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Phylogeny of Polycladida (Platyhelminthes) based on mtDNA data

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Abstract A phylogenetic analysis of Polycladida based on two partial mitochondrial genes (*cox1* and *16S*) is provided. The analysis includes 30 polyclad terminals that represent species from the two taxa which traditionally divide the groups Cotylea and Acotylea. Our phylogenetic analyses produced a well-supported hypothesis that confirms the monophyly of Polycladida, as well as Acotylea and Cotylea. Within Acotylea, there are two lineages not highly supported: on one hand, Leptoplanoidea (excluding *Hoploplana elisabelloi)* and one Stylochoidea member (*Pseudostylochus intermedius*) (classification sensu Faubel, 1983, 1984), and on the other hand, Stylochoidea members together with *Discocelis tigrina* and *H. elisabelloi*. The genera *Stylochus* and *Imogine* are not monophyletic. Within Cotylea, Pseudocerotidae and Euryleptidae are monophyletic, though not highly supported, while Prosthiostomidae is not. Euryleptoidea is paraphyletic. The genera *Pseudobiceros* and *Pseudoceros* are monophyletic and highly supported. Our results suggest that, within Acotylea, the prostatoid organs of *Discocelis* may have been derived from a prostatic vesicle. The genus *Hoploplana* could be included in Stylochoidea. Within Cotylea, the common ancestor of Euryleptidae and Pseudocerotidae might have been an aposematic animal with tentacles.

Keywords Cotylea · Acotylea · Systematics · Molecular phylogenetics

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Introduction

Polycladida is a group of free-living Platyhelminthes with predominantly benthic marine species. Polyclads are hermaphroditic animals with internal fertilization with direct or indirect development and a variety of life history strategies (Rawlinson 2014). Currently, there are around 800 described species (Tyler et al. 2006–2016). Even though taxonomists have profusely studied polyclads since they were firstly identified as a group (Lang 1884), there are only few reconstructions of their evolutionary history, and a stable classification is still desired.

Polycladida has been traditionally divided into two groups, Cotylea and Acotylea, based on the presence or absence of a ventral sucker of glandular nature behind the female genital pore, respectively (Lang 1884; Faubel 1984; Prudhoe 1985; Ax 1995). However, a ventral epidermal depression with adhesive function (genital sucker) has been also described for some acotylean species and although the homology of this structure with the ventral sucker of cotyleans remains unclear,

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the lack of any other apomorphy for each group has rendered their reciprocal monophyly controversial (Faubel 1984). Additionally, other morphological characters have been used, such as the pharynx shape, the presence and form of tentacles, shape of the branched intestine, direct or indirect embryonic development, or the shape and location of the genital system. However, these morphological features show great variation, and there are multiple exceptions within each lineage; hence, their utility for reconstructing the evolutionary history of the group has been debated (Faubel 1983; Ax 1995). Ax (1995) considered that this variation might be the result of multiple events of convergence during the radiation of the group. For instance, a ruffled pharynx is present in acotyleans and in some certain taxa of cotyleans, while rest of cotyleans usually show a cylindrical-shaped pharynx (Faubel 1983, 1984; Prudhoe 1985). The presence and shape of tentacles is also quite variable. Generally, when present, acotyleans have nuchal tentacles, while cotyleans pseudotentacles and marginal tentacles; but there are many exceptions (Prudhoe 1985). In addition, most species of Acotylea show direct development, while members of Cotylea show mainly an indirect development with larvae (Ax 1995; Rawlinson 2014). However, many acotyleans do have larvae and, in other cases, the embryonic development is completely unknown (Rawlinson 2014).

The classification within Acotylea and Cotylea was firstly established by Lang (1884). One century later, Faubel (1983, 1984) proposed a new classification system, in agreement with previous studies (Lang 1884; Bock 1913), and mostly based on internal morphological characters. One year later, Prudhoe (1985) proposed a different system mainly based on external anatomical features. Later on, several authors accepted the system proposed by Faubel (Cannon 1986; Tyler et al. 2006–2016), though the controversy between different

