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Base-substitution rates of nuclear and mitochondrial genes for polyclad flatworms

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Abstract

The increase in the use of molecular methodologies in systematics has driven the necessity for a comprehensive understanding of the limitations of different genetic markers. Not every marker is optimal for all species, which has led to multiple approaches in the study of the taxonomy and phylogeny of polyclad flatworms. The present study evaluates base-substitution rates of nuclear ribosomal (*18S rDNA* and *28S rDNA*), mitochondrial ribosomal (*16S rDNA*), and protein-codifying (*cytb*, *cox1*) markers for this taxonomic group, with the main objective of assessing the robustness of these different markers for phylogenetic studies. Mutation rates and Ti/Tv ratios of the other markers were assessed for the first time. We estimated substitution rates and found *cytb* to be the most variable, while *18S rDNA* was the least variable among them. On the other hand, the transition to transversion (Ti/Tv) ratio of the different genes revealed differences between the markers, with a higher number of transitions in the nuclear gene *28S* and a higher number of transversions in the mitochondrial genes. Lastly, we identified that the third codon position of the studied protein-codifying genes was highly variable and that this position was saturated in the *cox1* marker but not in *cytb*. We conclude that it is important to assess the markers employed for different phylogenetic levels for future studies, particularly in the order Polycladida. We encourage the use of mitochondrial genes *cytb* and *16S* for phylogenetic studies at suborder, superfamily, and family levels and species delimitation in polyclads, in addition to the well-known *28S* and *cox1*.

Key Words

Acotylea, codon, Cotylea, entropy, flatworm, molecular, purines, pyrimidines, saturation

Introduction

Fast and reliable DNA sequencing has become a routinely used methodology in the description and barcoding of new species. In particular, a fragment of the mitochondrial gene cytochrome oxidase *c* subunit 1 (*cox1*) has become the most frequently used marker for molecular identification-based DNA barcoding (Hebert et al. 2003) in the majority of species across all taxa, incentivised by the Barcode of Life initiative (www.barcodeoflife.org) (Ratnasingham and Hebert 2007). Recent studies show, however, that genome-wide nucleotide substitution patterns in coding sequences have species-specific features and are variable among evolutionary lineages (Zou and Zhang 2021), leading to the question of the ubiquity of their use of particular nuclear and mitochondrial genes for systematics.

To address this issue, we investigated transition bias, which involves analysing the frequency and nature of nucleotide changes between purines and pyrimidines

across species genomes. This information is crucial for understanding the behaviour of different markers commonly employed in phylogenetic studies. Nucleotide changes between purines (adenine, A, and guanine, G) and pyrimidines (cytosine, C, and thymine, T) are known as transitions, whereas changes between a purine and a pyrimidine are coined transversions. Due to the disparity in the number of types of each possible nucleotide change (four types of transitions compared to eight types of transversions), the expected number of transitions relative to that of transversions (Ti/Tv ratio) would be 0.5 in DNA sequence evolution, assuming all types of nucleotide changes had equal rates of occurrence. However, Ti/Tv often exceeds 0.5 or even 1, a phenomenon known as transition bias (Nei and Kumar 2000; Yang 2006). Ti/Tv bias is commonly considered for estimating nucleotide substitution rates, inferring molecular phylogenies, and testing for natural selection (Kimura 1980; Tamura and Nei 1993; Yang et al. 1998) and has been extensively studied in model organisms such as the yeast *Saccharomyces cerevisiae* (Liu and Zhang 2019), the common fruit fly *Drosophila melanogaster* (Schrider et al. 2013), the flowering plant *Arabidopsis thaliana* (Ossowski et al. 2010), and the nematode *Caenorhabditis elegans* (Denver et al. 2009). These studies suggest that transitions are less deleterious and less likely to be purged by natural selection than transversions, which could be a reason why transitions are more commonly found. Furthermore, studies on genome error correction show that, due to the structure of the genetic code, transversions often lead to non-synonymous mutations compared to transitions, which usually lead to synonymous mutations, thereby potentially affecting the function and phenotype of the encoded proteins (Zhang 2000; Schrider et al. 2013). Therefore, while transitions are more frequent than transversions, especially at lower taxonomic levels, transversions are considered less informative and more difficult to interpret, potentially leading to homoplasy effects (evolutionary convergence) when comparing distantly related species in parsimony-based phylogenies (Broughton et al. 2000).

