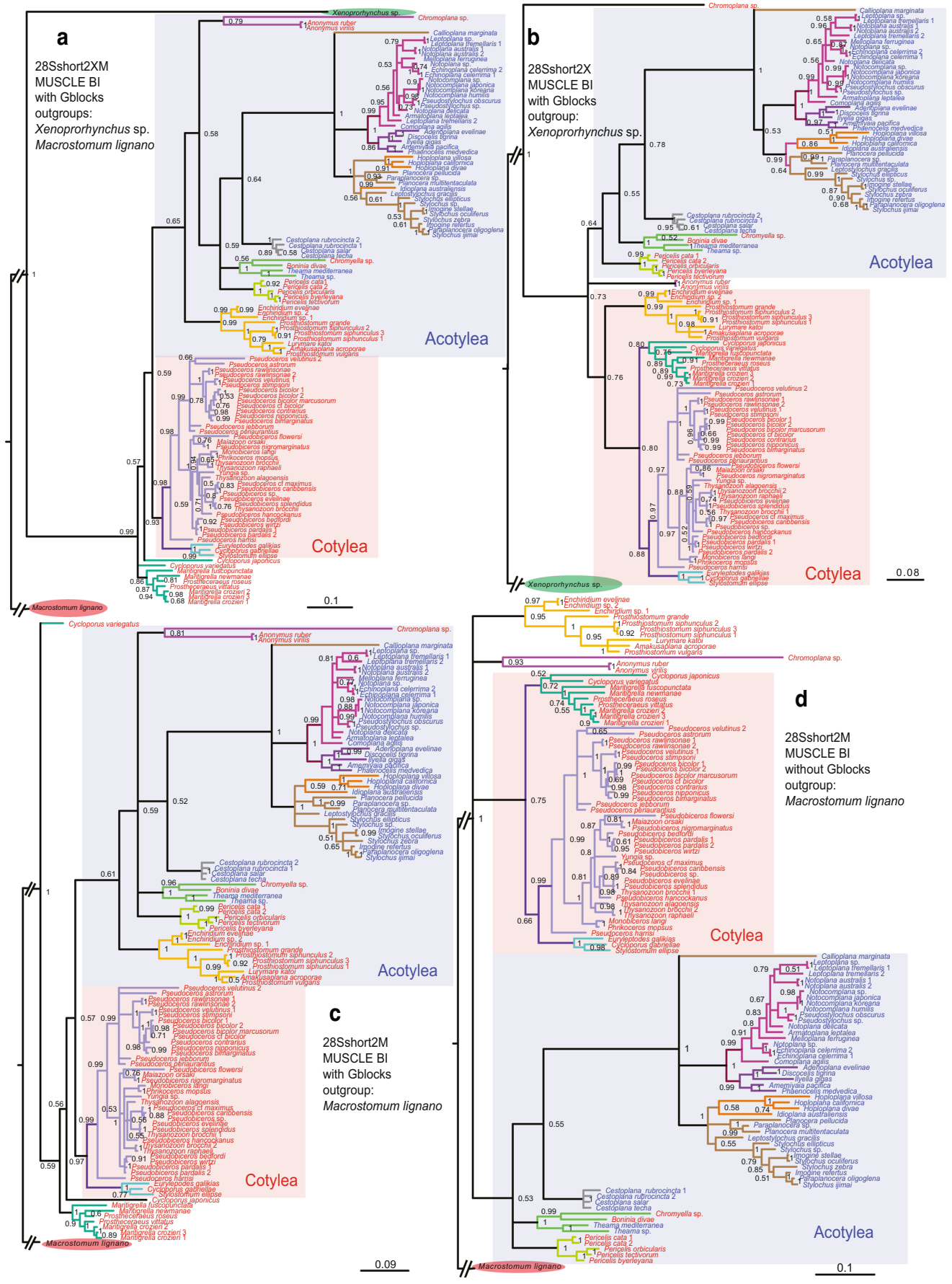


Fig. 2 Result of using versions 1 and 2 of sequences provided by Bahia et al. (2017). Bayesian inference tree reconstructions based on the 28Sshort1 (**a**) and 28Sshort3 (**b**) datasets, using MUSCLE alignments without Gblocks curation. The same taxa are used in (**a**) and (**b**), but with version 1 of sequences provided by Bahia et al. (2017) in (**a**) and version 2 in (**b**). Node numbers indicate posterior probabilities. Acotylea and Cotylea *sensu* Faubel 1983 and 1984 are written in blue and red fonts, respectively. Species recovered as Acotylea or Cotylea in our trees are displayed with blue and red background, respectively. Branches and nodes are given the same colour as their respective taxon


a
28Sshort2XM
MUSCLE BI
with Gblocks
outgroups:
Xenoprorhynchus sp.
Macrostomum lignano
Acotylea
Cotylea
0.1
b
28Sshort2X
MUSCLE BI
with Gblocks
outgroup:
Xenoprorhynchus sp.
Acotylea
Cotylea
0.08
c
28Sshort2M
MUSCLE BI
with Gblocks
outgroup:
Macrostomum lignano
Acotylea
Cotylea
0.09
d
28Sshort2M
MUSCLE BI
without Gblocks
outgroup:
Macrostomum lignano
Acotylea
Cotylea
0.1

◀ **Fig. 3** Effect of outgroup selection and alignment curation on tree topology. **a**–**d** Bayesian inference tree reconstructions based on the 28Sshort2 datasets, using MUSCLE alignments with (**a**–**c**) and without (**d**) Gblocks. **a** 28Sshort2XM dataset including both *Xenoprorhynchus* sp. and *Macrostomum lignano* as outgroups. **b** 28Sshort2X dataset only including *Xenoprorhynchus* sp. as outgroup. **c**, **d** 28Sshort2M dataset only including *Macrostomum lignano* as outgroup. Node numbers indicate posterior probabilities. Acotylea and Cotylea *sensu* Faubel 1983 and 1984 are written in blue and red fonts, respectively. Species recovered as Acotylea or Cotylea in our trees are displayed with blue and red background, respectively. Branches and nodes are given the same colour as their respective taxon

between *Callioplana*, clade 3 and clades 7 + 8, or clade 7 or 8 are not monophyletic, or several of these cases together.

In half of the 28Sshort6 trees, and also in the corresponding MRE tree, clade 7 is formed by *Hoploplana* clustering as sister group to *Idioplana*, but in one third of 28Sshort6 trees, *Hoploplana* is sister group to Planoceridae in clade 8. In our 18S28Slong trees, clade 7 is only represented by *Hoploplana californica*, in nine of twelve trees (and also in the 18S28Sshort MRE tree) as sister group to *Planocera pellucida*. In two cases, *Planocera pellucida* is clustering within clade 8, while in one case, it is unresolved as a polytomy.

Clade 8 resembles Stylochoidea *sensu* Faubel (1983) and in the 28Sshort6 dataset, includes *Leptostylochus*, *Stylochus*, *Imogine*, *Paraplanocera* and *Planocera*, but excludes Faubel's *Callioplana, Idioplana* and *Pseudostylochus*. This clade is monophyletic in a minority (two of twelve) 28Sshort6 trees and in the 28Sshort6 MRE tree. Clade 8 is polytomic in seven 28Sshort6 reconstructions and paraphyletic (including *Idioplana* and/or *Hoploplana*) in the remaining three 28Sshort6 trees. In the 18S28Slong dataset, clade 8 is represented by *Stylochus*, *Paraplanocera*, *Imogine* and *Planocera* and thus conforming to Faubel's (1983) definition. Clade 8 is supported by only two of twelve of the 18S28Slong trees as well, but not in the 18S28Slong MRE tree, where *Hoploplana* is sister group to *Planocera*.

