Polyclad phylogeny persists to be problematic

Isabel L. Dittmann 1 · Daniel Cuadrado 2 · Maria Teresa Aguado 3,4 · Carolina Noreña 2 · Bernhard Egger 1

Received: 12 April 2019 / Accepted: 14 August 2019 / Published online: 16 September 2019
© The Author(s) 2019

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 Tricladiida 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
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 Boniiniidae, 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 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 acotylean—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 taxa, namely Theama genes, the acotylean taxa. In a flatworm-wide phylogenetic study based on four cotyleans, thereby stressing the problematic position of these taxa, 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        | HJ659009.1 |            |                   |
| *salar*          | Marcus 1949   | Brazil           | KY263653.2 |            |                   |
|                  | Du Bois-Reymond Marcus 1957 | Brazil       | KY263652.2 |            |                   |
| *Phaeocelis* medvedica | Marcus 1952    | Brazil           | KY263701.2 |            |                   |
| *Echinoplana* celerrima 1 | Haswell 1907  | Tunis, Tunisia   | MN421930   | MN421936, MN421930 | SRS842092       |
| *celerrima* 2    |              | Australia        | HJ659020.1 |            |                   |
| *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    | MN384693   |            |                   |
| *Notoplana* australis 1 | (Laidlaw 1904) | Australia        | AY157153.1 | AJ228786.1, AY157153.1 |                   |
|                  |              |                  |           |            |                   |
| *Notocomplana* humilis | Stimpson 1857 | Japan            | LC100085.1 |            |                   |
|                   |              |                  |            |            |                   |
| *Notocomplana* japonica | Kato 1937a   | Japan            | LC100087.1 |            |                   |
|                  |              |                  |            |            |                   |
| *Notocomplana* koreana | Kato 1937b   | Japan            | LC100086.1 |            |                   |
| *sp.*            |              |                  |            |            |                   |
| *Melloplana* ferruginea | (Schmarda 1859) | Florida       | HJ659014.1 |            |                   |
|                  |              |                  |            |            |                   |
| *Comoplana* agilis | (Lang 1884) | Galicia, Spain   | MN384685   | MN334199, MN384685 |                   |
|                  |              |                  |            |            |                   |
| *Armatothecopsis* lepaticola | (Marcus 1947) | Brazil          | KY263648.2 |            |                   |
| *Amemiyaija* pacifica | Kato 1944   | Japan            | LC100077.1 |            |                   |
| *Thea* mediterranea 1 | Curini-Galletti et al. 2008 | Rovinj, Croatia | MN384705   | MN384707, MN384705 |                   |
|                  |              |                  | Panama     | KC869845.1, KC869792.1, KC869845.1 |                   |
| *Callioplana* marginata | Stimpson1857 | Japan            | LC100082.1 |            |                   |
| *Planocera* multitanuta | 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 |                   |
|                  |              |                  |            |            |                   |
| *Idioplana* australiensis | Woodworth 1898  | Australia        | HQ659008.1 |            |                   |
|                  |              |                  |            |            |                   |
| *Pseudostylochus* obscurus | (Stimpson 1857) | Japan          | LC100084.1 |            |                   |
|                  |              |                  |            |            |                   |
| *Stylochus* ellipticus | (Girard 1850) | Woods Hole, USA | Suppl. File 1 | Suppl. File 1 | SRS913554        |
| *ijima*          | Yeri and Kaburaki 1918 | Japan       | LC100079.1 |            |                   |
|                  |              |                  |            |            |                   |
| *Stylochus* oculiferus | (Girard 1953) | Florida         | HQ659007.1 |            |                   |
| *zebra*          | (Verrill 1882) | US Atlantic coast | AF342800.1 | AF342801.1, AF342800.1 |                   |
|                  |              |                  |            |            |                   |
| *Imaginaria* referens | (Schmarda 1859) | Brazil          | KY263694.2 |            |                   |

