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A molecular framework for the taxonomy and systematics of Japanese marine turbellarian flatworms (Platyhelminthes, Polycladida)

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ABSTRACT: The order Polycladida comprises a highly diverse and cosmopolitan group of marine turbellarian flatworms. Owing to the great morphological diversity and the absence of a molecular phylogeny, the classification of this group has always been controversial. Here we seek to add resolution by reporting the results of molecular phylogenetic analysis based on sequences of the 28S ribosomal RNA gene, thus providing a framework for understanding relationships and the evolution of characters within the group. The phylogeny provides strong support for 10 and 7 distinct families within the suborders Acotylea and Cotylea, respectively. In addition, an analysis based on the mitochondrial gene sequences (cytochrome *c*-oxidase subunit I) reveals further details of the relationships within Acotylea, which were classified by morphological analysis, but not by 28S rRNA sequence-based analyses. These analyses also showed that several species corresponded to previously described genera based on morphological features and character combinations. We conclude that a classification of genera in Acotylea and Cotylea based on molecular phylogeny reflects the morphological diversity of these polyclad flatworms.

KEY WORDS: Flatworms · Molecular systematics · Morphology · Polycladida · Taxonomy

INTRODUCTION

Polyclad turbellarian flatworms commonly occur in coastal and rocky reefs worldwide (Hyman 1951), and are a significant presence in marine ecosystems. For example, *Stylochus* spp. are known predators of commercially important oysters (Arakawa 1970, Galleni 1976, Newman et al. 1993) and the mussel *Mytilus galloprovincialis* (Galleni et al. 1980). In addition, several planocerid species accumulate high concentrations of tetrodotoxin in their eggs—as a possible defense against predators—and in the pharynx, where it has been shown to aid in capturing mobile prey (Miyazawa et al. 1986, 1987, Tanu et al. 2004, Ritson-Williams et al. 2006, Yamada et al. 2017). There have also been several reports of dogs in New Zealand having been affected after feeding on the opisthobranch *Pleurobranchaea maculata* which had washed up on the beach after having been toxified by the polyclad *Stylochoplana* sp. (McNabb et al. 2010, Wood et al. 2012, Salvitti et al. 2015).

Polyclad flatworms are acoelomate bilaterians, usually hermaphroditic, oviparous with densely ciliated epithelial cells, and are characterized by a highly branched intestine (Rieger et al. 1991). They also lack a circulatory system or specific respiratory

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organs, relying on the epidermis for gas exchange (Rieger et al. 1991). Polycladida contains 2 suborders, Acotylea and Cotylea, which are distinguished by the absence or presence of a ventral sucker, respectively (Lang 1884). Further classification below the suborder level has traditionally been based on morphological characteristics, such as the presence and arrangement of eyespots (e.g. cerebral, tentacular, clustered, and marginal eyespots), the presence of either true tentacles or pseudotentacles (i.e. mere folds of the anterior body margin), and the structure of the pharynx (Newman & Cannon 1994).

Several authors have maintained that color patterns, particularly in Cotylea, represent valid systematic characteristics, useful for classification, because many species in this suborder are conspicuously colored and exhibit striking color patterns (Hyman 1954, Prudhoe 1944, 1989, Newman & Cannon 1995). In contrast, Faubel (1983, 1984) believes that unequivocal species classification is best done, especially in acotyleans, based on the characteristics of the male reproductive organ, specifically the structure of the prostatic vesicle and its orientation and relationship to the ejaculatory duct, on serial sections of preserved specimens. Thus, 2 morphology-based classification systems are currently being used to understand polyclad taxonomy (Faubel 1983, 1984, Prudhoe 1985).

The existence of 2 classification methods is due to the almost exclusive reliance on morphological traits rather than molecular data for the classification of polyclad flatworms, except for a few studies (Katayama et al. 1996, Litvaitis & Newman 2001, Rawlinson et al. 2011, Vanhove et al. 2013). Consequently, molecular data for polyclad flatworms are sparse in DNA databases worldwide: only 312 and 59 nucleotide sequences of Acotylea and Cotylea, respectively, were available in the GenBank, EMBL, and DDBJ databases, including gene sequences and expressed sequence tags (as of 5 September 2016).