In 75% of our 28Sshort6 trees and also the corresponding MRE tree, *Planocera* is not monophyletic as *Paraplanocera*

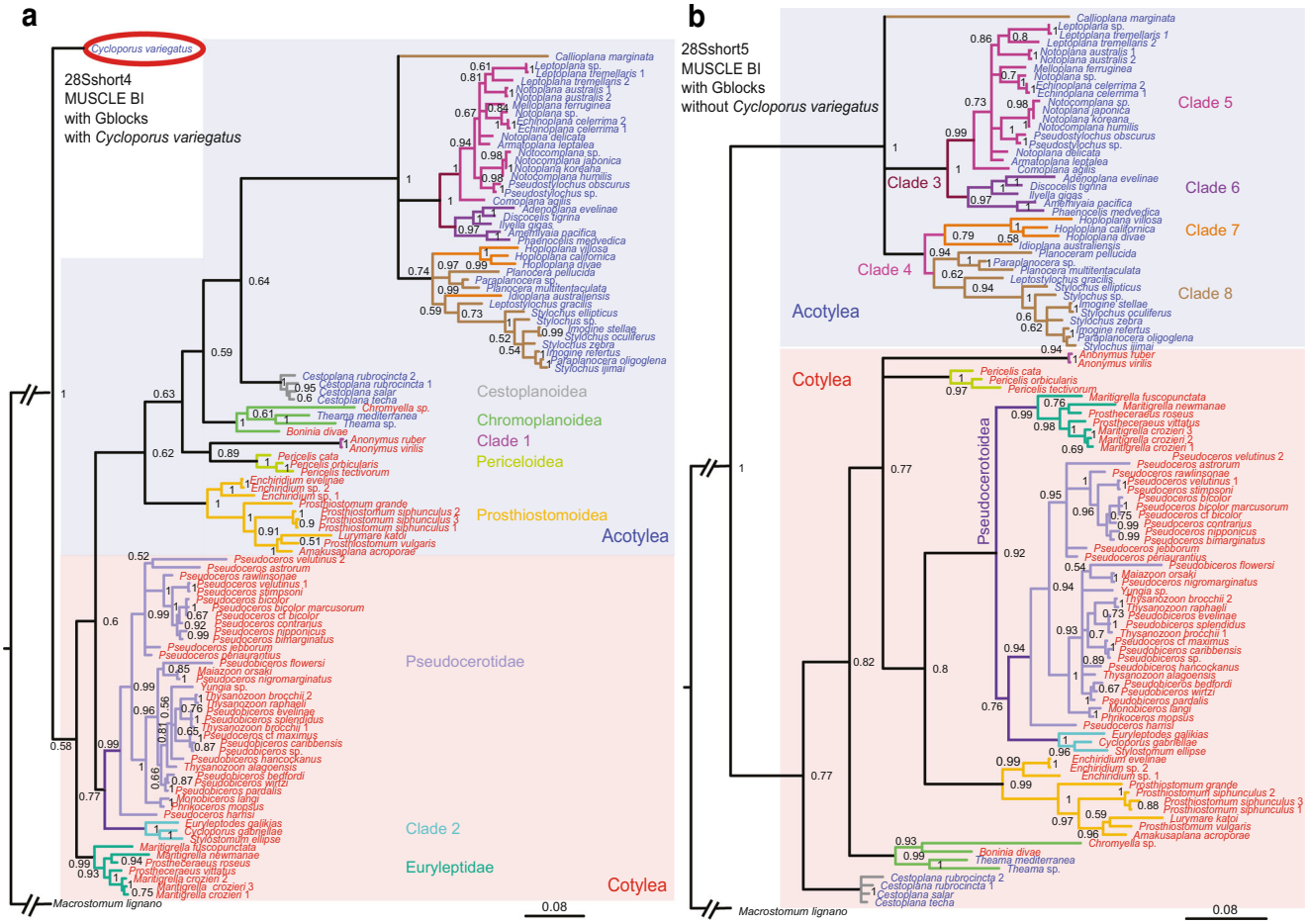


Fig. 4 Effect of taxon sampling on tree topology. **a**, **b** Bayesian inference tree reconstructions based on the 28Sshort4 dataset including *Cycloporus variegatus* (**a**) and the 28Sshort5 dataset without *Cycloporus variegatus* (**b**), using MUSCLE alignments and Gblocks for alignment curation. Node numbers indicate posterior probabilities. Acotylea and Cotylea *sensu* Faubel 1983 and 1984 are written in blue and red fonts, respectively. Species recovered as Acotylea or Cotylea in our trees are displayed with blue and red background, respectively. Branches and nodes are given the same colour as their respective taxon

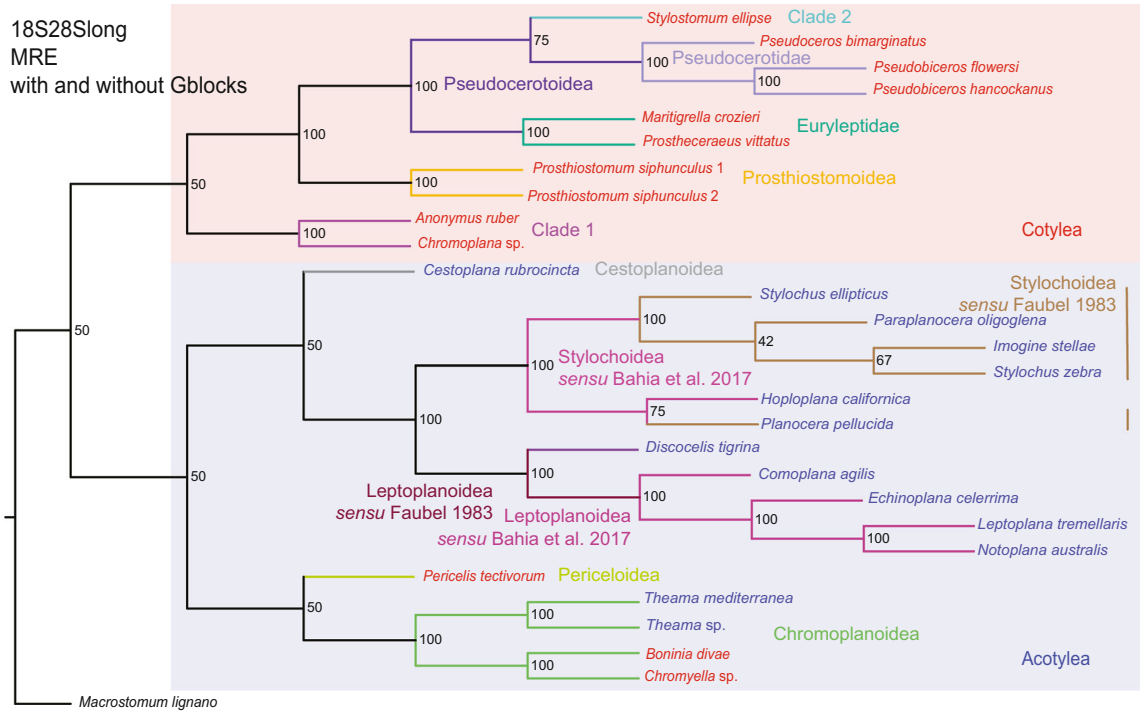


Fig. 5 Extended majority-rule consensus tree based on all 12 trees of the 18S28Slong dataset shown in Suppl. Figs. S1–12. Numbers indicate percentage of support. Acotylea and Cotylea *sensu* Faubel 1983 and 1984 are written in blue and red fonts, respectively. Species recovered as Acotylea or Cotylea in our trees are displayed with blue and red background, respectively. Branches and nodes are given the same colour as their respective taxon

sp. clusters within. Similarly, *Paraplanocera* is not monophyletic in any of our 28Sshort6 trees, and Planoceridae *sensu* Faubel 1983 is not recovered as monophyletic in any tree. In the majority (75%) of all trees, as well as in both MRE trees, the genus *Stylochus* is not monophyletic and in all 28Sshort6 trees, *Imogine* is not monophyletic.