Polyclad phylogeny persists to be problematic.
| Species                  | Authority                          | Location                          | 28Sshort6   | 18S28Slong    | SRA         |
|-------------------------|------------------------------------|-----------------------------------|-------------|---------------|-------------|
| *Du Bois-Reymond Marcus* 1965 | stellae Marquina et al. 2014       | Valencia, Spain                   | MN384692    | MN334201, MN384692 |
| *Leptostylochus* 1934 | gracilia Kato                       | Japan                             | LC100078.1  |
| *Cycloporus* 1950         | gabriellae 1 Marcus et al. 2014    | Brazil                            | KY263656.2  |
|                          | variegatus 1 Kato                   | Brazil                            | KY263657.2  |
|                          | variegatus 2                        | Spain                             | KY263659.2  |
|                          | variegatus 3                        | Brazil                            | KY263660.2  |
|                          | variegatus 4                        | Brazil                            | KY263661.2  |
| *Maritinella* 1944       | crozieri 1 (Hyman 1939)             | Florida Keys, USA                 | MN421933    | MN421939, MN421933, SRS844631 |
|                          | crozieri 2                          | Aquaria in Virginia, USA          | HQ659013.1  |
|                          | crozieri 3                          | Florida                            | KY263686.2  |
| *Prostheceraeus* 1884    | roseus Lang                         | Tenerife                          | KY263688.2  |
|                          | viattus (Montagui 1815)             | unknown                           | Suppl. File 1, Suppl. File 1, SRS913668 |
| *Stylostomum* 1853       | ellipse Pulcher et al. 2007         | Pulat, Croatia                    | MN384704    | MN334208, MN384704 |
| *Euryleptodes* 1853      | gallicia Noreña et al. 2014         | Galicia, Spain                    | MN384691    |
| *Prosthiostomum* 1887    | grande Stimpson                     | Japan                             | LC100090.1  |
|                          | sipunculus 1 (Delle Chiaie 1822)    | Barcelona, Spain                  | MN421934    | MN421940, MN421934, SRS842699 |
|                          | sipunculus 2                         | Asturias, Spain                   | MN384697    | MN334204, MN384697 |
|                          | sipunculus 3                         | Spain                             | HQ659012.1  |
| *Amakusaplan* 1938       | acroporae Rawlinson et al. 2011     | Aquaria US East Coast             | HQ659010.1  |
| *Lurymare* 1975          | katoi Pouler                         | Lizard Island (Australia)         | MN384694    |
| *Enchiridium* 1949       | evelinae Marcus                      | Brazil                            | KY263662.2  |
|                          | sp. 1                               | Lizard Island (Australia)         | MN384686    |
|                          | sp. 2                               | Santa Helena Island               | KY263665.2  |
| *Chromyella* 1884        | sp.                                 | Panama                            | KC869848.1  | KC869795.1, KC869848.1 |
| *Anonymus* 1853          | ruber Cuadrado et al. 2017          | Canary Island, Spain              | MN384687    | MN334197, MN384687 |
|                          | viridis Lang                        | Canary Island, Spain              | MN384688    |
| *Boninia* 1968           | divae Marcus and Marcus             | Panama                            | KC869846.1  | KC869793.1, KC869846.1 |
| *Chromoplana* 1884       | sp.                                 | Panama                            | KC869847.1  | KC869794.1, KC869847.1 |
| *Pericelis* 1876         | byerleyana (Collingswood 1876)      | Red Sea                           | MH047291.1  |
|                          | cata 1 Marcus and Marcus            | unknown                           | EU679114.1  |
|                          | cata 2                              | Brazil                            | KY263700.2  |
|                          | orbicularis (Schmarda 1859)         | unknown                           | EU679116.1  |
|                          | tectivorum Dittmann et al. 2019     | Aquaria Imbruck, Austria          | MK181524    | MN334202, MK181524 |
| *Pseudoceros* 2014       | astorum Bulnes and Torres           | Brazil                            | KY263737.2  |
|                          | bicolor 1 Verrill                   | Belize                            | GQ398095.1  |
|                          | bicolor 2                           | Brazil                            | KY263732.2  |
|                          | bicolor marcusorum                  | Belize                            | GQ398098.1  |
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

| Species | Authority | Location | 28Sshort1 | 18S-28Slong | SRA |
|---------|-----------|----------|-----------|-------------|-----|
| cf. bicolor | Meixner 1907 | Brazil | KY263729.2 | MN384700, MN334207, MN384700 |
| bimarginatus | Newman and Cannon 1995 | Papua New Guinea | KY263728.2 |
| contrarius | Bolaños et al. 2007 | Panama | EF514802.1 |
| harrisi | Newman and Cannon 1994 | Lizard Island (Australia) | MN384701 |
| jebborum | Lang 1884 | Spain | KY263708.2 |
| nipponicus | Kato 1944 | Japan | LC100096.1 |
| periarantius | Newman and Cannon 1994 | Lizard Island (Australia) | MN384702 |
| rawlinsonae 1 | Bolaños et al. 2007 | Bahamas | GQ398101.1 |
| rawlinsonae 2 | Newman and Cannon 1998 | Brazil | KY263733.2 |
| stimpsoni | Newman and Cannon 1998 | Lizard Island (Australia) | MN384703 |
| velatius 1 | (Blanchard 1847) | Spain | KY263726.2 |
| velatius 2 | (Laidlaw 1903) | Japan | LC100095.1 |
| Pseudobiceros bedfordi | Bolaños et al. 2007 | 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, MN384968 |
| 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 | (Blanchard 1847) | Brazil | KY263723.2 |
| splendidus | Lang 1884 | Florida | HQ659016.1 |
| wirtzi | Bahia and Schroedl 2016 | Senegal | KY263725.2 |
| sp. | Newman and Cannon 1996 | Santa Helena Island | KY263724.2 |
| Maiazoon orsaki | Bahia et al. 2015 | Papua New Guinea | KY263697.2 |
| Thysanozoon alagoensis | Bahia et al. 2015 | Brazil | KY263747.2 |
| brocchii 1 | (Rioso 1818) | Philip Island, Australia | HQ659017.1 |
| brocchii 2 | Lang 1884 | Brazil | KY263744.2 |
| raphaeli | Bolaños et al. 2007 | Panama | EF514809.1 |
| Yungia sp. | (Marcus 1950) | 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. | MN421932, MN421938, MN421932 SRS842645 |

**Table 1 (continued)**
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 gabiellae (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 Phyip 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 frequencies < 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 coteleans (Suppl. Figs. S5, S10, S11), while three trees rendered most of these taxa as acoteleans 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 coteleans (Suppl. Figs. S2, S8). 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 28Sshort3) 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 Prosthiostomoidae alternating between Acotylea and Cotylea (Fig. 3). With only Xenoprorynchus 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, Eurelyptidae and all other Polycladida was recovered (Fig. 3a). Prosthiostomoidae are basally branching Acotylea with Macrostomum + Xenoprorynchus, and only Macrostomum as outgroups. Xenoprorynchus 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.  