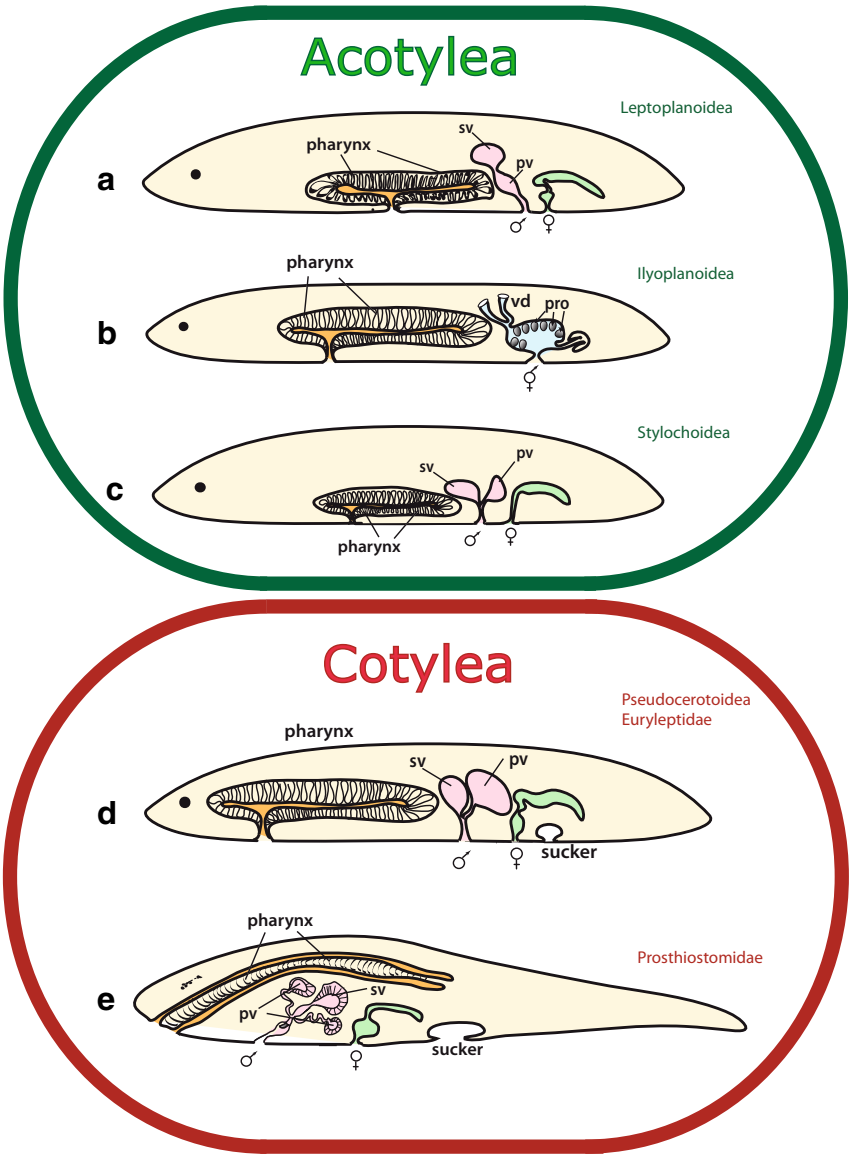

Fig. 1 Different configuration of the pharynx and the reproductive system in Cotylea and Acotylea. **a** Ruffled pharynx and interpolated prostatic vesicle [characteristic of Leptoplanoidea (Acotylea)]. **b** Ruffled pharynx and prostatoid organs [characteristic of Ilyplanoidea (Acotylea)]. **c** Ruffled pharynx and free prostatic vesicle [characteristic of Stylochoidea (Acotylea)]. **d** Ruffled pharynx, free prostatic vesicle and sucker [characteristic of Pseudocerotoidea and Eurylepidae (Cotylea)]. **e** Cylindrical pharynx, double prostatic vesicle and sucker [characteristic of Prosthiostomidae (Cotylea)]. po: prostatoid organs; pv: prostatic vesicle, sv: seminal vesicle. Note that same arrangement of pharynx and reproductive system are shared by **c** and **d**. The pharynx is anteriorly opened in Cotylea, while it opens approximately in the midbody in Acotylea. The reproductive systems are considerably increased in size

classifications is still a matter of debate (Rawlinson 2014). We will herein consider the classification proposed by Faubel (1983, 1984) with comments to the system of Prudhoe (1985).

Within Acotylea, Faubel (1983, 1984) included three superfamilies: Stylochoidea, Leptoplanoidea and Ilyplanoidea, which are defined by the presence/absence and the different location and opening of the prostatic vesicle (Fig. 1). While ilyplanoideans lack a prostatic vesicle (they have a prostatoid organ instead) (Fig. 1b), stylochoideans have a "free" prostatic vesicle where the prostatic duct joins the ejaculatory duct and together open at the base of the penis papilla (Faubel 1983, 1984) (Fig. 1c). Finally, leptoplanoideans have an interpolated prostatic vesicle, which is situated between the seminal vesicle and the penis papillae (Faubel 1983) (Fig. 1a). Within Cotylea, Faubel (1984) differentiated four superfamilies of which Pseudocerotoidea and Euryleptoidea include the overwhelming majority of the currently described species. Pseudocerotoidea was defined with a plicate ruffled pharynx and presence of marginal tentacles formed by the folding of the anterior margin called pseudotentacles, while Euryleptoidea members have mainly well-developed and prominent tentacles (with exception of Prosthiostomidae) as well as a plicate cylindrical (bell-shaped or tubular) pharynx (Faubel 1984). The taxonomy within these groups has been based on differences in the reproductive systems, alimentary system, presence of dorsal papillae, tentacles and colour patterns (Rawlinson and Litvaitis 2008).

There are few phylogenetic studies dealing with the relationships within Polycladida. In a non-cladistic study, Faubel (1984) explored the monophyly of the suborders and considered that both Acotylea and Cotylea were not monophyletic. On one hand, Ilyplanoidea (Acotylea) was proposed to be the sister group of the rest of polyclads, and, on the other hand, Opisthogenioidea (Cotylea) was more closely related to acotylean members than cotyleans. Later, very few 18S sequences of polyclad species (usually two per study) were included in phylogenetic analyses of Platyhelminthes (Campos et al. 1998; Littlewood et al. 1999; Litvaitis and Rhode 1999). Approximately 10 years later, Rawlinson et al. (2011) performed a molecular phylogenetic analysis of Polycladida based on a ~ 900 bp fragment of the nuclear 28S rRNA gene but included still only a limited number of cotylean (eight) and acotylean (six) species. This study provided evidence for the monophyly of Polycladida and Acotylea, while Cotylea was not recovered as monophyletic, since one cotylean taxon (*Pericelis*) represented the sister group of Acotylea. Rawlinson and Stella (2012) performed an analysis with the same genetic marker and terminals but included one more cotylean species. This time Cotylea was recovered as monophyletic with *Pericelis* basally branching. However, in this analysis, Acotylea was paraphyletic with *Cestoplana* as the sister group of Cotylea, though this relationship was poorly supported. The relationships within Cotylea have been studied based on morphology (Rawlinson and Litvaitis 2008), and molecular characters (nuclear *28S rRNA* gene) (Litvaitis et al. 2010). The latter study showed Pseudocerotoidea monophyletic but Euryleptoidea paraphyletic. Additionally, a phylogeny based on morphological characters was proposed for the acotylean genera *Discoplana* and *Euplana* (Doignon et al. 2003), and the nuclear *28S rRNA* gene was used to reconstruct the relationships within the cotylean family Pseudocerotidae (Litvaitis and Newman 2001). Recently, Laumer and Giribet (2014) worked with four molecular markers (*18S*, *28S*, *16S*, *cytb*) and included eight polyclads in their analyses of Plathyhelminthes. This study recovered polyclads monophyletic, and most members of Acotylea formed a clade, while *Theama* (Acotylea) was nested within Cotylea. Both taxa, Acotylea and Cotylea (including *Theama*), were therefore not supported. Egger et al. (2015), in a transcriptomic analysis of flatworms, included four polyclads and found the group, as well as Acotylea and Cotylea (represented by two taxa each) monophyletic and well-supported. Laumer et al. (2015) also worked with transcriptomes and included three polyclads, obtaining a tree in which all of them were in a well-supported clade and the two Acotylea showed a sister group relationship. More recently, Aguado et al. (2016) performed a phylogenetic analysis of Platyhelminthes based on complete mitochondrial genomes where Polycladida, Cotylea and Acotylea were recovered as monophyletic groups; however, that study only included two species of each group. Finally, Bahia et al. (2017) performed phylogenetic analyses based on a fragment of the *28S rRNA* gene of 57 polyclad species, 28 included for the first time. Their results recovered Polycladida monophyletic, though the suborders were not monophyletic. The acotyleans *Cestoplana* and *Theama* were nested within Cotylea.