Discussion

Taxon sampling and outgroup selection, as well as the choice of marker genes, the alignment method and the analysing statistical models affect the resulting phylogenetic reconstructions significantly (see e.g. Lockyer et al. 2003; Puslednik and Serb 2008; Aguado and Bleidorn 2010; Laumer and Giribet 2017). For polyclad interrelationships using mainly a rather short stretch of the 28S rDNA marker gene, but also a longer sequence comprised of both partial 18S and 28S rDNA, we show that the change of any of these parameters can vastly change the resulting tree topology (Figs. 1, 2, 3 and 4). A strong hypothesis about valid polyclad interrelationships is thus challenging, and we have therefore used majority-rule consensus trees to help us decide between different topologies (Figs. 5 and 6) and also manually analysed the support of different hypotheses (Table 2). To our knowledge, this is the first time that these difficulties and inconsistencies are discussed or even mentioned in regard to polyclad interrelationships.

Alignment is important

MUSCLE (Edgar 2004) was the alignment method of choice in both recently published polyclad phylogenies based on partial 28S sequences (Bahia et al. 2017; Tsunashima et al. 2017), and was also used in one of the best-scoring trees in both datasets shown here (Table 2, Suppl. Figs. S10, 13). We have also used two different variants of MAFFT (Katoh and Standley 2013); previously, MAFFT E-INS-i was selected for the polyclad phylogeny based on mitochondrial sequences (Aguado et al. 2017) and for an all-flatworm phylogeny working with the nearly complete nuclear ribosomal marker genes, 18S and 28S (Laumer and Giribet 2017). The other best-scoring 28Sshort6 tree according to our scoring in Table 2 is MAFFT E-INS-i aligned (Suppl. Figs. S15), and another MAFFT E-INS-i tree (Suppl. Fig. S18) is also closest to the topology shown in the MRE 28Sshort6 tree (Fig. 6). MAFFT Q-INS-i is by far the most computationally demanding alignment method, and was also employed quite extensively for resolving flatworm interrelationships on the level of orders based on partial 18S and 28S, e.g. macrostomorphs (Janssen et al. 2015), rhabdocoels (van Steenkiste et al. 2013; Tessens et al. 2014) and proseriates (Casu et al. 2014; Scarpa et al. 2015, 2016, 2017). The two best-scoring 18S28Slong trees are both based on a MAFFT Q-INS-i alignment (Table 2, Suppl. Figs. S2, 8).

However, the two worst-scoring 28Sshort6 trees are also based on MAFFT Q-INS-i alignments (Table 2, Suppl. Figs.

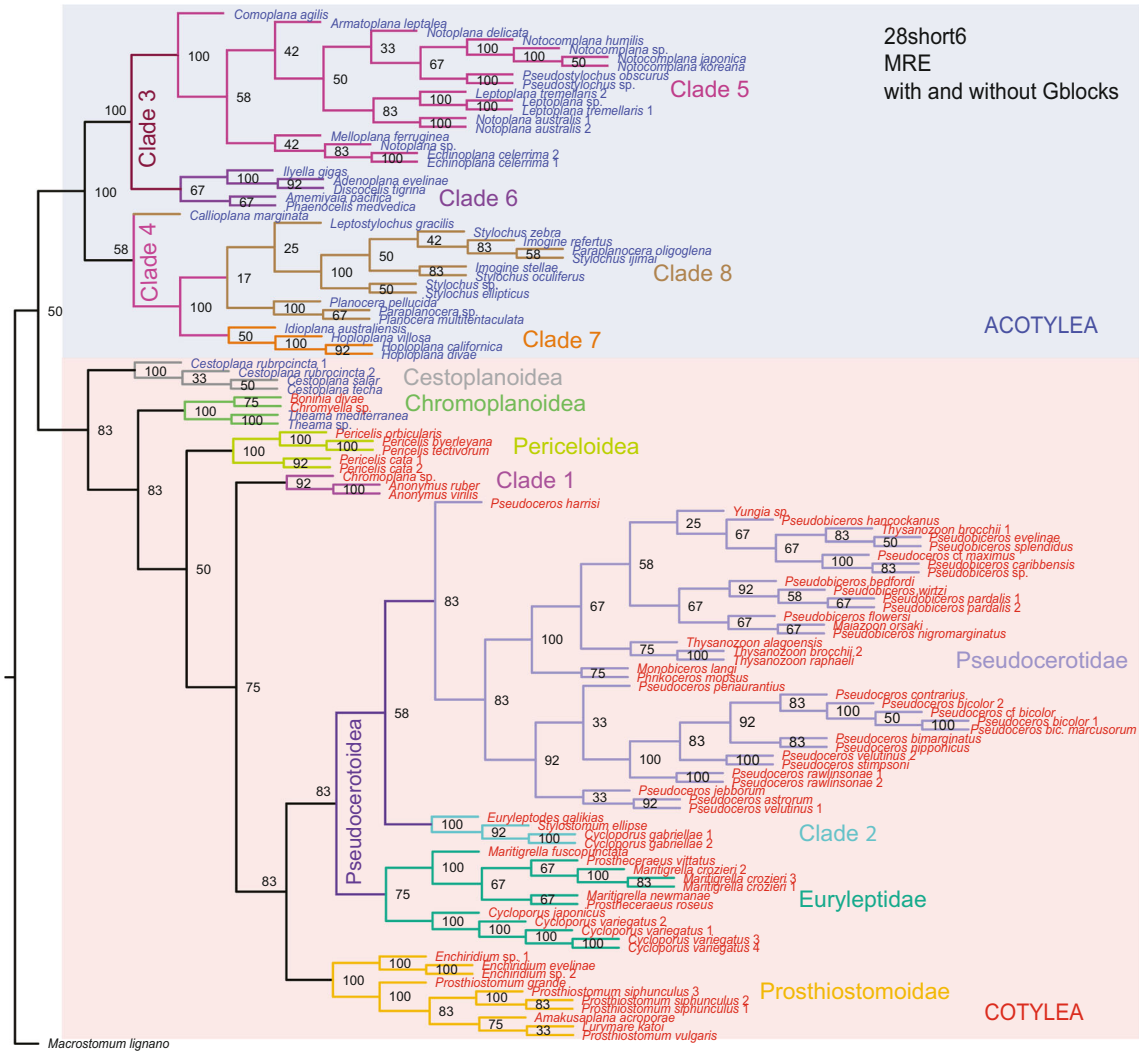


Fig. 6 Extended majority-rule consensus tree based on all 12 trees of the 28Sshort6 dataset shown in Suppl. Figs. S13–24. Numbers indicate percentage of support. Acotylea and Cotylea *sensu* Faubel 1983 and 1984 are written in blue and red fonts, respectively. Species recovered as Acotylea or Cotylea in our trees are displayed with blue and red background, respectively. Branches and nodes are given the same colour as their respective taxon

S17, 23), while the two worst-scoring 18S28Slong trees are MAFFT E-INS-i aligned (Table 2, Suppl. Figs. S6, 12).