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

Polyclad phylogeny persists to be problematic.
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).

| Suppl. Fig. S | 18S28Slong | 28Sshort6 | Support |
|---------------|------------|-----------|---------|
|               | 18S28Slong | 28Sshort6 |          |
|               | GB No GB   | GB No GB  | GB No GB |
|               | ML BI      | ML BI     | ML BI   |
|               | M Q E      | M Q E     | M Q E   |
| 1 2 3 4 5 6   | 7 8 9 10   | 11 12     |         |

Table 2 (continued)

21. Chromoplanoidea as sister group to all other Cotylea, except Cestoplanoidea

22. Theamatidae is sister group to a clade consisting of Boninia and Chromyella

23. Leptoplanoidea sensu Faubel 1983 (in whose definition Hoploplana and Theama are included) is supported

24. Clade 3 can be subdivided into two clades (clades 5 and 6)

25. Clade 5 is synonymous with Leptoplanoidea sensu Bahia et al. 2017

26. Pseudostylochus is part of clade 5

27. Leptoplana is monophyletic

28. Notoplanidae as a whole, as well as Notoplanidae are monophyletic, while Notocomplana is not monophyletic

29. Clade 6 appears monophyletic

30. Clade 6 appears as sister group to clade 5

31. Clade 4 can be subdivided into two clades, clades 7, 8 and Callioplana, where the latter is sister group to clades 7 + 8

32. A polytomy exists between Callioplana, clade 3 and clade 4

33. Clade 7 appears not monophyletic

34. Hoploplana clustering as sister group to Idioplana, as clade 7

35. Hoploplana is sister group to Planoceridae/Planocera pellucida

36. Clade 8 is monophyletic

37. Planocera is monophyletic

38. Paraplanocera is monophyletic

39. Planoceridae sensu Faubel 1983 are recovered as monophyletic

40. Stylochus is monophyletic

41. Imogenidae is monophyletic

Total score: 17 21 17 17 20 15 17 21 17 20 18 16 34 32 34 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)
Now we also removed *Chromoplana* from the dataset (28Sshort4) and once more had *C. variegatus* as sister group to all other Polycladida. Also, Cestoplanoidea, Chromoplanoida, Pericelioidea and Anonymidae emerged as Acotylea (Fig. 4a). With the additional removal of *C. variegatus* from the sequence collection (28Sshort5), we recovered Cestoplanoidea, Chromoplanoida, Pericelioidea 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 gabiellae*, instead of only using one sequence per species from the same authors (28Sshort6, Suppl. Figs. S1–24). 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. S1–24), 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 *Cestoplan* are cotyleans instead of acotyleans.

Cestopanoidea (Bahia et al. 2017) and thereby its only family, Cestopaneidae, 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 Cestopanoidea 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 Chromoplanoida (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 Chromoplanoida as sister group to all other Cotylea, except Cestopanoidea. In 88% of our trees, and in both MRE trees, *Theamatidae* is sister group to a clade consisting of *Boninia* and *Chromyella*.

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

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

Pseudocerotidea 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 gabiellae* 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.

Prosthiostoimoidea (Bahia et al. 2017) appears monophyletic in all trees, and in all but two trees as sister group to Pseudocerotoida. Within Prosthiostoimoidea, *Enchiridium* appears as sister group to a clade consisting of Prosthiostoimom, *Lurymare* and *Amakusaplana* in all
Polyclad phylogeny persists to be problematic

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

Leptoplanoida sensu Faubel 1983 (in whose definition Hoploplana and Theama are included) is not supported in any of our trees. We have termed Leptoplanoida 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 Leptoplanoida 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 Leptopla is monophyletic, and that Notoplanaeidae as a whole and also its genera Notoplana and Notocomplanae 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 Discocelidae, Adenoplanae, Ilyellae, Phaenocelidae and Amemiyae. In our 18S28Slong trees and the corresponding MRE tree, clade 6 is represented only by Discocelidae 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 Callioplae. 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...
The clad phylogeny is problematic. 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.

Clade 8 resembles Stylochoida sensu Faubel (1983) and is supported by only two of twelve 28Sshort6 trees as well, but not in the 18S28Sshort6 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.
sp. clusters within. Similarly, *Paraplanocera* is not monophyletic in any of our 28S short 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 28S short 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 28S short 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 28S short 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 prosieriates (Casu et al. 2014; Scarpa et al. 2015, 2016, 2017). The two best-scoring 18S28S long trees are both based on a MAFFT Q-INS-i alignment (Table 2, Suppl. Figs. S2, 8).

However, the two worst-scoring 28S short trees are also based on MAFFT Q-INS-i alignments (Table 2, Suppl. Figs. S10, 14),
et al. 2015, Laumer et al. 2015), suggesting that MAFFT E-INS-i provided a more robust alignment than RNAsalsa.

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 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 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. In their re-analysis, they used MAFFT E-INS-i instead of RNasalsa. In their re-analysis, they used MAFFT E-INS-i instead of RNasalsa.