To remedy this situation, we collected specimens of various marine polyclad species from the coastal areas of the Japanese Islands and conducted phylogenetic analyses based on nuclear gene sequences after having first used morphological criteria to classify the specimens. Additional phylogenetic analyses based on mitochondrial gene sequences were carried out to classify polyclad flatworms in Acotylea. Subsequently, the phylogenetic classification was compared with the morphological character-based classification.

MATERIALS AND METHODS

Animals

Polyclad flatworms were collected from inshore reefs at Hayama (35° 15′ N, 139° 34′ E) and Manazuru, Kanagawa (35° 8′ N, 139° 9′ E), and Shimoda, Shizuoka (Ebisu Island: 34° 39′ N, 138° 57′ E; Tsumekizaki: 34° 39′ N, 138° 59′ E), Japan (from 2 locations at Table S1 in the Supplement at www.int-res.com/articles/suppl/b026p159_supp.pdf). These areas harbor a variety of flatworm species found in the Japanese Islands (Kato 1944). Each specimen was photographed and its external features recorded (see 'Morphological analysis'), after which a small portion of the body tissue was excised for nucleotide sequencing. The rest of the specimen was fixed in Bouin's fluid (saturated picric acid, formaldehyde, and glacial acetic acid in a 15:5:1 ratio) for histological examination.

Morphological analysis

External features and reproductive organ structure were characterized following Kato (1944) and Hagiya (1992). Briefly, the fixed flatworms were removed from Bouin's fluid and transferred to 70% ethanol for preservation. They were then embedded in paraffin and subsequently serially sectioned (10 µm thickness) and stained with Delafield's hematoxylin and eosin. Slides were observed under a microscope to determine reproductive organ structure following the criteria of Kato (1944).

DNA extraction and PCR amplification

Total genomic DNA was extracted from the excised tissue sample for each specimen using the method of Sezaki et al. (1999) with some modifications. Briefly, proteinase K-treated samples were subjected to phenol-chloroform extraction with MaXtract High Density (Qiagen) following the manufacturer's protocol. Partial fragments of the 28S rRNA gene (approx. 1100 bp), including the D1–D2 region, were amplified by PCR using the universal primers HRNT-F2 (5′-AGTTC AAGAG TACGT GAAAC C-3′) and HRNT-R2 (5′-AACAC CTTTT GTGGT ATCTG ATGA-3′), which were designed based on the 28S rRNA gene sequence of the polyclad worm *Notocomplana humilis*, which in turn had been determined using a primer set for worms of the order Tricladida

(Alvarez-Presas et al. 2008). Partial fragments of the mitochondrial gene (approx. 750 bp), cytochrome *c*-oxidase subunit I (COI), were amplified by PCR using the universal primers HRpra2 (5′-AATAA GTATC ATGTA RACTD ATRTC T-3′) and HRprb2-2 (5′-GDGGV TTTGG DAATT GAYTA ATACC TT-3′), which were designed based on the COI gene sequences from the polyclad worms *N. humilis*, *Notoplana delicata* and *Pseudostylochus obscurus*, which again, in turn had been designed using a primer set for Tricladida (Alvarez-Presas et al. 2008). The reaction mixture for PCR amplification contained genomic DNA as a template, 0.625 units of TaKaRa ExTaq DNA polymerase (Takara Bio), 2 µl of 10× ExTaq DNA polymerase buffer (Takara Bio), 2.6 µl of 10 µM primers, 1.6 µl of 2.5 mM dNTP, and sufficient sterile water to bring the total volume up to 20 µl. The PCR was done with an initial denaturation at 95°C for 1 min followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min.