Understanding relationships among closely related taxa at a species level is essential for conserving biodiversity, maintaining ecosystem functioning, and understanding macroevolutionary processes (Oliver et al. 2015). External morphological characteristics are historically used as diagnostic features for species identification; however, contrasting results among morphological and molecular analyses appear across the entire animal kingdom, including nemerteans (Strand and Sundberg 2005), corals (Forsman et al. 2009), molluscs (Valdés et al. 2017; Fernández-Álvarez et al. 2020), polychaetes (Kupriyanova et al. 2023), fish (Park et al. 2020), insects (Selivon et al. 2005; Zhang et al. 2021), and also flatworms (Litvaitis et al. 2019).

Flatworms (order Polycladida) are free-living, carnivorous organisms that occur in a diversity of marine habitats, with over 800 species described worldwide (Tyler et al. 2006–2024). Exploring the diversity of polyclad species is critical, considering recent studies indicating the importance of the chemical and ecological roles of flatworms (Rawlinson and Stella 2012; Gammoudi et al. 2016; McNab et al. 2021, 2022; Tosetto et al. 2023). Traditionally, the taxonomy and phylogenetics of the order Polycladida have been based on morphological characteristics, where differences in tentacles, eyespots, ventral sucker, and genitalia are used to classify polyclads into different genera and families (Faubel 1983, 1984; Prudhoe 1985). External morphological characters are, however, not always an accurate reflection of the evolutionary relationships in flatworms. For example, different families of Leptoplanoidea (Acotylea) display very similar external morphologies but show different and distinguishable features internally and molecularly (Bahia 2016; Dittmann et al. 2019). Sometimes species with few morphological differences show large molecular discrepancies (Carrera-Parra et al. 2011), and the problem is exacerbated when different cryptic polyclad species live in sympatry, thereby complicating accurate identification and potentially resulting in the amalgamation of multiple species into a single one. It is therefore important to identify which molecular markers are best suited to resolving the evolutionary lineages of flatworms.

A variety of molecular markers have been used to date for the systematic analysis of polyclads. Resolution of deep nodes such as suborders (Cotylea and Acotylea) and assessment of differences in superfamilies and families have initially been based on the *28S rDNA* marker (Litvaitis and Newman 2001; Litvaitis et al. 2010; Rawlinson et al. 2011; Bahia et al. 2017; Cuadrado et al. 2021). Recent studies have, however, noted deficiencies in this marker (Dittmann et al. 2019; Litvaitis et al. 2019), because only a section of the phylogenetic tree topologies in Cotylea is consistently reconstructed. In the case of suborder Acotylea, despite recent studies (Oya and Kajihara 2020), there is a need for the inclusion of more taxa, additional genetic markers, complete markers, and/or searching for other alternatives to enhance understanding.

Other polyclad studies have used a range of different molecular markers, often employing specific primers due to performance issues with universal primers, such as *cox1*, the *16S* mitochondrial ribosomal subunit (*16S rDNA*), the mitochondrial cytochrome *b* (*cytb*), and the nuclear *18S rDNA* (Vella et al. 2016; Aguado et al. 2017; Tsunashima et al. 2017; Oya and Kajihara 2017, 2020; Oya et al. 2019; Tsuyuki et al. 2019, 2022; Cuadrado et al. 2021; Rodríguez et al. 2021), as well as complete mitochondrial genomes (Aguado et al. 2016; Kenny et al. 2019; Yonezawa et al. 2020) for both systematics and species delimitation.

This study evaluates the strength of support provided by *cox1*, *16S* rRNA, and *cytb* mitochondrial genes, as well as the *18S rDNA* and *28S rDNA* nuclear genes, on the phylogeny of the Polycladida through the study of nucleotide substitutions.

Materials and methods

Sampling sites and processing of materials

Polyclad flatworms were collected from different sites along the coasts of eastern Australia, the Iberian Peninsula, the Canary Islands, Cape Verde, Costa Rica, Cyprus, and Martinique Island (Table 2). This broad distribution range included representation of the majority of superfamilies across the order Polycladida, including Pseudocerotoidea Faubel, 1984; Prosthiostomoidea Bahia, Padula, & Schrödl, 2017 for the suborder Cotylea; Leptoplanoidea Faubel, 1984; Stylochoidea Poche, 1925; and Discoceloidea Laidlaw, 1903 for the Acotylea suborder. These species stem from a compilation of available biological material from recent studies (Noreña et al. 2014, 2015; Marquina et al. 2015a, 2015b; Aguado et al. 2017; Pérez-García et al. 2019; Cuadrado et al. 2021; Rodríguez et al. 2021; Soutullo et al. 2021), with the aim of achieving the greatest possible representativeness and sequencing of all available samples.