In order to test the monophyly of Polycladida, but mainly the relationships within the order, we perform herein a phylogenetic analysis based on the mitochondrial *cox1* and *16S* genes. Both genes have been widely used to infer phylogenetic hypotheses for many different groups of animals; however, they have been scarcely sequenced for Polycladida. This has been probably due to the problem that available primer combinations, which work well for most animals (Folmer et al. 1994; Palumbi 1996) did not work for Polycladida. We have designed specific primers and were able to perform the first phylogenetic analysis for the group based only on mitochondrial markers. A total of 30 species that represent most of the main lineages within Cotylea and Acotylea are included; 22 of them are incorporated for the first time in a phylogenetic analysis.

Material and methods

Taxon sampling

Studied specimens were collected from several localities in the Iberian Peninsula, Ireland, Argentina and Australia (Table 1).

Polyclads from the Iberian Peninsula were collected in the north and north-western coasts (Galicia and Asturias, Atlantic Ocean), as well as in the south-eastern coast (Murcia and Granada, Mediterranean Sea). Specimens from Galicia and from the Mediterranean Sea were collected at about 5–30 m deep. Samples from Australia and Argentina were collected in the intertidal and sub-littoral regions. Specimens were first photographed, collected by hand using a brush or net and stored in plastic containers. In the laboratory, a small piece of tissue of the lateral margin of the body was separated for DNA analyses, and the rest was fixed for histological studies. The Spanish material is deposited in the Invertebrate Collection of the Museo Nacional de Ciencias Naturales (MNCN, Spain). The Argentinean material is deposited in the Museo de La Plata (MLP, La Plata, Argentina). Australian specimens are deposited in the Invertebrate Collection of the Australian Museum, Sydney (AM). Additionally, available transcriptome data and complete mitochondrial genomes available on NCBI GenBank have been mined (Table 1). As outgroups, representatives of Macrostomorpha and Prorhynchidae are included. The latter taxon has been suggested as a possible sister group of Polycladida (Egger et al. 2015; Laumer et al. 2015).

In total, 13 acotylean species and 17 cotylean species were included in our phylogenetic analyses (Table 1). Within Acotylea, representatives of the three superfamilies Stylochoidea, Leptoplanoidea and Ilyplanoidea are included. Stylochoidea and Leptoplanoidea are represented by families with the highest number of species. Within Cotylea, the two major superfamilies, Pseudocerotoidea and Euryleptoidea are included, and like within Acotylea, those families with highest number of species are represented (Table 1).

Morphological analysis

Species identification was based on histological analysis of the internal morphology. Photographs of the external morphology were taken using a stereomicroscope with a Zeiss AxioCam ICc 1 camera attached. Material for morphological studies was fixed in Bouin's solution (Romeis 1989) and later preserved in 70% ethanol. The samples were progressively dehydrated and embedded in paraplast and serially sectioned sagittally at intervals of 7 μm and stained with AZAN (trichrome staining method). Detailed morphological descriptions for most of the species used in this study can be found in Brusa and Damborenea (2011), Marquina et al. (2014a, b, 2015) and Noreña et al. (2014, 2015).

DNA extraction, amplification and sequencing

The tissue for DNA extraction was fixed in absolute ethanol. Total genomic DNA was extracted from each sample following the phenol-chloroform protocol (Sambrook et al. 1989). DNA concentration and purity of the extraction was measured using a NanoDrop Fluorospectrometer (Thermo Fisher Scientific). The protein coding genes *cox1* and *16S* of the investigated polycladid species were found contiguous in the mitochondrial genome, in many taxa with the *trnG* in the middle. The latter gene, when present was not included in the analyses. A final fragment of approximately 1700 bp including partial sequences of both genes was amplified using different sets of primers in subsequent steps (Tables 2 and 3). Firstly, sequences of approximately 200 bp of *cox1*, and 400 bp of *16S*, were amplified with degenerated primers (Table 2). The PCR consisted in an initial denaturation at 95 °C (10 min), followed by 40 cycles of denaturation at 96 °C (1 min), annealing at 50 °C (1 min) and extension at 72 °C (1 min), with a final extension of 10 min at 72 °C. Secondly, species-specific primers (Table 3) were designed from the sequences already obtained (forward primer form the *cox1* and reverse primer from the *16S* fragments) and used in subsequent PCR amplification processes. The second PCRs amplified a fragment of around 1500 bp, and consisted in an initial denaturation at 95 °C (10 min), followed by 40 cycles of denaturation at 96 °C (1 min), annealing temperatures variable for each sample (Table 3) (1 min and 30 s), extension at 72 °C (1 min), with a final extension of 10 min at 72 °C. Finally, the sequences obtained from the first and the second steps were combined manually using the program Sequencher 4.1.4 (Gene Codes Corporation). All PCRs were performed using Taq DNA polymerase following the manufacturers' protocol in a total volume of 25 μl. PCR products were visually tested and purified using ExoSAP-IT (Affymetrix), following manufacture's protocol, prior to sequencing both strands on a 3730 DNA Analyzer (Applied Biosystems).