From this work, we cannot give an unambiguous 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 *Xenoproryynchus* sp., a basally branching prohyynchid—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 *Xenoproryynchus*, only *Xenoproryynchus* or only *Macrostomum* as outgroups (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 *Amakusaplanapia 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*; 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 mis-determination of its 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 re-determination 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 Prosthioistomoidae and Pseudocerotoidae. 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 Cestoplanida 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 Periceloidae, Anonymus, Chromoplana, Prosthioistomoidae and Pseudocerotoidae (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 28Sshort tree topology, although the 18S28Slong alignment with ca. 3000 nt is almost four times as long as the 28Sshort 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 28Sshort 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 (28Sshort) 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 variiegatus 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):

Pseudocerotoidae 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 variiegatus 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 variiegatus 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).
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 Pseudocerotoidae 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.
The position of Chromoplanoidea within Cotylea is supported by most of our analyses (Table 2), although in the 18S28S long MRE tree, Chromoplanoidea is recovered as acotylean (Fig. 5). The superfamily always is monophyletic, but the interrelationships between the three included cotylean 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 Chromoplanaeidae. Theama is a member of Theamatidae Marcus 1949, Chromyella is a member of either Amyellidae Faubel 1983 or Chromoplanaeidae Bock 1922, but Boninia is a member of Boniniidae Bock 1923. Also, the eponymous genus of Chromoplanaeidae, 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 Chromoplanaeidae 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, Boninioidae superfam. nov., with the morphological definition of Chromoplanaeidae sensu Bahia et al. 2017, but including the families Theamatidae, Amyellidae and Boniniidae.

Cryptoceloidae 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 Ameniyaiia in our 28Sshort6 trees). While Faubel (1983) puts the genus Ameniyaiia into the family Stylochoplanidae, Prudhoe (1985) considers it to be a Cryptocelidae, 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 Cryptocelidae sensu Prudhoe (1985), and with Ilyella gigas an Ilyplanidae (Faubel 1983). We therefore reject Cryptoceloidae sensu Bahia et al. (2017) as it contains Cryptocelidae sensu Faubel (1983) and redefine the superfamiliy with the inclusion of the family Cryptocelidae sensu Prudhoe (1985), and the families Ilyplanidae and Discocelidae. This in turn means that Discocelidae LaID (1903) is the oldest family constituting the superfamiliy, and accordingly, the superfamiliy is named Discoceloidae, including the families Cryptocelidae, Discocelidae and Ilyplanidae.

Stylochoidae 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 superfamiliy Stylochoidae. Hoploplana was sister to Planocera within Stylochoidae in Bahia et al. (2017) and Litvaitis et al. (2019). Also Aguado et al. (2017) proposed the inclusion of Hoploplana in Stylochoidae based on the morphological differences of the prostatic vesicle (also see Noriea et al. 2015) between leptoplanooids and that of Hoploplana, as well as on their molecular phylogeny. Our 28Sshort 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 Idioplanida 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, Idioplena 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 Idioplena, 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 superfamly as Leptoplanaidae, 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 superfamly Stylochoidea (sensu Bahia et al. 2017) consisting of Hoploplanaidae, Idioplanidae nov. fam., Stylochidae and Planoceridae, but without Pseudostylochidae.

Within the family Stylochidae (represented by the genera Stylochus, Imagine, 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 Imagine, 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 Imagine (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 Amemiyaiia, Comoplana and Armatoplana) appears polyphyletic in all of our 28Sshort6 trees (see Discussion about Cryptoceloida), 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.
Acknowledgements We are grateful to Miquel Vila-Farré, Alexandra Grosbush, Lucy Neumann, Tamara Schadt, Tania Holtzem, Florian Holler and Philip Bertemes for their assistance with sampling. We additionally thank the Research Focal Point Scientific Computing at the University of Innsbruck for providing computing infrastructure for assemblies, and the University of Innsbruck for supporting ILD with a ‘Stipendium für kurzfristige wissenschaftliche Arbeiten im Ausland’ and a PhD fellowship, and BE with a grant for young academics. This research received support from the SYNTHESYS Project http://www.syntheses.info/ (ES-TAF-3940 and ES-TAF-4482) which is financed by European Community Research Infrastructure Action under the FP6 “Structuring the European Research Area” Programme and the FP7 “Capacities” Program at the Museo Nacional de Ciencias Naturales Madrid (CSIC).

Funding Information Open access funding provided by University of Innsbruck and Medical University of Innsbruck.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

Aguado, M. T., & Bleidorn, C. (2010). Conflicting signal within a single gene confounds syllid phylogeny (Syllidae, Annelida). Molecular Phylogenetics and Evolution, 55(3), 1128–1138.

Aguado, M. T., Noreña, C., Acalarz, L., Marquina, D., Brusa, F., Damborenea, C., Almon, B., Bleidorn, C., & Grande, C. (2017). Phylogeny of Polycladida (Platyhelminthes) based on mtDNA data. Organisms Diversity & Evolution, 17(4), 767–778.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. Journal of Molecular Biology, 215, 403–410.

Alvarez-Pressas, M., Baguñá, J., & Riutort, M. (2008). Molecular phylogeny of land and freshwater planarians (Tricladida, Platyhelminthes): From freshwater to land and back. Molecular Phylogenetics and Evolution, 47(2), 555–568.

Baiha, J., Padula, V., Correia, M. D., & Sovierzoski, H. H. (2015). First records of the order Polycladida (Platyhelminthes, Rhabditophora) from reef ecosystems of Alagoas State, north-eastern Brazil, with the description of Thysanozoon alagoensis sp. nov. Journal of the Marine Biological Association of the United Kingdom, 95(8), 1653–1666.

Baiha, J., & Schroedl, M. (2016). Pseudobiceros wirzii sp. nov. (Polycladida: Cotylea) from Senegal with revision of valid species of the genus. Zootaxa, 4097(1), 101–117.