Sequencing and phylogenetic analysis

Both strands of the PCR products were directly sequenced with a 3130*xl* Genetic Analyzer (Applied Biosystems) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). 28S rRNA gene sequences for the following species were obtained from DDBJ, EMBL, or GenBank: *Notoplana australis* (accession number HQ659015), *Melloplana ferruginea* (HQ659014), *Echinoplana celerrima* (HQ659020), *Idioplana australiensis* (HQ659008), *Stylochus oculiferus* (HQ659007), *Stylochus zebra* (AF342800), *Paraplanocera oligoglena* (KC869849), *Chromoplana* sp. (KC869847), *Amakusaplana acroporae* (HQ659010, JQ791553), *Prosthiostomum siphunculus* (HQ659012), *Pericelis cata* (EU679114), *Pericelis orbicularis* (EU679116), *Boninia divae* (KC869846), *Chromyella* sp. (KC869848), *Maritigrella newmanae* (EF514799), *Maritigrella crozieri* (HQ659013), *Maritigrella fuscopunctata* (KU674837), *Pseudoceros bicolor* (GQ398097), *Pseudoceros bicolor marcusorum* (GQ398100), *Pseudoceros rawlinsonae* (GQ398102), *Pseudoceros harrisi* (EF514802), *Pseudobiceros splendidus* (HQ659016), *Pseudobiceros caribbensis* (EF514806), *Pseudobiceros pardalis* (EF514807), *Thysanozoon brocchii* (HQ659017), *Thysanozoon raphaeli* (EF514810), and *Yungia* sp. (HQ659018). The COI nucleotide sequence was obtained for *Pseudostylochus intermedius* (AB049114). *Paratoplana renatae* (28S rRNA: AJ270176) and *Dugesia japonica* (COI: AB618487) were used as the respective outgroup species for the 28S and COI trees.

The nucleotide sequences of the partial 28S rRNA and COI genes for all the flatworms were aligned using the program MUSCLE in MEGA version 6.0.6 (Tamura et al. 2013). After alignment, 28S sequences with poorly aligned regions were trimmed using trimAL, with the following parameters: minimum conservation threshold of 60%, gap threshold of 80%, or a similarity score lower than 0.001 (Capella-Gutiérrez et al. 2009).

The maximum likelihood method was used to determine the phylogeny, with the GTR+G model (as determined in MEGA) with 1000 bootstrap replications using MEGA ver. 6.0.6 (Tamura et al. 2013). The bootstrap consensus tree with the highest log likelihood inferred from 1000 replicates was generated.

RESULTS

Classification by morphological characters

In total, 84 specimens were classified using morphological features into the genera *Pseudoceros*, *Thysanozoon*, *Prosthiostomum*, and *Cycloporus* in the suborder Cotylea (Fig. 1), and the genera *Notoplana*, *Notocomplana*, *Pseudostylochus*, *Amemiyaia*, *Discoplana*, *Callioplana*, *Planocera*, *Leptostylochus*, and *Hoploplana* in the suborder Acotylea (Fig. 2).

They were further identified at the species level under each suborder using external morphological traits, features of the sexual organs, or both, with one exception (Table 1). As Table 1 shows, 4 specimens were identified at the genus level (*Notocomplana*) but could not be further classified into species. While the external characters of the unidentified *Notocomplana* specimens matched those of *N. japonica* or *N. koreana*, the reproductive organs matched those of *N. humilis*.

Comparison of phylogenetic relationships and their match with morphological character-based classification

Application of the maximum likelihood method to the 28S rRNA data resulted in a clear separation of cotylean and acotylean genera, with high bootstrap support values (Fig. 3). The genera *Notoplana*, *Pseudostylochus*, *Notocomplana*, *Amemiyaia*, *Discoplana*, *Paraplanocera*, *Stylochus*, *Hoploplana*, *Leptostylochus*, *Planocera*, and *Callioplana* collected in

Table 1. Summary of morphological classification of polyclad flatworms used in this study. COI = cytochrome *c*-oxidase subunit I