Phylogenetic analysis

DNA sequences of both *cox1* and *16S* were edited separately with Sequencher 4.1.4 and alignments for both genes were performed separately using the program Mafft (Katoh et al. 2002) with the default parameters and the iterative refinement method E-INS-i, and default gap open and extension values. Both genes were combined into a supermatrix using FASCONCAT-G (Kück and Longo 2014). For maximum likelihood (ML) analysis a partition scheme based on four partitions (*16S*, *cox1* first codon position, *cox1* second codon position, *cox1* third codon position) was optimized using the partition finder algorithm (Lanfear et al. 2014) as implemented in IQ-TREE (Nguyen et al. 2015; Trifinopoulos et al. 2016) and best fitting models for each partition were selected by the same program (Cox1_codon1 TVM + G, (Cox1_codon2 HKY + I + G, Cox1_codon3 TN + I + G, 16S TN + G). Each partition was allowed to have its own set of branch lengths (−sp. option). ML analysis of the combined dataset was conducted with IQ-TREE, and support values were

Table 1 Species included in the present study. For each species, classification, sample locality, sample coordinates, GenBank accession number and reference with the detailed morphological description are specified

Out-group		Family	Species	Locality	Coordinates	GeneBank accession number (COI)	GeneBank accession number (16S)	Reference
Macrostomorpha		Macrostomidae	*Macrostomum lignano*[a]	Genbank	_	KP308282.1	KP308283	_
Lecithoepitheliata		Prorhynchidae	*Prorhynchus* sp.[a]	Genbank	_	KC869758.1		_
			Geocentrophora sphyrocephala[a]	Genbank	_	SRX871533	KC869762.1	_
In-group								
Polycladida	Superfamily (Faubel 1883–84)	Family	Species	Locality	Coordinates	GeneBank Accession Number		Reference
Acotylea	Ilyplanoidea	Discocelidae	*Discocelis tigrina* (Blanchard, 1847)	Calpe, Alicante (Spain)	38° 38′ 08″ N 0° 04′ 13″ E	MF371159	MF398475	_
	Leptoplanoidea	Leptoplanidae	*Hoploplana elisabelloi* Noreña, Rodríguez, Almón and Pérez, 2015[a]	Ría de Arousa, Galicia (Spain)	42° 32′ 59″ N 8° 56′ 28″ W	NC_028200		Noreña et al. 2015
			Leptoplana tremellaris (Müller OF, 1773)[a]	Genbank	_	SRX872321		_
		Stylochoplanidae	*Stylochoplana maculata* (Quatrefage, 1845)[a]	Genbank	_	KP965863		_
		Notoplanidae	*Notocomplana palta* (Marcus, 1954)	Puerto Lobos, Chubut (Argentina)	42° 00′ 04″ S 65° 04′ 15″ W	MF371142	MF398458	Brusa and Damborenea 2011
		Stylochoplanidae	*Comoplana agilis* (Lang, 1884)	Ría de Arousa, Galicia (Spain)	42° 32′ 3″ N 8° 59′ 18″ W	MF371145	MF398461	_
	Stylochoidea	Callioplanidae	*Crassiplana albatrossi* Hyman, 1955	Puerto Lobos, Chubut (Argentina)	42° 00′ 04″ S 65° 04′ 15″ W	MF371157	MF398473	Brusa and Damborenea 2011
		Stylochidae	*Imogine stellae* Marquina, Osca, Rodríguez, Fernández-Despiau and Noreña, 2014	Mar Menor, Murcia (Spain)	37° 46′ 37″ N 0° 45′ 17″ W	MF371143	MF398459	Marquina et al. 2014a
			Imogine fafai Marquina, Fernández-Álvarez and Noreña, 2014	Muros de Nalón, Asturias (Spain)	43° 33′ 36″ N 6° 6′ 18″ W	MF371138	MF398454	Marquina et al. 2014b
			Imogine cf. *pardalotus* Jennings and Newman, 1996	Lizard Island, Queensland (Australia)	14° 41′ 52″ S 145° 27′ 50″ E	MF371158	MF398474	_
			Stylochus neapolitanus (Delle Chiaje, 1841–1844)	Ría de Arousa, Galicia (Spain)	42 ° 33′ 46″ N 8° 59′ 20″ W	MF371141	MF398457	Noreña et al. 2015
			Stylochus ellipticus (Girard, 1850)[a]	Genbank	_	SRX999628		_
			Pseudostylochus intermedius Kato, 1939[a]	Genbank	_	AB049114		_
Cotylea	Euryleptoidea	Euryleptidae				MF371139	MF398455	

Table 1 (continued)