The tree topologies resulting from these widely used alignment methods are not consistent (Fig. 1, Suppl. Figs. S1–24), corroborating the findings of Laumer and Giribet (2017), in which they re-analysed their differently aligned dataset from their earlier publication (Laumer and Giribet 2014) and also recovered trees with several major differences. In their re-analysis, they used MAFFT E-INS-i instead of RNAsalsa (Stocsits et al. 2009), and then recovered a tree very similar to two independently made transcriptomic analyses of flatworm interrelationships (Egger et al. 2015, Laumer et al. 2015), suggesting that MAFFT E-INS-i provided a more robust alignment than RNAsalsa.

From this work, we cannot give an unambigious recommendation for the most suitable alignment method, but recommend to use at least two different methods to check for consistency.

Model choice is important

In the work presented here, we consistently recovered inconsistent BI and ML topologies using the same datasets and alignments (Table 2, Fig. 1). In the most recently published polyclad phylogeny, both BI and ML trees gave congruent results (Litvaitis et al. 2019). In other recent polyclad phylogenies based on partial 28S, only either BI (Rawlinson and Stella 2012) or ML (Bahia et al. 2017, Tsunashima et al. 2017) were used, so no comparisons between different models can be made. In two polyclad phylogenies, both BI and ML analyses were run, and the trees show the same

topology in Rawlinson et al. (2011) and are 'highly congruent' in a mitochondrial gene analysis with several switches within families, but not of the overall topology (Aguado et al. 2017). Both models were used to resolve interrelationships within other flatworm orders, and reported with very similar or identical results using combined 18S and 28S datasets (Casu et al. 2014; Tessens et al. 2014; Janssen et al. 2015; Scarpa et al. 2015, 2016, 2017), in one case also including mitochondrial markers (Janssen et al. 2015). While these studies usually use a matrix of more than 3000 nt, our own large matrix with more than 3000 nt positions gives less congruent results among different models and alignments than our short matrix (ca. 800 nt) (Table 2, Figs. 5 and 6), indicating that taxon sampling may be even more important than matrix length.

Again, we recommend to use both models (BI and ML) to check for consistency between the models. In our case, results were not consistent, indicating that taxon sampling and matrix length were not sufficient yet.

Outgroup selection is important

We have tested the influence of outgroup choice on tree topology with *Macrostomum lignano*, a basally branching rhabditophoran, and *Xenoprorhynchus* sp., a basally branching prorhynchid—Prorhynchida being sister group of Polycladida (Egger et al. 2015, Laumer et al. 2015), using the same alignment (MUSCLE) and alignment curation (Gblocks), as well as the same model (BI) and the same dataset (28Sshort2). We found markedly different tree topologies between using both *Macrostomum* and *Xenoprorhynchus*, only *Xenoprorhynchus* or only *Macrostomum* as ougroups (Fig. 3a–c). Especially the sister group relationships of either *Chromoplana* sp. or *Cycloporus variegatus* with all other polyclads (Fig. 3b, c) were the reason to also test the influence of taxon sampling on the polyclad tree topology (Fig. 4).

An almost identical dataset, aligned with the same algorithm and tree reconstruction done with the same model and by the same leading author yielded two different topologies: in the first account, both *Cestoplana* and *Pericelis* are basally branching Acotylea (Rawlinson et al. 2011), while these two taxa switch to basally branching Cotylea in the second account (Rawlinson and Stella 2012). The only two differences in the reconstructions are one instead of two outgroups and a third sequence of *Amakusaplana acroporae* in the second paper (Rawlinson and Stella 2012), indicating that a higher number of outgroups gives more reliable results in their case. In our own datasets, we found no clear preference for outgroup selection (Fig. 3), making us default on a single, basally branching outgroup (*Macrostomum lignano*) for our main datasets (28Sshort6 and 18S28Slong).

Taxon sampling is important

Not only the long-branching *Chromoplana* (therefore excluded from the analysis in Bahia et al. 2017), but also *Cycloporus variegatus* was prone to upend the tree topology in the 28S trees (Figs. 3b, c and 4). Interestingly, both the complete removal of *Chromoplana* and all *Cycloporus* sequences, and the addition of more variants of *Cycloporus* species yielded similar tree topologies (Figs. 4b and 6). We have not tested removing taxa from the 18S28Slong dataset, but at least in theory, it should be more robust to taxon sampling artefacts than the much shorter 28Sshort dataset. In general, and as stated above, taxon sampling seems to be more important for resolving a stable polyclad phylogeny than matrix length at this point.

Correct determination is important

The correct identification of species is far-reaching for the interpretation of phylogenetic trees. During our analysis, we realised several inconsistencies in species determination of so far published sequences. In several of our 28Sshort6 trees, as well as in the corresponding MRE (Fig. 6), a sequence tagged as *Paraplanocera* sp. (KY263699.2) on GenBank clusters within *Planocera*. Therefore, *Planocera* does not appear monophyletic (Table 2, Fig. 6). However, according to Bahia et al. (2017), this sequence and the associated accession number belongs to *Planocera* sp.; hence, *Planocera* would be monophyletic also in our trees. We found several similar problems with sequences listed as '*Leptoplana* sp. or *Notoplana* sp.' in Table 1 of Bahia et al. (2017). In their table, these sequences have the accession numbers KY263695, KY263650, KY262696, KY263698 and KY263651. KY262696 is apparently a typo and should read KY263696, which together with KY263695 and KY263698 is tagged as '*Leptoplana tremellaris*' on GenBank, while KY263650 and KY263651 are labelled as 'Notoplana sp.' on GenBank. In their tree, Bahia et al. (2017) also show an unlisted *Notocomplana* sp., but it is not clear to which accession number this species refers to. As usual, we only took one sequence of the same species from the same authors, and we have used KY263695 (*Leptoplana tremellaris*) and KY263650 (*Notoplana* sp.) in our phylogenetic reconstructions (Table 1). Interestingly, in our 28Sshort6 MRE tree (Fig. 6), this *Notoplana* sp. by Bahia et al. (2017) does not cluster with any other *Notoplana*, *Notocomplana* (Notoplanidae) or *Leptoplana* (Leptoplanidae) species, but with *Melloplana* (Pleioplanidae) and *Echinoplana* (Gnesiocerotidae).

Also *Pseudoceros* is not monophyletic in our analyses, as two species, *Pseudoceros harrisi* and *Pseudoceros* cf. *maximus* are clustering outside the other 13 included *Pseudoceros* species (Fig. 6). *Pseudoceros harrisi* is consistently recovered as sister group to all other Pseudocerotidae in

our trees and also by Bahia et al. (2017) and Tsunashima et al. (2017). In its species description, which is based on a single damaged specimen, it is stated that 'This species does not resemble any other species of *Pseudoceros*. However, *P. harrisi* may be confused with members of *Cycloporus* [...]' (Bolaños et al. 2007). Hence, the phylogenetic position of *Pseudoceros harrisi* might be the result of a misdetermination of its genus in the original description. The *Pseudoceros* cf *maximus* sequence (KY263708) we used was published by Bahia et al. (2017) and it appears with high support within *Pseudobiceros* in our reconstructions (Fig. 6). We noticed that the species name *Pseudoceros* cf *maximus* does not appear in Bahia et al.'s tree. On the other hand, they show two branches labelled '*Pseudobiceros* spp.' in their tree, but only list a single *Pseudobiceros* sp. sequence in their Table 1. Taking into account our own results, we believe it is possible that the sequence published as *Pseudoceros* cf *maximus* on GenBank is one of the '*Pseudobiceros* sp.' in their tree.