Flatworms were collected from under rocks in coastal environments, either by hand for intertidal and shallow individuals or using SCUBA in deeper areas, and placed in separate containers filled with seawater (specific information on species is available in the bibliography of Table 2). After being transported to a laboratory, a small piece of tissue (<1 g) was removed from the body margin of each individual using a sterile scalpel blade. The tissue of each animal was fixed in absolute ethanol and stored for DNA extraction. Each animal was then coaxed onto a piece of paper and transferred to a Petri dish containing clean, frozen seawater, where it was fixed with either 10% formalin or Bouin's liquid. Once the fixation process was complete, specimens were stored in 70% ethanol for species identification through morphological techniques, as per Rodríguez et al. (2021).

DNA extraction and amplification

Total genomic DNA was extracted from each tissue sample using an Isolate II Genomic DNA Kit (Meridian Bioscience®) following the manufacturer's protocol. Amplicons from two nuclear (*28S rDNA*, *18S rDNA*) and three mitochondrial (*16S* rRNA, *cox1*, and *cytb*) target genes from each polyclad species were sequenced. All polymerase chain reactions (PCRs) were performed using Taq DNA polymerase (Qiagen). The reaction mix included: H_2O – 10.92 µl; 10x buffer − 2 µl; 25 mM $MgCl_2$ − 4 µl; 0.5 mM dNTP − 1 µl; 10 µM primer – 0.25 µl /primer; Taq 5 U/µl − 0.08 µl; DNA – 1.5 µl. This gave a reaction volume of 20 µl.

Sequences of approximately 1100 base pairs (bp) (*28S*), 800 pb (*18S*), 500 bp (*16S*), 1000 bp (*cox1*), and 400 bp (*cytb*) were amplified using the primers listed in Table 1. The PCR consisted of an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 47 °C (*cytb*), 49 °C (*cox1*), 59 °C (*28S rDNA*, *18S rDNA*, *16S* rRNA) for 30 sec, and extension at 72 °C for 1 min, with a final extension of 10 min at 72 °C.

The PCR products were observed using TBE gel electrophoresis in 1.5% agarose gel stained with SYBER Safe and visualised under UV light. PCR products were sent to Macrogen Korea for clean-up and sequencing. Lastly, the obtained forward and reverse sequences were combined using the programme Geneious Prime 2020.2.4 (http://www.geneious.com, Kearse et al. 2012) using the alignment-transition/transversion with the consensus sequence tool and manually curated.

The species with the highest possible number of correctly sequenced genes was selected to compare the analyses performed on the different markers. All sequences obtained in the present study have been deposited in the GenBank database under the accession numbers listed in Table 2.

Comparison of genetic markers

Alignments of each molecular marker were performed with the Clustal W algorithm (Larkin et al. 2007) using the programme Geneious Prime 2020.2.4. Ambiguously aligned and variable regions were recognised and excluded using the programme Gblocks version 0.91b

Table 1. Primers used in this study.

Gene	Primer name	Sequence	Reference
18S	*18S*F2	ACTTTGAACAAATTTGAGTGCTCA	Morgan et al. (2003)
	1800mod	GATCCTTCCGCAGGTTCACCTACG	Raupach et al. (2009)
28S	Platy*28S*_F	AGCCCAGCACCGAATCCT	Cuadrado et al. (2021)
	Platy*28S*_R	GCAAACCAAGTAGGGTGTCGC	Cuadrado et al. (2021)
16S	PLATYS*16S*F1	ACAACTGTTTATCAAAAACAT	Aguado et al. (2017)
	PLATYS*16S*R1	ACGCCGGTYTTAACTCAAATCA	Aguado et al. (2017)
cox1	HRpra2	AATAAGTATCATGTARACTDATRTCT	Tsunashima et al. (2017)
	HRprb2-2	GDGGVTTTGGDAATTGAYTAATACCTT	Tsunashima et al. (2017)
	Acotylea_COI_F	ACTTTATTCTACTAATCATAAGGATATAGG	Oya and Kajihara (2017)
	Acotylea_COI_R	CTTTCCTCTATAAAATGTTACTATTTGAGA	Oya and Kajihara (2017)
cytb	*cytb*424-444	CAGGAAACAGCTATGACCGGWTAYGTWYTWCCWTGRGGWCARAT	Jondelius et al. (2002)
	*cytb*876-847	TGTAAAACGACGGCCAGTGCRTAWGCRAAWARRAARTAYCAYTCWGG	Jondelius et al. (2002)

Table 2. List of species and sequences studied (material from previous studies, see table list of references).