		Eurylepta cornuta (Müller OF, 1776)	Ría de Arousa, Galicia (Spain)	42° 33′ 24″ N 8° 57′ 50″ W			Noreña et al. 2014
		Eurylepta sp.	Moira Mounds (off Ireland, 1069 m depth)	51° 26′ 19″ N 11° 49′ 23″ W	MF371144	MF398460	
		Prostheceraeus vitattus (Montagu, 1815)	Ría de Arousa, Galicia (Spain)	42° 32′ 49″ N 8° 57′ 59″ W	MF371140	MF398456	Noreña et al. 2014
		Maritigrella crozieri (Hyman, 1939)[a]	Genbank	_	SRX875739		_
	Prosthiostomidae	*Enchiridium* sp.	Lizard Island, Queensland (Australia)	14° 41′ 39″ S 145° 27′ 56″ E	MF371137	MF398453	_
		Lurymare clavocapitata Marquina, Aguado and Noreña, 2015	Lizard Island, Queensland (Australia)	14° 41′ 52″ S 145° 27′ 50″ E	MF371153	MF398469	Marquina et al. 2015
		Prosthiostomum siphunculus (Delle Chiaje, 1822)[a]	Almuñecar, Granada (Spain)	36° 43′ 18″ N 3° 43′ 43″ W	NC_028201		_
		Lurymare sp.	Lizard Island, Queensland (Australia)	14° 38′ 46″ S 145° 27′ 13″ E	MF371156	MF398472	_
Pseudocerotoidea	Pseudocerotidae	*Pseudobiceros hancockanus* (Collingwood, 1876)	Lizard Island, Queensland (Australia)	14° 34′ 20″ S 145° 36′ 54″ E	MF371154	MF398470	Marquina et al. 2015
		Pseudobiceros uniarborensis Newman and Cannon, 1994	Lizard Island, Queensland (Australia)	14° 38′ 36″ S 145° 27′ 09″ E	MF371146	MF398462	Marquina et al. 2015
		Pseudoceros bimarginatus Meixner, 1907	Lizard Island, Queensland (Australia)	14° 40′ 53″ S 145° 28′ 12″ E	MF371152	MF398468	Marquina et al. 2015
		Pseudoceros jebborum Newman and Cannon, 1994	Lizard Island, Queensland (Australia)	14° 39′ 07″ S 145° 27′ 02″ E	MF371151	MF398467	Marquina et al. 2015
		Pseudoceros paralaticlavus Newman and Cannon, 1994	Lizard Island, Queensland (Australia)	14° 40′ 53″ S 145° 28′ 12″ E	MF371150	MF398466	Marquina et al. 2015
		Pseudoceros periaurantius Newman and Cannon, 1994	Lizard Island, Queensland (Australia)	14° 40′ 53″ S 145° 28′ 12″ E	MF371149	MF398465	Marquina et al. 2015
		Pseudoceros prudhoei Newman and Cannon, 1994	Lizard Island, Queensland (Australia)	14° 39′ 07″ S 145° 27′ 02″ E	MF371155	MF398471	Marquina et al. 2015
		Pseudoceros stimpsoni Newman and Cannon, 1998	Lizard Island, Queensland (Australia)	14° 39′ 07″ S 145° 27′ 02″ E	MF371147	MF398463	Marquina et al. 2015
		Pseudoceros zebra (Leuckart, 1828)	Lizard Island, Queensland (Australia)	14° 41′ 52″ S 145° 27′ 50″ E	MF371148	MF398464	Marquina et al. 2015

[a] Sequences downloaded from Genbank

estimated based on 1000 bootstrap pseudoreplicates. For Bayesian inference (BI) analyses, we conducted a new test of nucleotide models for all four partitions in IQ-TREE, including only those models which are available in MrBayes (−mset option). For BI, two independent runs of 1,000,000 generations and four chains (one cold, three heated each) each were run in MrBayes 3.2.2 (Ronquist et al. 2012) and trees were sampled every 1000 generations. Convergence of chains was diagnosed using a deviation of standard frequencies below 0.05 and of the 1001 sampled trees, 250 trees were discarded as burn-in. A majority-rule consensus tree was constructed from the remaining 751 trees to approximate posterior possibilities.

Results

The partial nucleotide sequences of the aligned mitochondrial *cox1* and *16S* genes were combined into a single matrix with 1530 positions. Results from the ML and BI analyses are highly congruent (Fig. 2 and Fig. 1 Supp. Mat., respectively). Our analyses found high support for the monophyly of Polycladida (0.92 posterior probability (pp), 99% bootstrap (B)), as well as both Acotylea and Cotylea (1 pp., 83% B and 1 pp., 84% B, respectively) (Fig. 2). Within Acotylea, there are two lineages highly supported in the BI, while lower in ML. The first one (1 pp., 77% B) comprises most of the sampled Leptoplanoidea (excluding *Hoploplana elisabelloi*) and one Stylochoidea member (*Pseudostylochus intermedius*) (classification sensu Faubel, 1983, 1984). The second one (1 pp., 54% B) includes the Stylochoidea members (*Crassiplana albatross*i and Stylochidae), *H. elisabelloi* (Leptoplanoidea) and *Discocelis tigrina* (Ilyoplanoidea) (Fig. 2). The family Stylochidae is monophyletic and well supported (96% B) (Fig. 2). Genera *Imogine* and *Stylochus* are not monophyletic, though the support values of the clades in which the species are organized are very low. Sequences of *Stylochoplana maculata* and *Leptoplana tremellaris* are nearly identical.

Within Cotylea, Prosthiostomidae is paraphyletic, while Pseudocerotidae and Euryleptidae are monophyletic, though generally poorly supported (1 pp., 49% B for Pseudocerotidae and 0.6 pp., 32% B for Euryleptidae). Euryleptoidea is paraphyletic (Fig. 2). However, the support values of Prosthiostomidae (excluding *Enchiridium* sp.), Euryleptidae and the clade comprising *Enchiridium* sp., Euryleptidae and Pseudocerotidae are generally low in the ML results. The genera *Pseudobiceros* and *Pseudoceros* are monophyletic and well supported (1 pp., 98%B and 1 pp., 99%B, respectively). The relationship of sister group between *Lurymare* sp. and *Prosthiostomum siphunculus* is not supported, resulting in slightly different topologies in the BI and ML results (*Lurymare* monophyletic in the BI, while it is not in ML).