Suborder/family	Genus	Species	Collection site	No. of ind.	Accession number	
					28S rRNA	COI
Acotylea						
Leptoplanidae	*Hoploplana*	*villosa*	Shimoda, Shizuoka (Ebisu Island)	2	LC100076	–
Stylochoplanidae	*Amemiyaia*	*pacifica*	Shimoda, Shizuoka (Ebisu Island)	1	LC100077	–
Stylochidae	*Leptostylochus*	*gracilis*	Shimoda, Shizuoka (Ebisu Island)	1	LC100078	–
	Stylochus	*ijimai*	Hayama, Kanagawa	1	LC100079	–
Ilyplanidae	*Discoplana*	*gigas*	Hayama, Kanagawa	1	LC100080	LC190985
Planoceridae	*Planocera*	*multitentaculata*	Hayama, Kanagawa	3	LC100081	LC190986 LC190987
Callioplanidae	*Callioplana*	*marginata*	Shimoda, Shizuoka (Tsumekizaki); Hayama, Kanagawa	2	LC100082	–
Pseudostylochidae	*Pseudostylochus*	*obscurus*	Shimoda, Shizuoka (Ebisu Island and Tsumekizaki); Hayama and Manazuru, Kanagawa	11	LC100084	LC190983 LC190984
	Pseudostylochus	*elongatus*	Hayama, Kanagawa	1	LC100083	–
Notoplanidae	*Notoplana*	*delicata*	Shimoda, Shizuoka (Ebisu Island and Tsumekizaki); Hayama, Kanagawa	4	LC100088	LC190982
	Notocomplana	*humilis*	Shimoda, Shizuoka (Ebisu Island and Tsumekizaki); Hayama, Kanagawa	23	LC100085	LC190978
	Notocomplana	*koreana*	Shimoda, Shizuoka (Ebisu Island)	2	LC100086	LC190980
	Notocomplana	*japonica*	Shimoda, Shizuoka (Tsumekizaki)	2	LC100087	LC190979
	Notocomplana	sp.	Shimoda, Shizuoka (Tsumekizaki)	4	LC100089	LC190981
Cotylea						
Prosthiostomidae	*Prosthiostomum*	*grande*	Shimoda, Shizuoka (Ebisu Island); Hayama, Kanagawa	5	LC100090	–
		vulgaris	Shimoda, Shizuoka (Tsumekizaki)	2	LC100091	–
Pseudocerotidae	*Cycloporus*	*japonicus*	Shimoda, Shizuoka (Tsumekizaki)	1	LC100092	–
	Thysanozoon	*brocchii*	Shimoda, Shizuoka (Ebisu Island and Tsume kizaki); Hayama, Kanagawa	4	LC100093	–
	Thysanozoon	*japonicum*	Shimoda, Shizuoka (Tsumekizaki)	1	LC100094	–
	Pseudoceros	*velutinus*	Manazuru, Kanagawa	2	LC100095	–
	Pseudoceros	*nipponicus*	Manazuru, Kanagawa	2	LC100096	–
	Pseudobiceros	*nigromarginatus*	Shimoda, Shizuoka (Ebisu Island and Tsumekizaki); Manazuru, Kanagawa	3	LC100097	–
	Pseudoceros	*atropurpureus*	Shimoda, Shizuoka (Tsumekizaki); Hayama, Kanagawa	4	LC100098	–
	Pseudobiceros	*flavomarginatus*	Hayama, Kanagawa	1	LC100099	–
	Pseudobiceros	*hancockanus*	Shimoda, Shizuoka (Tsumekizaki)	1	LC100100	–