Baiha, J., Padula, V., & Schrödl, M. (2017). Polycladida phylogeny and evolution: Integrating evidence from 28S rDNA and morphology. Organisms Diversity & Evolution, 17, 653–678.

Blanchard, E. (1847). Recherches sur l’organisation des vers. Annales des Sciences Naturelles. Troisième série. Zoologie, 8, 271–275.

Bock, S. (1913). Studien über Polycladen. Zoologiska Bidrag från Uppsala, 2, 31–344.

Bock, S. (1922). Two new Cotylean genera of Polyclads from Japan and remarks on some other Cotyleans. Ark. Zool. v. 14 n. 13, p. 1–31 t. 1–2. Stockholm.

Bock, S. (1923). Boninia, a new polyclad genus from the Pacific. Nov Act R Soc Sci Uppsala, 6, 1–32.

Bolaños, D. M., Quiroga, S. Y., & Litvaitis, M. K. (2007). Five new species of cotylean flatworms (Platyhelminthes: Polycladida) from the wider Caribbean. Zootaxa, 1650(1), 1–23.

Bolune, N. V., & Torres, Y. (2014). Pseudoceros astrorum, a new species of Polycladida (Cotylea, Pseudocerotidae) from northeastern Brazil. Zootaxa, 3881(1), 94–100.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular Biology and Evolution, 17, 540–552.

Casu, M., Scarpa, F., Delogu, V., Cossu, P., Lai, T., Sanna, D., & Curini-Galletti, M. (2014). Biodiversity patterns in interstitial marine microturbellaria: A case study within the genus Parotoplana (Platyhelminthes: Rhabditophora) with the description of four new species. Journal of Zoological Systematics and Evolutionary Research, 52(3), 190–202.

Chen, H., Rangasamy, M., Tan, S. Y, Wang, H., & Siegfried, B. D. (2010). Evaluation of five methods for total DNA extraction from western corn rootworm beetles. PLoS One, 5(8), e11963.

Collingwood, D. (1876). VI. On thirty-one species of marine planarians, collected partly by the late Dr. Kelaart, FLS, at Trincomalee, and partly by Dr. Collingwood, FLS, in the eastern seas. Transactions of the Linnee Society of London. 2nd Series: Zoology, 1(3), 83–98.

Cuadrado, D., Moro, L., & Noreña, C. (2017). The Polycladida (Platyhelminthes) of the Canary Islands. New genus, species and records. Zootaxa, 4312(1), 038–068.

Curini-Galletti, M., Campus, P., & Delogu, V. (2008). Thea mediterranea sp. nov. (Platyhelminthes, Polycladida), the first interstitial polyclad from the Mediterranean. Italian Journal of Zoology, 75(1), 77–83.

Dalyell, J. P. (1853). Observations on some interesting phenomena in animal physiology, exhibited by several species of planaria. Archibald Constable, Edinburgh.

Delle Chiage, S. (1822–1829). Memorie sulla storia e notomia degli animali senza vertebre del regno di Napoli. Atlas of 109 Tables (1822); Vol. I (1823): 1–184; Vol. II (1825): 1–444; Vol. III (1828): 1–232; Vol. IV (1829) 1–214 Fratelli Fernandes, Napoli.

Dittmann, I. L., Dibiasi, W., Noreña, C., & Egger, B. (2019). Description of the snail-eating flatworm in marine aquaria, Pericelis tectivorum sp. nov. (Polycladida, Platyhelminthes). Zootaxa, 4563(3), 383–397.

Du Bois-Reymond Marcus, E. (1957). On Turbellaria. Anais da Academia Brasileira de Ciencias, 29(1), 153–191.

Du Bois-Reymond Marcus, E. (1965). Drei neue neotropische Turbellaria. Sitzungsberichte der Gesellschaft naturforscher und Freunde zu Berlin, 5, 129–135.

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids research., 32(5), 1792–1797.

Egger, B., Lapraz, F., Tomiczek, B., Müller, S., Dessimoz, C., Girstmair, J., Skunca, N., Rawlinson, K. A., Cameron, C. B., Beli, E., Todaro, M. A., Gammoudi, M., Noreña, C., & Telford, M. J. (2015). A transcriptomic-phylogenetic analysis of the evolutionary relationships of flatworms. Current Biology, 25, 1–7.

Ehrenberg, C. G. (1831). Phytotzoa Turbellaria africanæ et asiatica. In: Hemprich und Ehrenberg "Symbolae physicae." Animalia evertrebata exclusis insectis recensuit Dr. CG Ehrenberg. Series prima cum tabularum decade prima. Berolini, Fol. Phytotzoa Turbellaria folia a–d, 74–5 [plates 4, 5 published in 1828]

Faubel, A. (1983). The Polycladida, Turbellaria; proposal and establishment of a new system. Part I. The Acotylea. Mitteilungen aus dem Hamburgischen Zoologischen Museum und Institut, 80, 17–121.

Faubel, A. (1984). The Polycladida, Turbellaria proposal and establishment of a new system. Part II. The Cotylea. Mitteilungen aus dem Hamburgischen Zoologischen Museum und Institut, 81, 189–259.

Girard, C. (1850). Descriptions of several new species of marine planariae from the coast of Massachusetts. In Proceedings of the Boston Society of Natural History, 3, 251–256.
Girard, C. F. (1853). Descriptions of new nemerteans and planarians from the coast of the Carolinas. *Proceedings of the Academy of Natural Sciences of Philadelphia*, 6, 365–367.