Table 2 Degenerated primers

PLATYSCOIF2	TGGGCNCAYCAYATGTAYACNGT
PLATYSCOIR3	GCNACNACRTARTANGTRTCRTG
PLATYS16SF1	ACAACTGTTTATCAAAAACAT
PLATYS16SR1	ACGCCGGTYTTAACTCAAATCA

Discussion

According to our analyses, the monophyly of Polycladida, as well as both Acotylea and Cotylea is well supported (Fig. 2). Thereby our results are consistent with the traditional classification of Polycladida. The monophyly of both groups was firstly questioned by Faubel (1984) who considered that Ilyplanoidea (Acotylea) was sister to the rest of polyclads, and the monospecific Opisthogenioidea (Cotylea) was sister to Acotylea. Rawlinson et al. (2011) found that *Pericelis* (a Cotylea member) was the sister taxon of the acotyleans. Rawlinson and Stella (2012) found *Pericelis* closely related to the rest of cotyleans, but *Cestoplana* (Acotylea) as the sister group of Cotylea. Laumer and Giribet (2014) found the cotylean *Theama* nested within Acotylea. Due to lack of overlap in the sampled genes we could not include these data in our analysis. Finally, Bahia et al. (2017) found *Cestoplana* the sister group of Cotylea and *Theama* nested within Cotylea. Although our results support the monophyly of Acotylea and Cotylea, the inclusion of *Opisthogenia*, *Pericelis*, *Cestoplana* and *Theama* in future combined phylogenetic analyses of mitochondrial, as well as nuclear markers, would help to finally conclude about the monophyly of both groups and the members each group contains.

Within Acotylea, *Pseudostylochus intermedius*, downloaded from GenBank (Sato et al. 2001) is nested within Leptoplanoidea when it is considered a Stylochoidea member (sensu Faubel, 1983). This species may have been misidentified; though this cannot be checked since the authors (Sato et al. 2001) did not provide evidence of their identification. Another possible explanation would be that *P. intermedius* should belong to Leptoplanoidea; this is supported by the presence of an interpolated prostatic vesicle (characteristic of Leptoplanoidea) (C.N. pers. obs.) instead of the free prostatic vesicle as in members of Stylochoidea. Additionally, the GenBank sequences of *Stylochoplana maculata* (Golombek et al. 2015) are nearly identical with those of *Leptoplana tremellaris* (sequenced for this study, see Table 1). The identification of the latter has been verified based on morphological sections. Animals sampled at the same area of Golombek et al. (2015) were made available by

Table 3 Specific primers

Species	Primer	Sequence	T° annealing
Comoplana agilis	COI-44-F	GCTGGTCCTATATGAGCTACAGG	58°
	16S-44-R	CCCATTTAAACGGTGTATATTCCG	
Notocomplana palta	COI-9-F	GGACGTCCTTTATCTCAGGATAGA	58°
	16S–ESP-R	ACCTTAGTGCAGTTAAGATACCGC	
Discocelis tigrina	COI-72-F	TGGATAGCGCAGGTCCAATATGAGC	60°
	16S–ESP-R	ACCTTAGTGCAGTTAAGATACCGC	
	COI-72F-2	AGGATTGAGAGGGATGCCCCGACG	
Crassiplana albatrossi	COI-10-F	ACAAGTCCTATGTGAGCTACAGG	58°
	16S-10-R	ACCTTAGTGCAGCTAGTATACCGC	
Imogine cf. *pardolotus*	COI-65-F	ATAGCAGTTCCTACGGGAATTAAG	58°
	16S–ESP-R	ACCTTAGTGCAGTTAAGATACCGC	
Imogine stellae	COI-15-F	TAGATAATCTTGGACCCCTGTGGG	60°
	16S–ESP-R	ACCTTAGTGCAGTTAAGATACCGC	
Imogine fafai	COI-3-F	GTTGGAACGTTATGAGCTACTGGG	58°
	16S–ESP-R	ACCTTAGTGCAGTTAAGATACCGC	
Stylochus neapolitanus	COI-8-F	TAGATAATGTAGGACCTCTTTGGG	58°
	16S–ESP-R	ACCTTAGTGCAGTTAAGATACCGC	
Enchiridium sp.	COI-47-F	TGAAGTACCAGGAGCTTATGTGG	58°
	16S–ESP-R2	ACCTTCGCGCAGTTAAAATACCGC	
Lurymare clavocapitata	COI-54-F	ACGAARTCCCAGCTGCAATTTGGT	58°
	16S–ESP-R2	ACCTTCGCGCAGTTAAAATACCGC	
Lurymare sp.	COI-63-F	ACGGGAGCAACGATGGTAATTGCC	62°
	16S-63-R	TTTAGCCGTTCAAACAAGTCACCA	
Euryleptidae sp.	COI-28-F	CTACTTTACATGGTCGTCCTC	55°
	16S-28-R	GATTAGCCCTCATTAAGCCAT	
Eurylepta cornuta	COI-5-F	CGGGACTTATTTGAGGTACTCGCA	58°
	16S-7-R	ACCTTCGCGCACTTAAAATAGCGC	
Prostheeraeus vittatus	COI-7-F	CATATCATGGGGGGCCACTTCGAC	58°
	16S-7-R	ACCTTCGCGCACTTAAAATAGCGC	
Pseudobiceros uniarborensis	COI-45-F	TTGAAGTACCTGGAGCAATGTGAT	58°
	16S–ESP-R2	ACCTTCGCGCAGTTAAAATACCGC	
Pseudobiceros hancockanus	COI-56-F	TGAAGTGCCCGCGGCTATGTGATCATTAGGC	60°
	16S–ESP-R2	ACCTTCGCGCAGTTAAAATACCGC	
Pseudoceros periaurantius	COI-50-F	ATGGTCGGCCTTTGCGGGATT	60°
	16S-50-R	ACCCAAGATAATAAATTCAATAGG	
Pseudoceros prudhoei	COI-57-F	TGAAGTTCCAGCCGCTTTGTGGTCAT	57°
	16S-16-R	ACCTTCGCGCAGTTAAGATACCGC	
Pseudoceros jebborum	COI-52-F	TGAAGTTCCTGCAGCGTTGTGATCT	58°
	16S–ESP-R2	ACCTTCGCGCAGTTAAAATACCGC	
Pseudoceros bimarginatus	COI-53-F	ATGGTCGTCCATTACGAGATT	58°
	16S–ESP-R2	ACCTTCGCGCAGTTAAAATACCGC	
Pseudoceros zebra	COI46/49/51F	TGAAGTTCCTGCAGCRTTATGATCRT	58°
	16S–ESP-R2	ACCTTCGCGCAGTTAAAATACCGC	
Pseudoceros stimpsoni	COI46/49/51F	TGAAGTTCCTGCAGCRTTATGATCRT	58°
	16S-46-R	CCTCATTGAGCCATTCAAACAAG	
Pseudoceros paralaticlavus	COI46/49/51F	TGAAGTTCCTGCAGCRTTATGATCRT	58°
	16S–ESP-R2	ACCTTCGCGCAGTTAAAATACCGC	