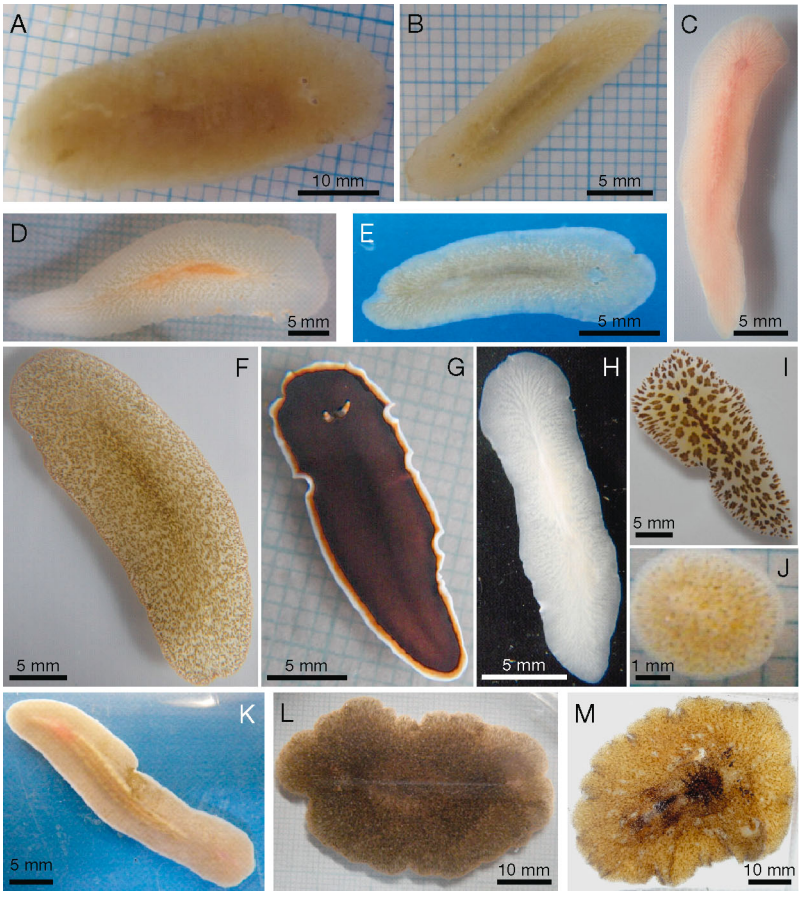

Fig. 1. Wholemounts of polyclad flatworms in the suborder Acotylea. (A) *Notocomplana humilis*; (B) *Notocomplana koreana*; (C) *Notocomplana japonica*; (D) *Notoplana delicata*; (E) *Notocomplana* sp.; (F) *Pseudostylochus obscurus*; (G) *Callioplana marginata*; (H) *Amemiyaia pacifica*; (I) *Discoplana gigas*; (J) *Hoploplana villosa*; (K) *Leptostylochus gracilis*; (L) *Stylochus ijimai*; (M) *Planocera multitentaculata*

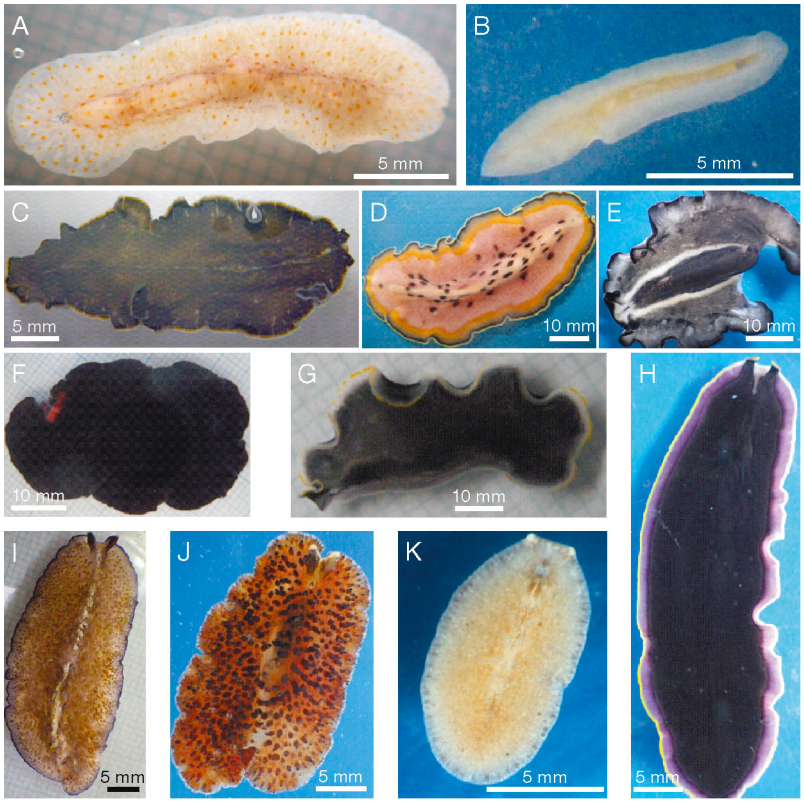

Fig. 2. Wholemounts of polyclad flatworms in the suborder Cotylea. (A) *Prosthiostomum grande*; (B) *Prosthiostomum vulgaris*; (C) *Pseudoceros velutinus*; (D) *Pseudoceros nipponicus*; (E) *Pseudobiceros nigromarginatus*; (F) *Pseudoceros atropurpureus*; (G) *Pseudobiceros flavomarginatus*; (H) *Pseudobiceros hancockanus*; (I) *Thysanozoon brocchii*; (J) *Thysanozoon japonicum*; (K) *Cyclo porus japonicus*