Several sequences have undergone name changes after redetermination efforts by the authors, or have dubious affiliations. For example, *Cestoplana rubrocincta* from Australia (*C. rubrocincta* 2 in our tree, HQ659009.1) is labelled as *C. australis* in the tree provided by Rawlinson et al. (2011), but called *C. rubrocincta* in their table, and also on GenBank. Other sequence names were updated without changing their accession number versions. We originally downloaded the following sequences published in Tsunashima et al. (2017) from GenBank in June 2017, but they were subsequently renamed: *Discoplana* sp. to *Ilyella gigas* (LC100080), *Notoplana koreana* to *Notocomplana koreana* (LC100086), *Melloplana japonica* to *Notocomplana japonica* (LC100087), *Cycloporus* sp. to *Cycloporus japonicus* (LC100092), *Thysanozoon* sp. 1 to *Thysanozoon brocchii* (LC100093), *Thysanozoon* sp. 2 to *Thysanozoon japonicum* (LC100094), *Pseudoceros* sp. 1 to *Pseudoceros velutinus* (LC100095), *Pseudoceros* sp. 2 to *Pseudoceros nipponicus* (LC100096), and *Pseudoceros* sp. 3 to *Pseudobiceros nigromarginatus* (LC100097).

Sequence problems

When we started with this study in 2017, we noticed gaps in all newly generated sequences uploaded to GenBank by Bahia et al. (2017). The first set of 28Sshort trees we made was based on a dataset including these sequences. We later realised that the gaps in the sequences were caused by alignment curation using Gblocks (J. Bahia, pers. comm.), and all other trees (using the 28Sshort2-6 sequence collections) were based on the updated sequences (version 2 on GenBank). We provided reconstructions based on both, Gblocks curated and original alignments, and often recovered different topologies if all other parameters stayed the same (Table 2, Fig. 3). According to a recent publication, phylogeny may be even better without using Gblocks or similar alignment curation programs (Tan et al. 2015). In our own study, however, we find that the best-scoring trees were made with datasets using Gblocks for alignment curation (Table 2).

Some of the sequences published by Tsunashima et al. (2017) appear to be quite different to all other polyclad sequences published, especially in the 5′ region: among these are the above-mentioned *Cycloporus japonicus* (LC100092), *Thysanozoon brocchii* (LC100093) and *Thysanozoon japonicum* (LC100094). We initially removed all of these sequences from further analyses, but later added *Cycloporus japonicus* (28Sshort2 and 28Sshort6) despite the divergent sequence. Also *Chromoplana* sp. from Laumer and Giribet (2014) was an unusual sequence and was therefore removed from the tree of Bahia et al. (2017), but is included in most of our reconstructions (except 28Sshort4-5).

Although termed as 'clones' on GenBank, there is a considerable difference between the four published *Cycloporus variegatus* sequences by Bahia et al. (2017); we believe these sequences are not derived from clones, but from different specimens of the same species.

Polyclad phylogenies based on partial 28S rDNA published by different authors used different primers, making the integration of all sequences a challenge, as the overlapping regions get smaller. Especially Tsunashima et al. (2017) used a region of the 28S gene more towards the 3′ end than all other studies, but we have still included most of their sequences, because they provide important taxa not covered by our own or other previously published sequences. For future studies, we recommend amplifying 28S starting with expansion segment D1 and stretching as long as possible, to maximise compatibility with published sequences.

Classification on suborder and superfamily level

On suborder level, our 28Sshort6 trees are mostly compatible with the molecular phylogenetic hypothesis of Bahia et al. (2017), supporting their redefinition of Cotylea and Acotylea (see Table 2 and Fig. 6). There, two traditional actoylean families, Cestoplanidae and Theamatidae, switched from Acotylea to Cotylea.

The majority of the 18S28Slong trees, on the other hand, support Cestoplanidae and Theamatidae as acotyleans. Also, the traditionally cotylean genera *Pericelis*, *Boninia* and *Chromyella* are recovered as acotyleans (Table 2, Fig. 5). In this scenario, a sucker would be a character at the base of Polycladida and would have been lost at least five times: in the traditional Acotylea, in some Cestoplanidae, in the anonymid *Simpliciplana marginata* (Kaburaki 1923), in Theamatidae, in *Amakusaplana* (Rawlinson et al. 2011), and possibly in *Chromyella* (Fig. 5 and Faubel 1983, 1984, Prudhoe 1985). In the 28Sshort6 scenario, a sucker would

be present at the base of Cotylea and would have been lost one time less, i.e. not in Acotylea (Fig. 6). According to Bahia et al. (2017), a 'true sucker' may have gradually evolved and may be an apomorphy of Prosthiostomoidea and Pseudocerotoidea. A true sucker is muscular and characterised by a modified epithelium with a thin basement membrane, while the adhesive disc or pad found in *Boninia* and *Cestoplana* is just a shallow depression of the epithelium not differentiated from the parenchyma (Prudhoe 1985; Rawlinson and Litvaitis 2008). Both true sucker and adhesive disc/pad are always located posterior of the genital openings. Several *Pericelis* species (excluded from having a true sucker in Bahia et al. 2017, but listed as having a true sucker in Rawlinson and Litvaitis 2008) are described with a 'distinct sucker' (Dittmann et al. 2019), so we suggest that the true sucker behind the genital openings already is an apomorphy for the unnamed group including Pericelodea, *Anonymus*, *Chromoplana*, Prosthiostomoidea and Pseudocerotoidea (Fig. 6). The acotylean genus *Leptoplana* has a sucker (a so-called genital pit) between the genital openings (Prudhoe 1985); therefore, it is excluded from the definition of a cotylean sucker.

Based on this scenario of sucker evolution in polyclads, it is more parsimonious to support the 28Sshort6 tree topology, although the 18S28Slong alignment with ca. 3000 nt is almost four times as long as the 28Sshort6 alignment with ca. 900 nt. Also, the support values of the trees rejecting Cotylea and Acotylea *sensu* Bahia et al. (2017) are consistently lower than those supporting them (Suppl. Figs. S1–24). In five of the twelve 18S28Slong trees, Cotylea and Acotylea *sensu* Bahia et al. (2017) are actually supported, and also in the 18S28Slong MRE tree without Gblocks (Suppl. Fig. S26). Only the 18S28Slong dataset using Gblocks skews the picture towards a weakly supported topology making Cestoplanidae, Theamatidae, *Pericelis*, *Boninia* and *Chromyella* acotyleans (Suppl. Fig. S25), also in the combined 18S28Slong MRE tree (Fig. 5).