Torsten Struck (Oslo) and were sectioned, studied and identified as *Leptoplana tremellaris*. We hence suggest that a misidentification for *S. maculata* might have also been possible.

Our results do not support the monophyly of the included superfamilies within Acotylea (sensu Faubel 1983, 1984). Most terminals of Leptoplanoidea are included in a clade, excepting *Hoploplana elisabelloi*. The classification of *Hoploplana* within Polycladida has been controversial. Faubel (1984) considered this genus to be placed within leptoplanoideans, mainly due to the presence of an

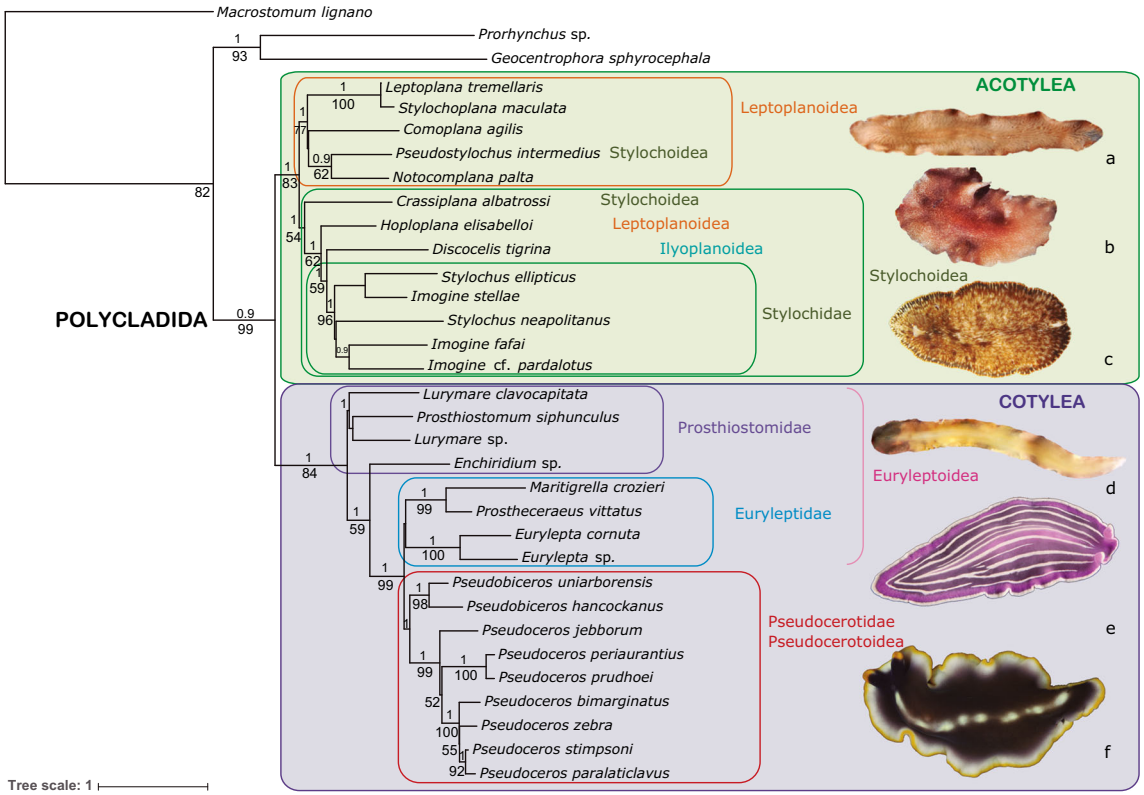

Fig. 2 Maximum likelihood tree obtained from the combined dataset (*16S* + *cox1*). Posterior probability support values (> 0.9 pp) from the Bayesian inference analysis above nodes. Bootstrap support values (> 50%B) are below nodes. The coloured boxes highlight main groups among Polycladida (sensu Faubel, 1983, 1984). Pictures from above to below: **a** *Leptoplana tremellaris*. **b** *Discocelis tigrina*. **c** *Stylochus neapolitanus*. **d** *Prosthiostomum siphunculus*. **e** *Prostheceraeus roseus*. **f** *Pseudoceros paralaticlavus*