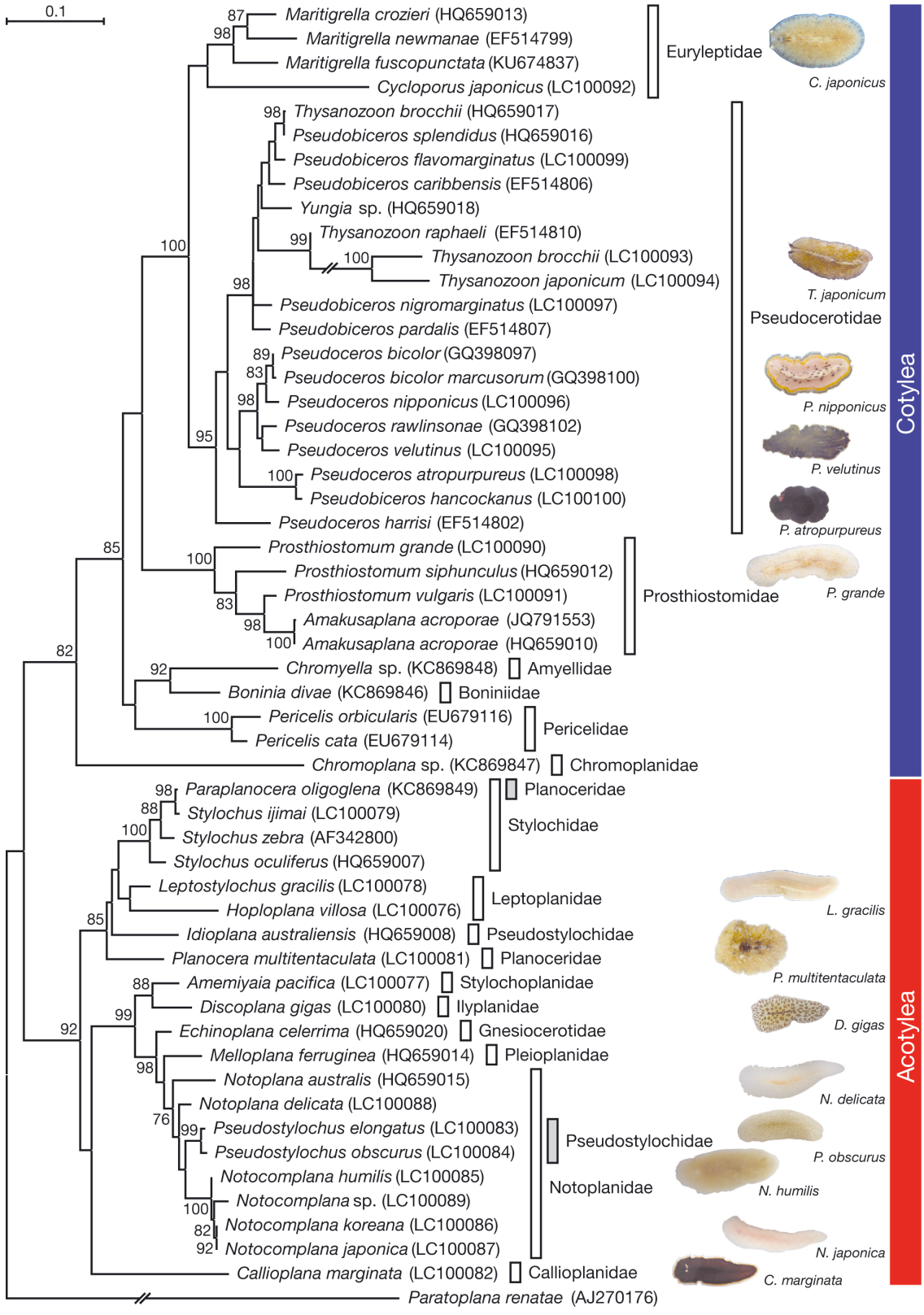

Fig. 3. Maximum likelihood phylogenetic tree of polyclad flatworms based on the partial nucleotide sequences of the 28S rRNA gene. Numbers above the branches denote the bootstrap percentages from 1000 replicates. The accession numbers for the sequences are shown in parentheses. The accession numbers LC100076–LC100100 refer to those deposited in the DDBJ, EMBL, and GenBank databases used in this study. *Paratoplana renatae* (AJ270176) was used as the outgroup in the analysis. Only bootstrap probabilities >70% are represented. The scale at the top of the tree refers to nucleotide substitutions per site