In all but two 28Sshort6 trees, Cotylea and Acotylea *sensu* Bahia et al. (2017) are well supported (Fig. 6, Suppl. Figs. S13–16, 18–22, 24). On the other hand, we have shown that this topology is very much dependant on taxon sampling, outgroup selection, alignment method and curation, and model choice (Figs. 1, 2, 3 and 4). Possibly, the most important parameter is taxon sampling, and this would explain why a much larger alignment (18S28Slong) with 27 polyclad terminals and 26 different polyclad species gives less consistent results than the shorter matrix (28Short6) with 118 polyclad terminals and 100 different polyclad species. Bahia et al. (2017) show 136 polyclad terminals, but only 55 different polyclad species, and Tsunashima et al. (2017) use 53 polyclad terminals and 50 polyclad species in their phylogenetic trees. While we have not tested their original datasets with different parameters here, their results suggest that neither the number of taxa, nor sequences are decisive for tree topology, but that some sequences are prone to change tree topology, among them *Chromoplana*, *Cycloporus variegatus* and *Cycloporus japonicus* (Figs. 3 and 4). As long as single taxa included or excluded can drastically change tree topology even in the overall more consistent 28S-only trees, polyclad phylogeny remains only preliminarily resolved, calling for larger datasets like in transcriptomic phylogenies.

However, apart from the position of Cestoplanidae, Theamatidae, *Pericelis*, *Boninia*, *Chromyella*, Anonymidae and *Chromoplana* in the tree, we find that most polyclad taxa are included in very well-supported clades.

Our data support the following new superfamilies *sensu* Bahia et al. (2017):

Pseudocerotoidea *sensu* Bahia et al. (2017); this superfamily includes Pseudocerotidae and two clades of Euryleptidae in their reconstruction. In this work, we termed one of these clades 'clade 2' as all relevant trees show this non-monophyly (Table 2). This division can also be observed in the study of Bahia et al. (2017), where *Cycloporus gabriellae* represents our clade 2 of Euryleptidae, while *Cycloporus variegatus* and *Cycloporus japonicus* are part of the remaining Euryleptidae. Also in a cladistic analysis, Euryleptidae was not recovered as monophyletic (Rawlinson and Litvaitis 2008). As already suggested before, the genus *Cycloporus* needs to be revised, but no obvious characters to distinguish between described *Cycloporus* species could be determined so far (Bahia et al. 2017). Our data show that the separation of the *Cycloporus* species not only results from potential inconsistencies within the genus *Cycloporus*, as also *Stylostomum* and *Euryleptodes* appear within clade 2. Therefore, we propose the revision of the whole family of Euryleptidae. As *Eurylepta* has been shown to cluster as sister group of other Euryleptidae in a phylogeny based on mitochondrial genes (Aguado et al. 2017), the family name Euryleptidae should be retained for the group containing *Maritigrella*, *Prostheceraeus*, *Cycloporus variegatus* and *Cycloporus japonicus* (Fig. 7). *Cycloporus japonicus* has been shown to group with *Maritigrella* in Tsunashima et al. (2017) as well. We propose the new family name Stylostomidae fam. nov. for clade 2, including at least *Stylostomum*, *Euryleptodes* and *Cycloporus gabriellae*. In the recently published work by Litvaitis et al. (2019), both Euryleptidae and *Cycloporus* appear as monophyletic, but neither *Stylostomum*, nor *Euryleptodes* are included in their study. As in our study, Litvaitis et al. (2019) have recovered both, *Prostheceraeus* and *Maritigrella*, as non-monophyletic and consequently they have synonymised *Maritigrella* as junior synonym with *Prostheceraeus*.

Pseudoceros, Pseudobiceros and *Thysanozoon* are not recovered as monophyletic in our study, agreeing with Bahia et al. (2017) and Tsunashima et al. (2017), stressing the need of further revision of the family Pseudocerotidae (Litvaitis and Newman 2001; Rawlinson and Litvaitis 2008).

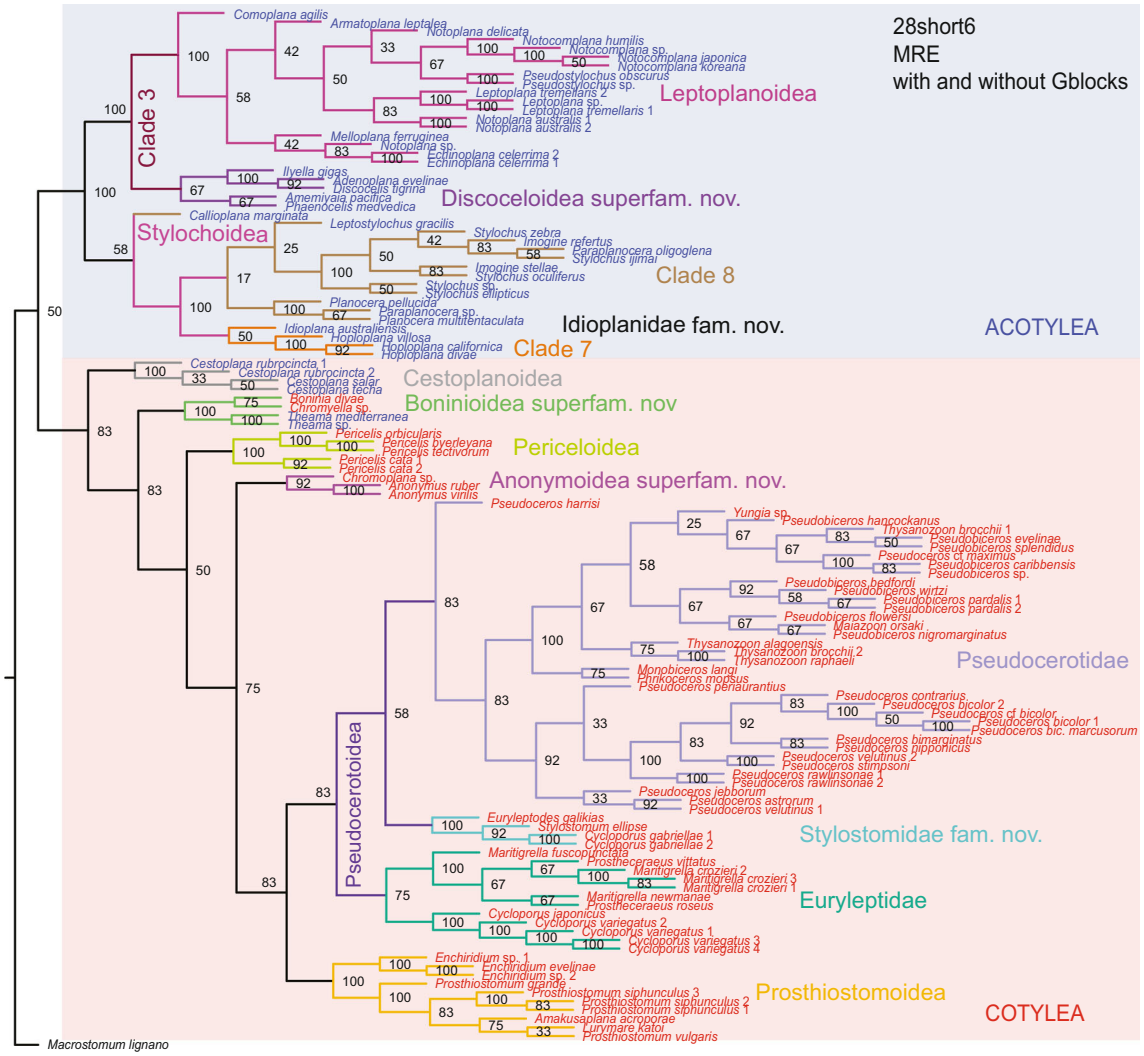


Fig. 7 Same tree as shown in Fig. 6, but with newly defined and named groups indicated. Acotylea and Cotylea *sensu* Faubel 1983 and 1984 are written in blue and red fonts, respectively. Species recovered as Acotylea or Cotylea in our trees are displayed with blue and red background, respectively. Branches and nodes are given the same colour as their respective taxon