interpolated prostatic vesicle (Fig. 1a). However, it has tentacles, as members of Stylochoidea. Prudhoe (1985) considered the genus to be part of Planoceridae and thus in the superfamily Stylochoidea. As well, Bahia et al. (2017) found that *Hoploplana* was sister to *Planocera*, nested within Stylochoidea. Additionally, Noreña et al. (2015) noticed that *Hoploplana* possess an atypical granular prostatic gland with the ejaculatory duct and the granular secretions packed at the beginning of the stylet. Our results argue for a distinction between the prostatic vesicle of leptoplanoideans and that of *Hoploplana*, and the inclusion of the latter in Stylochoidea.

The taxonomy of *Discocelis tigrina* has previously been a matter of debate. Prudhoe (1985) considered *Discocelis* as a member of Stylochoidea, while Faubel (1984) considered it within Ilyplanoidea. Additionally, Faubel (1984), as mentioned before, proposed the acotylean Ilyplanoidea to be the sister group of the remaining polyclads, because he considered that the absence of prostatic vesicle and the presence instead of prostatoid organs (Fig. 1b) might be the plesiomorphic feature for the group. However, the topology of our phylogenetic hypothesis (Fig. 2) suggests that the prostatoid organs (Fig. 1b) may have been derived from a prostatic vesicle, as that of Stylochoidea (Fig. 1c). However, the clade containing Stylochoidea, *Discocelis* and *Hoploplana* is not well supported. Future phylogenetic analyses including more markers and terminals representing Ilyoplanoidea are needed to resolve the relationships of this group with Stylochoidea. Within the family Stylochidae, *Imogine* and *Stylochus* were raised from subgenera of *Stylochus* to genus level by Jennings and Newman (1996). Bahia et al. (2017) found *Imogine* paraphyletic. Our phylogenetic analysis shows that both genera are not monophyletic, though the support values within Stylochidae are generally low (Fig. 2).

Within Cotylea, Pseudocerotoidea and Euryleptoidea include the largest number of species. Pseudocerotoidea is characterized by the presence of a plicate ruffled pharynx and pseudotentacles, while Euryleptoidea is defined with a plicate cylindrical pharynx and prominent marginal tentacles (Faubel 1984). However, phylogenetic analyses based on morphological and molecular characters do not support the monophyly of one or both taxa (Rawlinson and Litvaitis 2008; Litvaitis et al. 2010; Rawlinson et al. 2011; Rawlinson and Stella 2012; Bahia et al. 2017). Our study, including members of both groups, does not support the monophyly of Euryleptoidea (Fig. 2). Our phylogenetic analysis recovered two main clades within Cotylea. The first one included *Prosthiostomum* and *Lurymare* species, members of Prosthiostomidae (Fig. 2). All these taxa share the presence of a cylindrical pharynx,

the duplication of the prostatic vesicle (Fig. 1e), and the absence of tentacles. The second clade included *Enchiridium* sp. (Prosthiostomidae) as the sister group of a clade with members of Euryleptidae and Pseudocerotidae, both monophyletic though poorly supported, mainly in the ML results. Pseudocerotidae belongs to the superfamily Pseudocerotoidea, while Euryleptidae belongs together with Prosthiostomidae to Euryleptoidea. These results suggest that Euryleptoidea and Prosthiostomidae are not monophyletic, though the clades in which they are organized are poorly supported. Prosthiostomidae is a group with species that are mostly not aposematic and lack tentacles. In contrast, Euryleptidae and Pseudocerotidae both have, in general, aposematic colours. Additionally, Pseudocerotidae members share the presence of pseudotentacles, which are folds of the frontal body edge, and most euryleptidae have "true" tentacles, a different feature to the body edge (Faubel 1984; Newman and Cannon 1994). Our results suggest that the ancestor of Euryleptidae and Pseudocerotidae might have been an aposematic animal with tentacles.

Additionally, the evolution of different developmental strategies within polyclads is another interesting topic to investigate. Within polyclads there are many taxa that show direct development (mainly acotyleans), but different larvae can also be found. The polyclad larvae have been divided into two types, Götte and Müller, depending on the number of lobes, though this division is considered inconsistent with the phylogeny (Rawlinson 2014). Unfortunately, the number of terminals included, as well as the available information regarding polyclads development was very limited. Our phylogenetic analysis, though including a larger number of polyclad species than most previous studies, is still far from being enough to trace the evolution of developmental strategies of these animals. Among the 30 included species, the development is known only for 10. Those are: *Leptoplana tremellaris*, *Discocelis tigrina*, *Stylochus neapolitanus*, *Stylochoplana maculata* and *Pseudostylochus intermedius* with direct development (Gammoudi et al. 2012; Remane 1929; Teshirogi et al. 1981); *Stylochus ellipticus* with indirect development and Götte larva (Lang 1884; Girard 1854; Rawlinson 2014; Allen 2017); *Prosthiostomum siphunculus*, *Prostheceraeus vittatus*, *Eurylepta cornuta* and *Maritigrella crozieri* (Lang 1884; Newman et al. 2000; Rawlinson 2010; Lapraz et al. 2013) with indirect development and Müller larva. The species *Maritigrella crozieri* has been recently proposed as a model for evo-devo studies (Lapraz et al. 2013). More efforts in the investigation of developmental strategies, since the mode of development in only known for 8% of the described species (Rawlinson 2014), as well as more terminals and markers are still needed to be included in phylogenetic in order to perform a more clear hypothesis about the evolution of this group of organisms.

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