Glez-Peña, D., Gómez-Blanco, D., Reboiro-Jato, M., Fdez-Riverola, F., Posada, D. (2010). ALTER: Program-oriented format conversion of DNA and protein alignments. Nucleic Acids Research. Web Server issue. ISSN: 0305-1048.

Grube, A. E. (1840). Actinien, Echinodermen und Würmer des Adriatischen- und Mittelmeers, nach eigenen Sammlungen beschrieben. JH Bon, Königsberg, 92 pp.

Haswell, W. A. (1907). Observations on Australian polyclads. *Transactions of the Linnean Society, London*, 9, 465–485.

Hyman, L. H. (1939). Acoel and polyclad turbellarians from Bermuda and the Sargassum. Bulletin of the Bingham Oceanographic Collection, 7: 1–26. Art.I.

Hyman, L. H. (1951). The Invertebrates: Vol. II. Platyhelminthes and Flora des Golfes von Neapel. W. Engelmann, Leipzig.

Hyman, L. H. (1953). The polyclad flatworms of Tsunathe Pacific coast of North America. *Bulletin of the American Museum of Natural History*, 100, 265–392.

Janssen, T., Vizoso, D. B., Schulte, G., Littlewood, D. T. J., Waeschenbach, A., & Schärer, L. (2015). The first multi-gene phylogeny of the Macrostomorpha sheds light on the evolution of sexual reproduction in basal Platyhelminthes. *Molecular Phylogenetics and Evolution*, 92, 82–107.

Jennings, K. A., & Newman, L. J. (1996). Two new stylochid flatworms (Platyhelminthes: Polycladida) from the Southern Great Barrier Reef, Australia. *Raffles Bulletin of Zoology*, 44(1), 135–142.

Jacubowa, L. (1906). Polycladen von Neu-Britannien und Neu-Caledonien. *Jenaische Zeitschrift für die gesammten Naturwissenschaft*, 41, 113–158.

Kaburaki, T. (1923) The polyclad turbellarians from the Philippine Islands. Smithsonian Institution United States National Museum Bulletin 100 (volume I, part 10):635-649.

Kato, K. (1934). *Leptostylochus gracilis*, a new polyclad turbellarian. *Proceedings of the Imperial Academy*, 10(6), 374–377.

Kato K. (1937a). Polyclads collected in Idu, Japan. *Journal of Zoology, 7*, 21–232.

Kato K. (1937b). Polyclads from Korea. *Japanese Journal of Zoology, 7*, 233–240.

Kato, K. (1938). Polyclads from Seto, Middle Japan. *Japanese Journal of Zoology, 7*, 577–592.

Kato, K. (1944). Polycladida of Japan. *The Journal of the Sigenkagaku Kenkyuso*, 1, 257–318.

Kato, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, 30, 772–780.

Ladurner, P., Scharer, L., Salvenmoser, W., Rieger, R. M. (2005). A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: Macrostomum lignano, n. sp. (Rhabditophora, Macrostomophora). *Journal of Zoological Systematics and Evolutionary Research* 43 (2):114–126.

Laidlaw, F. F. (1903). Suggestions for a revision of the classification of the polyclad Turbellaria. *Memoirs and proceedings of the Manchester Literary & Philosophical Society*, 48(4), 1–16.

Laidlaw, E.F. (1904). Notes on some polyclad Turbellaria in the British Museum. Memoirs and Proceedings of the Manchester Literature and Philosophical Society 48 (Art. 15) 1 6.

Lang, A. (1884). Die Polycladen (Seeplanieren) des Golfs von Neapel und der angrenzenden Meeresabschnitte. Eine Monographie. Fauna und Flora des Golfs von Neapel. W. Engelmann, Leipzig.

Larsson, K., & Jonndelius, U. (2008). Phylogeny of Catenulida and support for Platyhelminthes. *Organisms Diversity & Evolution*, 8(5), 378–387.

Laumer, C. E., & Giribet, G. (2014). Inclusive taxon sampling suggests a single, stepwise origin of ectolecithality in Platyhelminthes. *Biological Journal of the Linnean Society*, 111(3), 570–588.

Laumer, C. E., Hejnol, A., & Giribet, G. (2015). Nuclear genomic signals of the ‘microturbellarian’ roots of platyhelminth evolutionary innovation. *eLife*, 4(05503), 1–31.

Laumer, C. E., & Giribet, G. (2017). Phylogenetic relationships within Adiaphanida (phylum Platyhelminthes) and the status of the crustacean-parasitic genus Genostoma. *Invertebrate Biology*, 136(2), 184–198.

Litvaitis, M. K., & Newman, L. J. (2001). A molecular framework for the phylogeny of the Pseudocerotidae (Platyhelminthes, Polycladida). *Hydrobiologia*, 444(1–3), 177–182.

Litvaitis, M. K., Bolaños, D. M., Quiroga, S. Y. (2010) When names are wrong and colours deceive: unravelling the species complex (Turbellaria: Polycladida). *Journal of Natural History* 44(13–14), 829–845.

Litvaitis, M. K., Bolaños, D. M., & Quiroga, S. Y. (2019). Systematic congruence in Polycladida (Platyhelminthes, Rhabditophora): Are DNA and morphology telling the same story? *Zoological Journal of the Linnean Society*, 186, 865–891.

Lockyer, A. E., Olson, P. D., & Littlewood, D. T. J. (2003). Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): Implications and a review of the concermer theory. *Biological Journal of the Linnean Society*, 78(2), 155–171.