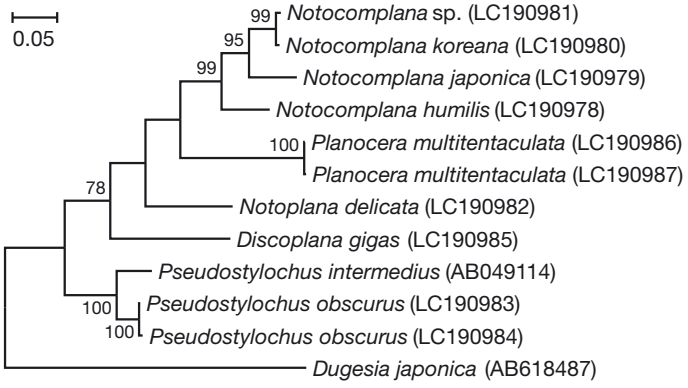

Fig. 4. Maximum likelihood phylogenetic tree of polyclad flatworms (suborder Acotylea) based on the partial nucleotide sequences of the COI gene. Numbers above the branches denote the bootstrap percentages from 1000 replicates. The accession numbers for the sequences are shown in parentheses. The accession numbers for LC190978–LC190987 refer to those deposited in the DDBJ, EMBL, and GenBank databases used in this study. *Dugesia japonica* (AB618487) was used as the outgroup in the analysis. Only bootstrap probability >70% are represented. The scale at the to of the tree refers to nucleotide substitutions per site

this study formed a clade with acotylean species, including *Melloplana ferruginea* (HQ659020), *Echinoplana celerrima* (HQ659020), and *Idioplana australiensis* (HQ659008), whereas *Pseudoceros*, *Pseudobiceros*, *Thysanozoon*, *Cycloporus*, and *Prosthiostomum* formed a clade with cotylean species, including *Maritigrella* spp. (HQ659013, EF514799), *Amakusaplana acroporae* (JQ791553, HQ659010), *Chromyella* sp. (KC869848), *Boninia divae* (KC869846), *Pericelis* spp. (EU679114, EU679116), *Yungia* sp. (HQ659018), and *Chromoplana* sp. (KC869847).

The cotylean genera *Pseudobiceros*, *Thysanozoon* and *Yungia* formed a clade separate from the genus Pseudoceros, in the family *Pseudocerotidae* (Fig. 3). The only exception is the species *Pseudobiceros hancockanus*, which grouped with *Pseudoceros atropurpureus*. The genera *Cycloporus* and *Maritigrella* (Euryleptidae), *Amakusaplana* and *Prosthiostomum* (Prosthiostomidae), *Chromyella* (Amyellidae), *Boninia* (Boniniidae), *Pericelis* (Pericelidae), and *Chromoplana* (Chromoplanidae) formed separate clades. The Prosthiostomidae clade included the genera *Amakusaplana* and *Prosthiostomum*. The *Prosthiostomum* specimens collected in this study clearly separated into 2 species, *P. vulgaris* and *P. grande*, as in the morphology-based classification.

In the part of the tree contained by acotylean taxa, the genera *Callioplana*, *Leptostylochus*, *Hoploplana*, *Idioplana*, *Planocera*, *Amemiyaia*, *Discoplana*, *Echinoplana*, and *Melloplana* formed separate clades, whereas the family Stylochidae clade included the genera *Stylochus* and *Paraplanocera*, and the family Notoplanidae clade included the genera *Notoplana*, *Notocomplana*, and *Pseudostylochus* (Fig. 3). That combined cluster formed a clade with the cluster that included the unclassified specimens (n = 4) in the genus *Notocomplana* based on the morphology (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/b026p159_supp.pdf). The genus *Notocomplana* was studied in greater detail by means of phylogenetic analysis based on the COI sequence, which revealed that *N. japonica* and *N. koreana* formed separate clusters, and *Notocomplana* sp. grouped with *N. koreana* (Fig. 4).

DISCUSSION

Polyclad flatworms have been traditionally classified using phenotypic data of external body traits and the reproductive organ (Kato 1944, Faubel 1983, 1984, Prudhoe 1985), although there are a few reports of classifications based on molecular data (Katayama et al. 1996, Litvaitis & Newman 2001, Rawlinson et al. 2011). One possible reason for the dearth of molecular studies is the difficulty of designing universal primers for polyclad worms due to their extreme sequence diversity. Although both mitochondrial and nuclear markers have been utilized to classify and identify various eukaryotic species (Woese 2000, Hebert et al. 2003, Eickbush & Eickbush 2007), it is generally expected that mitochondrial genes and regions, such as the COI gene, provide better resolution at the species level in phylogenies because neutral mutations reach fixation faster in the mitochondria (Hebert et al. 2003). In contrast, nuclear markers, such as rRNA genes and their spacer regions, are useful for resolving phylogenies at various taxonomic levels: the phylum or class level (e.g. 18S rRNA gene; Nielsen 2003), at the species level (e.g. 26S and 28S rRNA genes; Kurtzman & Robnett 1998, Fell et al. 2000, Sonnenberg et al. 2007) and within species (e.g. internal transcribed spacers, intergenic spacers; Álvarez & Wendel 2003).