Prosthiostomoidea was erected by Bahia et al. (2017) and only contains a single family, Prosthiostomidae. All our data support the monophyly of this family/superfamily and most data (Table 2) also their sister group relationship to Pseudocerotoidea as described by Bahia et al. (2017). Similar to our results, also in the study of Tsunashima et al. (2017), *Prosthiostomum* is not monophyletic, as *Amakusaplana* (and in our case, also *Lurymare*) clusters within. In Aguado et al. (2017), two different species of *Lurymare* do not form an adelphotaxon. The fact that *Amakusaplana* clusters within *Prosthiostomum* is not very surprising, as Faubel (1984) remarks that the genus *Amakusaplana* has to be eliminated, as it is too similar to *Prosthiostomum*. The genus *Amakusaplana* is distinguished from *Prosthiostomum* mainly by body shape and the arrangement of eyes (Kato 1938; Faubel 1984), and also by the absence of the ventral sucker (Kato 1938; Rawlinson et al. 2011). Only in two of twelve 28Sshort6 reconstructions is *Prosthiostomum* monophyletic (Suppl. Figs. S17, 23), and Litvaitis et al. (2019) synonymise *Amakusaplana* with *Prosthiostomum*. Our data support this decision. The position of *Lurymare* within *Prosthiostomum* was already assumed by Poulter (1975). He proposed a subdivision of the genus *Prosthiostomum* into the subgenera *P.* (*Lurymare*) and *P.* (*Prosthiostomum*), distinguishable by the constitution of the prostatic vesicle (Poulter 1975). Faubel (1984) remarks that this definition also includes *Enchiridium* and elevates both subgenera back as genera. At least *Enchiridium* may be monophyletic, as suggested by Bahia et al. (2017), Litvaitis et al. (2019) and our own trees. Together, the molecular phylogenies do not support any of the previously proposed genera (Kato 1938; Poulter 1975; Faubel 1984) except *Enchiridium*, i.e. the revision of the genera *Prosthiostomum* and *Lurymare* is required.

Our clade 1, consisting of *Anonymus* and *Chromoplana*, is extremely well supported and always monophyletic, except in one case, where it appears polytomic (Suppl. Fig. S21). We propose a new superfamily Anonymoidea superfam. nov.

(Fig. 7), including the families Anonymidae (Lang 1884) and Chromoplanidae (Bock 1922).

Cestoplanoidea was defined by Poche (1926), emended by Prudhoe (1985) and supported by Bahia et al. (2017) and Litvaitis et al. (2019); a majority of the 28Sshort6, but only a minority of the 18S28Slong analyses support its sister group relationship with all other Cotylea, as suggested by Rawlinson and Stella (2012) and Bahia et al. (2017), even if the family was originally assigned to Acotylea (Lang 1884; Faubel 1983; Prudhoe 1985) and appears as an acotylean in a majority of the 18S28Slong analyses, as well as in Rawlinson et al. (2011). In his monograph, Faubel remarks that organisation features of *Cestoplana*, like the forward direction of the male complex, the multiplication of the female apparatus in *Cestoplana polypora*, or the presence of an adhesive organ in some (but not all) *Cestoplana* species, could imply that Cestoplanidae have possibly arisen from a cotylean ancestor (Laidlaw 1903; Bock 1922; Faubel 1983; Bahia et al. 2017).

In all of our reconstructions, Cestoplanoidea are monophyletic (Table 2), although the only representing genus is *Cestoplana*.

Pericelloidea was also erected by Bahia et al. (2017) and also contains a single, monotypic family, Pericelidae. Our data support the monophyly of this group. Additionally, our 28Sshort6 MRE tree (Fig. 6) supports its sister group relationship with all remaining Cotylea except Cestoplanidae, as already assumed by Bahia et al. (2017) and Rawlinson and Stella (2012). In Tsunashima et al. (2017), *Pericelis* is also recovered as a cotylean, but as sister group to *Boninia* + *Chromyella* (Theamatidae), although *Cestoplana* is absent in Tsunashima et al.'s reconstruction. In Rawlinson et al. (2011) and our 18S28Slong MRE tree (Fig. 5), Pericelloidea are grouping with Acotylea, however. Litvaitis et al. (2019) include *Diposthus* in their phylogenetic reconstruction, which emerges as sister group of *Pericelis*, and they argue for abolishing both Pericelidae and Pericelloidea in favour of the family Diposthidae.

Our data do not support the following superfamilies *sensu* Bahia et al. (2017):

The position of Chromoplanoidea within Cotylea is supported by most of our analyses (Table 2), although in the 18S28Slong MRE tree, Chromoplanoidea is recovered as acotylean (Fig. 5). The superfamily always is monophyletic, but the interrelationships between the three included chromoplanoid genera are differently resolved. In Bahia et al. (2017), *Theama* + *Chromyella* form a sister group to *Boninia*, while in almost all of our trees, including the MRE trees, *Chromyella* + *Boninia* are sister group to *Theama*. Curiously, in the only trees of our dataset supporting *Theama* + *Chromyella* (Suppl. Figs. S13, 15, 21), we used the same alignment method (MUSCLE), the same reconstruction method (RAxML), a partial 28S matrix and Gblocks, just like Bahia et al. (2017). In Laumer and Giribet (2014, 2017), the remaining possibility is realised, i.e. *Theama* + *Boninia* are sister group to *Chromyella*.

Moreover, the name of the superfamily has been erected based on the oldest family of the three included genera, *Theama*, *Chromyella* and *Boninia* (Bahia et al. 2017). According to Bahia et al. (2017), the corresponding families of these genera are Theamatidae, Amyellidae and Chromoplanidae. *Theama* is a member of Theamatidae Marcus 1949, *Chromyella* is a member of either Amyellidae Faubel 1983 or Chromoplanidae Bock 1922, but *Boninia* is a member of Boniniidae Bock 1923. Also, the epynomous genus of Chromoplanidae, *Chromoplana*, is not clustering with *Chromyella* in any tree containing both of the genera (see also Laumer and Giribet 2014; Tsunashima et al. 2017). Therefore, the family name Chromoplanidae should stay with *Chromoplana*, and *Chromyella* should be retained in the family Amyellidae, making Boniniidae the oldest family of the three clustering genera. Here, we propose a new superfamily, Boninioidea superfam. nov., with the morphological definition of Chromoplanoidea *sensu* Bahia et al. 2017, but including the families Theamatidae, Amyellidae and Boniniidae.

Cryptoceloidea *sensu* Bahia et al. (2017) include the families Discocelidae (represented by *Adenoplana* in Bahia et al. 2017 and by *Discocelis* and *Adenoplana* in our 28Sshort6 trees), and Cryptocelidae (represented by *Phaenocelis* in Bahia et al. 2017, and by *Cryptocelis*, *Phaenocelis* and *Amemiyaia* in our 28Sshort6 trees). While Faubel (1983) puts the genus *Amemiyaia* into the family Stylochoplanidae, Prudhoe (1985) considers it to be a Cryptocelididae, the latter being consistent with our results (Figs. 6 and 7). Thus, we reject the family Cryptocelidae *sensu* Faubel (1983). Our clade 6 contains members of Discocelidae and Cryptocelididae *sensu* Prudhoe (1985), and with *Ilyella gigas* an Ilyplanidae (Faubel 1983). We therefore reject Cryptoceloidea *sensu* Bahia et al. (2017) as it contains Cryptocelidae *sensu* Faubel (1983) and redefine the superfamily with the inclusion of the family Cryptocelididae *sensu* Prudhoe (1985), and the families Ilyplanidae and Discocelidae. This in turn means that Discocelidae Laidlaw (1903) is the oldest family constituting the superfamily, and accordingly, the superfamily is named Discoceloidea, including the families Cryptocelididae, Discocelidae and Ilyplanidae.