Marcus, E. (1947). Turbellarios Marinhos do Brasil (5). *Boletim da Facultade de Filosofia, Ciências e Letras Zoologia*, 12, 99–215.

Marcus, E. (1949). Turbellaria brasileiros (7). *Boletim da Facultade de Filosofia, Ciências e Letras Zoologia*, 14, 7–155.

Marcus, E. (1950). Turbellaria brasileiros (8). *Boletim da Facultade de Filosofia, Ciências e Letras Zoologia*, 15, 5–191.

Marcus, E. (1952). Turbellaria brasileiros (10). *Boletim da Facultade de Filosofia, Ciências e Letras Zoologia*, 17, 5–86.

Marcus, E., & Marcus, E. (1966). Systematische Übersicht der Polycladen. *Zoologische Beiträge*, 12, 319–343.

Marcus, B. R., & Marcus, E. (1968). Polycladida from Curaçao and other Caribbean Islands, 26(3), 1–133.

Marquina, D., Osca, D., Rodriguez, J., Fernandez-Despiau, E., & Noreña, C. (2014). State of knowledge of the Acotylea (Polycladida, Platyhelminthes) from the Mediterranean coasts of Spain: New records and new species. *Zootaxa*, 3780(1), 108–134.

Martin-Durán, J. M., & Egger, B. (2012). Developmental diversity in free-living flatworms. *EvoDevo*, 3(1), 1.

Meixner, A. (1907). Polycladen von der Somaliküste, nebst einer Revision der Stylochinen. *Zeitschrift für Wissenschaftliche Zoologie, 88*, 385–498.

Mertens, H. (1833). Untersuchungen über den inneren Bau verschiedener in der See lebender Planarien. *Memories Academy Science St. Petersbourg, Series 6*, 2, 3–17.

Montagu, G. (1813). I. Descriptions of several new or rare animals, principally marine, discovered on the South Coast of Devonshire. *Transactions of the Linnean Society of London*, 11(1), 1–26.

Müller, O.F. (1773). Vermivm terrestrial et fluviatilium, seu animalium Infusorium, Helminthicorum et Testaceorum, non marinorum succincta historia. Havniae et Lipsiae: Apud Heineck et Faber, Typis Martinus Hallager, 1773–1774, Vol.1, Part 1, 1–80.

Newman, L. J., & Cannon, L. R. G. (1994). *Pseudocercus* and *Pseudobicerus* (Platyhelminthes, Polycladida, Pseudocerotidae) from eastern Australia and Papua New Guinea. *Memoirs of the Queensland Museum*, 37(1), 205–266.

Newman, L. J., & Cannon, L. R. G. (1995). The importance of the fixation of colour, pattern and form in tropical Pseudocerotidae (Platyhelminthes, Polycladida). *Hydrobiologia*, 305(1), 141–143.
Newman, L. J., & Cannon, L. R. G. (1996). New genera of pseudocerotid flatworms (Platyhelminthes; Polycladida) from Australian and Papua New Guinean coral reefs. *Journal of Natural History, 30*(10), 1425–1441.

Newman, L. J., & Cannon, L. R. G. (1997). Nine new species of *Pseudobiceros* (Platyhelminthes: Polycladida) from the Indo-Pacific. *The Raffles Bulletin of Zoology, 45*(2), 341–368.

Newman, L. J., & Cannon, L. R. G. (1998). *Pseudoceros* (Platyhelminthes; Polycladida) from the Indo-Pacific with twelve new species from the Australia and Papua New Guinea. *The Raffles Bulletin of Zoology, 46*(2), 293–323.

Noreña, C., Marquina, D., Perez, J., & Almon, B. (2014). First records of *Cotylea* (Polycladida, Platyhelminthes) for the Atlantic coast of the Iberian Peninsula. *ZooKeys, 404*, 1–22.

Noreña, C., Rodríguez, J., Perez, J., & Almón, B. (2015). New *Acotylea* (Polycladida, Platyhelminthes) from the east coast of the North Atlantic Ocean with special mention of the Iberian littoral. *Zootaxa, 4039*(1), 157–172.

Poche, F. (1926). Das System der Platodaria. *Archiv für Naturgeschichte, 91*, 1–458.

Poultier, J. L. (1975). Hawaiian polyclad flatworms: *Prosthiostomids. Pacific Science, 29*, 317–339.

Posada, D. (2008). *jModelTest:* Phylogenetic model averaging. *Molecular Biology and Evolution, 25*(7), 1253–1256.

Prudhoe, S. (1978). Some polyclad turbellarians new to the fauna of the Australian coasts. *Records of the Australian Museum, 31*, 586–604.

Prudhoe, S. (1985). A monograph on *Polyclad Turbellaria*. London: British Museum of Natural History and Oxford University Press 259 pp.

Puslednik, L., & Serb, J. M. (2008). Molecular phylogenetics of the Cotylea (Polycladida, Platyhelminthes) from Australian and Papua New Guinean coral reefs. *Coral Reefs, 30*(3), 693.

Rawlinson, K. A. (2008). Biodiversity of coastal polyclad flatworm assemblages in the wider Caribbean. *Marine Biology, 153*, 769–778.

Rawlinson, K. A., & Litvaitis, M. K. (2008). *Cotylea* (Polycladida): A cladistic analysis of morphology. *Invertebrate Biology, 127*(2), 121–138.