In this study, since our attempts to amplify a mitochondrial DNA encoded-gene (COI) using reported universal primers (Vanhove et al. 2013) failed to amplify all the polyclad samples (especially cotylean taxa), we designed new primers to amplify the partial 28S rRNA nuclear gene sequence including the D1–D2 region and applied them to our samples, resulting in DNA fragments that were successfully amplified in all the samples with the predicted size.

Phylogenetic analysis supported the results derived from the morphology-based classification at the genus level. However, it raised several questions on the validity of the classification of the cotylean genera *Thysanozoon*, *Pseudoceros*, and *Pseudobiceros*, and of the acotylean genera *Notoplana*, *Pseudostylochus*, and *Notocomplana*. These results suggest that it might be necessary to revise the criteria used for classifying polyclads based on morphology.

We show that a combination of the molecular phylogenetic analyses based on the sequences from nuclear and mitochondrial markers results in a better classification of polyclads. Relying only on morphology, we classified cotylean specimens assigned to the genera *Pseudoceros*, *Pseudobiceros*, and *Thysanozoon*. In the molecular phylogenetic tree, however, *Pseudobiceros nigromarginatus* and *P. flavomarginatus* formed a clade with *P. splendidus* (HQ659016), *P. caribbeansis* (EF514806), and *P. pardalis* (EF514807) in the database, and group with the genera *Thysanozoon* and *Yungia*. Faubel (1983) separated the genus *Pseudobiceros* from the genus *Pseudoceros* based on the difference in the structure of the male copulatory apparatus. However, our phylogenetic analysis included *Pseudobiceros hancockanus* in the *Pseudoceros* clade rather than grouping it with *Pseudobiceros*, thus corroborating the phylogenetic analysis of Rawlinson et al. (2011), which did not support the monophyly of *Pseudoceros* and *Pseudobiceros* and *Thysanozoon* either (Fig. 3). These results suggest that a future revision of the classification criteria in their morphological traits for *Pseudoceros*, *Pseudobiceros*, *Thysanozoon*, and *Yungia* will be necessary.

In acotylean taxa, the genera *Pseudostylochus* and *Notocomplana* group with *Notoplana* being an outgroup. Faubel (1984) proposed that the genera *Notocomplana* and *Notoplana* are classified, respectively, by the absence or presence of a penis stylet. Although our results support this proposal, the resolution of our tree based on 28S rRNA gene sequences was insufficient to classify the specimens at the species level within the genus *Notocomplana*, which were classified by morphological analysis as *Notocomplana japonica* and *Notocomplana koreana* (Fig. 3; Kato 1937a,b). These 2 species also grouped with *Notocomplana* sp., which could not be classified into species based on morphological characters because they were sexually immature. The discrepancy between a phylogenetic topology based on morphology and one based on the 28S rRNA gene were resolved by a phylogeny based on the COI genes encoded by mitochondrial DNA, suggesting that the molecular phylogenetic analyses using 28S rRNA gene and mitochondrial gene sequences would be a powerful tool for the resolution of the polyclad classifications at the levels of genus and species, respectively.

In contrast, the genus *Pseudostylochus* was included in the Notoplanidae cluster of the tree based on the 28S rRNA gene sequence, but they have been classified into Callioplanidae based on the 18S rRNA gene sequence (Katayama et al. 1993). A similar discrepancy was observed in the families Stylochidae and Planoceridae: although the genus *Paraplanocera* has been included in the family Planoceridae (Laumer & Giribet 2014), our molecular analysis showed that *Paraplanocera oligoglena* grouped with the genus *Stylochus* (Stylochidae). These discrepancies need further investigations with more data.

In conclusion, our results showed that the classification of polyclads based on the 28S rRNA gene was approximately consistent with the morphological classification previously reported by Faubel (1983, 1984) and Kato (1944). We also show that the COI gene helped to resolve the phylogenetic relationships within Acotylea, which could not be resolved in the phylogeny based on 28S rRNA alone. The sequences of the 28S rRNA and COI genes, as used in this study to construct a phylogeny, would be a valuable tool in the classification of polyclads.

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