Stylochoidea *sensu* Bahia et al. (2017) has nuchal tentacles in common and includes the families Hoploplanidae, Stylochidae, Pseudostylochidae and Planoceridae. Faubel (1984) placed the genus *Hoploplana* within Leptoplanoidea, mainly due to the presence of an interpolated prostatic vesicle. This is in contrast to Prudhoe (1985), who considered the genus to be part of Planoceridae and thus in the superfamily Stylochoidea. *Hoploplana* was sister to *Planocera* within Stylochoidea in Bahia et al. (2017) and Litvaitis et al. (2019). Also Aguado et al. (2017) proposed the inclusion of *Hoploplana* in Stylochoidea based on the morphological differences of the prostatic vesicle (also see Noreña et al. 2015) between leptoplanoids and that of *Hoploplana*, as well as on their molecular phylogeny. Our 28Sshort6 MRE tree supports the sister group relationship of *Hoploplana* with the pseudostylochid *Idioplana* (Fig. 6), while

there is strong support of *Hoploplana* + *Planocera* in our 18S28Slong trees, where *Idioplana* is lacking (Fig. 5), but also in some of the 28Sshort6 trees (Suppl. Figs. S14, 17, 20, 23).

We reject the superfamily Stylochoidea *sensu* Bahia et al. (2017) in the current form, as all our 28Sshort6 trees show that the pseudostylochids *Pseudostylochus* sp. as well as *Pseudostylochus obscurus* appear within Leptoplanoidea *sensu* Bahia et al. (2017), thus forming our clade 5, whereas the remaining pseudostylochid, *Idioplana australiensis,* recovers within Stylochoidea (*sensu* Bahia et al. 2017), see above. *Pseudostylochus* is the type genus of Pseudostylochidae, so the family name is retained with the genus; consequently, we erect a new family for *Idioplana,* Idioplanidae fam. nov., currently with the diagnosis of the genus.

A further indication that Pseudostylochidae belongs within Leptoplanoidea *sensu* Bahia et al. (2017), rather than within Stylochoidea *sensu* Bahia et al. (2017), can be found in the study of Aguado et al. (2017). There, *Pseudostylochus intermedius* clusters within Leptoplanoidea (Aguado et al. 2017). The authors trace this position back to a misidentified species by Sato et al. (2001). However, we think a misidentification is unlikely, as all of our phylogenetic trees including *Pseudostylochus* sp. as well as *Pseudostylochus obscurus* confirm the position of *Pseudostylochus* within Leptoplanoidea—with different sampling material, and different genes than provided by Sato et al. (2001). Also, *Pseudostylochus* is always recovered as monophyletic. Already in the original description of the genus *Pseudostylochus*, it was placed within the same superfamily as Leptoplanidae, Schematommata (Yeri and Kaburaki 1918). In the study of Tsunashima et al. (2017), *Pseudostylochus* is shown within Notoplanidae, and hence within Leptoplanoidea as well. As *Pseudostylochus* has nuchal tentacles, albeit 'small and indistinct' (Yeri and Kaburaki 1918), the placement of the genus within the Leptoplanoidea (a group without nuchal tentacles) contradicts the hypothesis that nuchal tentacles have only evolved once in Polycladida, at the base of Stylochoidea (Bahia et al. 2017).

As a result, we redefine the superfamily Stylochoidea (*sensu* Bahia et al. 2017) consisting of Hoploplanidae, Idioplanidae nov. fam., Stylochidae and Planoceridae, but without Pseudostylochidae.

Within the family Stylochidae (represented by the genera *Stylochus*, *Imogine*, *Leptostylochus*), only the minority of our 28Sshort6 trees recovers the genus *Stylochus* as monophyletic (two of twelve), and none of our trees supports a monophyletic *Imogine*, corroborating the results of Aguado et al. (2017) and Bahia et al. (2017). This is not surprising, as both genera were formerly included as subgenera of *Stylochus* (Jennings and Newman 1996; Aguado et al. 2017). We therefore recommend to combine them in one genus—*Stylochus*—once more, as the name *Stylochus* (Ehrenberg 1831) predates the name *Imogine* (Girard 1853).

Additionally, Planoceridae *sensu* Faubel (1983) are never monophyletic in any of our 28Sshort6 trees, because *Paraplanocera oligoglena* always clusters within Stylochidae, even in our 18S28Slong trees (Table 2, Fig. 5). This phylogenetic position of *Paraplanocera oligoglena* corresponds to the finding of Tsunashima et al. (2017) and Bahia et al. (2017). As stated under the section 'Correct determination is important', *Paraplanocera* sp. is confusingly labelled as *Planocera* sp. in their paper (Bahia et al. 2017), but published as *Paraplanocera* sp. in GenBank. This *Paraplanocera* sp. sequence renders the genus *Planocera* paraphyletic in most of our 28Sshort6 trees (Table 2).

Leptoplanoidea *sensu* Bahia et al. (2017) includes Pleioplanidae, Leptoplanidae, Notoplanidae and Stylochoplanidae. As discussed above (in Stylochoidea *sensu* Bahia et al. 2017), we also have to reject this superfamily in its current form, as Pseudostylochidae (represented by *Pseudostylochus*) clusters in all of our 28Sshort6 trees within Leptoplanoidea. Hence, the group including Pleioplanidae, Leptoplanidae *sensu* Prudhoe 1985 (excluding *Hoploplana*), Notoplanidae, Stylochoplanidae and Pseudostylochidae is to be called Leptoplanoidea.

Within Leptoplanoidea, Stylochoplanidae *sensu* Faubel (1983) (including *Amemiyaia*, *Comoplana* and *Armatoplana*) appears polyphyletic in all of our 28Sshort6 trees (see Discussion about Cryptoceloidea), strongly suggesting the need of revision of the family. The only other molecular study including more than one member of Stylochoplanidae is Aguado et al. (2017), in which mitochondrial sequences of *Stylochoplana maculata* and *Comoplana agilis* were used, which did not appear as sister groups in their phylogenetic reconstruction. However, the published sequence of *S. maculata* was found to be almost identical to the sequence of *Leptoplana tremellaris*, leading the authors to suggest that *S. maculata* was possibly misidentified and is actually *L. tremellaris* (Aguado et al. 2017).

All our 28Sshort6 trees show that Leptoplanidae (*sensu* Faubel 1983 or Prudhoe 1985), Notoplanidae (*sensu* Faubel 1985) and *Notoplana* are not monophyletic, while *Notocomplana* and *Leptoplana* are always monophyletic. In Tsunashima et al. (2017), as well as in Bahia et al. (2017), Notoplanidae are not monophyletic as well. In their recently published phylogenetic reconstruction, Litvaitis et al. (2019) revised several families and genera within this superfamily.

Conclusions

Success in resolving polyclad interrelationships was hampered so far by different approaches using different genes or different parts of the same gene, making a combination of published data difficult. Polyclad interrelationships are still only tentatively resolved using single or two gene phylogenies. We have identified some stable parts of the phylogeny, and also groups which need to be revisited with better taxon sampling and with longer alignments, ideally using a transcriptomic-phylogenomic approach.

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