Rawlinson, K. A., Gillis, J. A., Billings, R. E., & Borneman, E. H. (2011). Taxonomy and life history of the Acropora-eating flatworm *Amakusaplana acroporae* nov. sp. (Polycladida: Prosthiostomiidae). *Coastal Reefs, 30*(3), 693.

Rawlinson, K. A., & Stella, J. S. (2012). Discovery of the corallivorous polyclad flatworm, *Amakusaplana acroporae*, on the Great Barrier Reef, Australia: The first report from the wild. *PLoS One, 7*, e42240.

Risso, A. (1818). Mémoire sur quelques gastéropodes nouveaux, nudi-branches et tectibranches observés dans la Mer de Nice. *Journal de Physique, de Chimie, d'Histoire Naturelle et des Arts, 87*, 368–377.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Lartet, B., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systems Biology, 61*, 539–542.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular cloning: A laboratory manual (2nd ed.). Cold Spring Harbor: Cold Spring Harbor Laboratory Press 1626 pp.

Sato, K., Sugita, T., Kobayashi, K., Fujita, K., Fujii, T., Matsumoto, Y., Mikami, T., Nishizuka, N., Nishizuka, S., Shoijima, K., Suda, M., Takahashi, G., Himeno, H., Muto, A., & Ishida, S. (2001). Localization of mitochondrial ribosomal RNA on the chromatin bodies of marine planarian polyclad embryos. *Development, Growth & Differentiation, 43*, 107–114.

Scarpa, F., Cosso, P., Sanna, D., Lai, T., Norenborg, J. L., Curini-Galletti, M., & Casu, M. (2015). An 18S and 28S-based clock calibration for marine *Proseriata* (Platyhelminthes). *Journal of Experimental Marine Biology and Ecology, 463*, 22–31.

Scarpa, F., Cosso, P., Lai, T., Sanna, D., Curini-Galletti, M., & Casu, M. (2016). Meiofaunal cryptic species challenge species delimitation: The case of the *Monocelis lineata* (Platyhelminthes: Proseriata) species complex. *Contributions to Zoology, 85*(2), 123–145.

Scarpa, F., Cosso, P., Delogu, V., Lai, T., Sanna, D., Leasi, F., Norenborg, J. L., Curini-Galletti, M., & Casu, M. (2017). Molecular support for morphology-based family-rank taxa: The contrasting cases of two families of *Proseriata* (Platyhelminthes). *Zoologica Scripta, 46*(6), 753–766.

Schmarda, L. K. (1859). Neue wirbellose Thiere beobachtet und gesammelt auf einer Reise 1853 bis 1857. Band I. *Turbellarien. Rotatorien und Anneldren. Erste Hälfte. W. Engelmann, Leipzig, 66 pp, tab 1-8.

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics, 30*, 1312–1313.

Stosic, R. R., Letsch, H., Hertel, J., Misof, B., & Stadler, P. F. (2009). Accurate and efficient reconstruction of deep phylogenies from structured RNAs. *Nucleic Acids Research, 37*, 6184–6193.

Stimpson, W. (1857). *Prodromus descriptionis animalium evertorbitum quae in Expeditione ad Oceanum Pacificum Septentriolalem, Johanne Rodgers Duce a Republica Federata missa, observavit et descriptis. Pars. I. Turbellaria Dendrocoela. Proceedings of the Academy of Natural Sciences of Philadelphia, 9*, 19–31.

Tan, G., Muffato, M., Ledergerber, C., Herrero, J., Goldman, N., Gil, M., & Desimoz, C. (2015). Current methods for automated filtering of multiple sequence alignments frequently worsen single-gene phylogenetic inference. *Systematic Biology, 64*(5), 778–791.

Tessens, B., Janssen, T., & Artois, T. (2014). Molecular phylogeny of the *Kalyptorhyncha* (Rhabdocoela, Platyhelminthes) inferred from ribosomal sequence data. *Zoologica Scripta, 43*(5), 519–530.

Tsunashima, T., Hagiya, M., Yamada, R., Koito, T., Tsuyuki, N., Izawa, S., Kosoba, K., Itoi, S., & Sugita, H. (2017). A molecular framework for the taxonomy and systematics of Japanese marine turbellarian flatworms (Platyhelminthes, Polycladida). *Aquatil Biology, 26*, 159–167.

Van Steenkiste, N., Tessens, B., Willems, W., Backeljau, T., Jondelius, U., & Artois, T. (2013). A comprehensive molecular phylogeny of Dalytyphloplanida (Platyhelminthes: Rhabdocoela) reveals multiple escapes from the marine environment and origins of symbiotic relationships. *PLoS One, 8*(3), e59917.

Verrill, A. E. (1882). Notice of the remarkable marine fauna occupying the outer banks off the southern coast of New England, No. 7, and of some additions to the fauna of Vineyard Sound. The American Journal of Science 24: 360 pages.

Verrill, A. E. (1900). Additions to the Turbellaria, Nemertina, and Annelida of the Bermudas, with revisions of some New England genera and species. *Connecticut academy of arts and sciences. Transactions of the Connecticut Academy of Arts and Sciences, 11*, 15–62.

Woodworth, W. M. (1898). Some Planarians from the Great Barrier Reef of Australia. *Museum of Comparative Zoology.*

Yer, M., & Kaburaki, T. (1918). Description of some Japanese polyclad Turbellaria. *The Journal of the College of Science, Imperial University of Tokyo, Japan, 39*, 1–54.

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.