Family	Species	*18S*	*28S*	*16S*	*cox1*	*cytb*	Locality	Reference
Discoceloidea								
Cryptocelidae	*Cryptocelis* sp.	MZ292810	MZ292829	MZ292858	MZ273073	PP856191	Galicia, Spain	Noreña et al. (2015)
Discocelidae	*Discocelis tigrina*	MZ292799	MK299370	-	-	PP856182		Cuadrado et al. (2021)
Leptoplanoidea								
Gnesiocerotidae	*Echinoplana celerrima*	MW376754	MW377507	MW376599	MW375911	MW392971	New South Wales, Australia	Rodríguez et al. (2021)
	Ceratoplana falconerae	MW376740	MW377493	MW376585	MW375897	MW392973	Victoria, Australia	Rodríguez et al. (2021)
	Parabolia megae	MW376744	MW377497	MW376589	MW375901	MW392974	New South Wales, Australia	Rodríguez et al. (2021)
Leptoplanidae	*Leptoplana* sp.	-	MZ292828	MZ292853	MZ273072	-	Cape Verde Island	Cuadrado et al. (2021)
	Parviplana geronimoi	MZ292807	-	MZ292855	-	-	Cádiz, Spain	Pérez-García et al. (2019)
Notoplanidae	*Notoplana australis*	MW376750	MW377503	MW376595	MW375907	MW392986	New South Wales, Australia	Rodríguez et al. (2021)
	Notoplana felis	MW376753	MW377506	MW376598	MW375910	MW392985	Victoria, Australia	Rodríguez et al. (2021)
Pleioplanidae	*Pleioplana atomata*	MZ292820	MZ292832	MZ292866	MZ273074	PP856198	Asturias, Spain	Marquina et al. (2015a)
	Pleioplana sp.	MZ292808	MZ292840	MZ292856	MZ273079	PP856189	Cádiz, Spain	This study
Pseudostylochidae	*Tripylocelis typica*	MW376752	MW377505	MW376597	MW375909	MW392983	New South Wales, Australia	Rodríguez et al. (2021)
Stylochoplanidae	*Stylochoplana clara*	MW376741	MW377494	MW376586	MW375898	MW392972	Victoria, Australia	Rodríguez et al. (2021)
Stylochoidea								
Callioplanidae	*Callioplana marginata*	MW376747	MW377500	MW376592	MW375904	MW392984	New South Wales, Australia	Rodríguez et al. (2021)
	Neostylochus ancorus	MW376748	MW377501	MW376593	MW375905	-	New South Wales, Australia	Rodríguez et al. (2021)
Latocestidae	*Eulatocestus australis*	MW376749	MW377502	MW376594	MW375906	-	New South Wales, Australia	Rodríguez et al. (2021)
	Latocestus plehni	MZ292806	MK299376	MZ292852	-	PP856187	Cape Verde Island	Cuadrado et al. (2021)
Planoceridae	*Paraplanocera marginata*	MW376745	MW377498	MW376590	MW375902	MW392981	New South Wales, Australia	Rodríguez et al. (2021)
	Paraplanocera sp.	MZ292818	MZ292833	MZ292868	MZ273075	PP856200	Cyprus	This study
	Planocera edmondsi	MW376755	MW377508	MW376600	MW375912	MW392979	Victoria, Australia	Rodríguez et al. (2021)
	Planocera pellucida	MZ292797	MK299355	-	-	PP856180	Canary Island, Spain	Cuadrado et al. (2021)
Idioplanidae	*Idioplana australiensis*	MW376746	MW377499	MW376591	MW375903	MW392980	New South Wales, Australia	Rodríguez et al. (2021)
Stylochidae	*Imogine fafai*	MZ292817	MZ292835	MZ292865	MF371138	PP856197	Asturias, Spain	Aguado et al. (2017)
	Leptostylochus victoriensis	MW376742	MW377495	MW376587	MW375899	MW392982	New South Wales, Australia	Rodríguez et al. (2021)
	Stylochus neapolitanus	MZ292800	MZ292841	MZ292846	MF371141	PP856183	Galicia, Spain	Aguado et al. (2017)
Boninioidea								
Boniniidae	*Boninia* sp.	MZ292819	MZ292834	MZ292869	-	PP856201	Costa Rica	Soutullo et al. (2021)
Cestoplanidae	*Cestoplana rubrocincta*	MW376751	MW377504	MW376596	MW375908	MW392977	New South Wales, Australia	Rodríguez et al. (2021)
Pericelidae	*Pericelis beyerleyana*	MZ292801	MK299374	MZ292847	-	PP856184	Martinique Island	Cuadrado et al. (2021)
	Pericelis cata	MZ292805	MK299352	MZ292851	-	-	Cape Verde Island	Cuadrado et al. (2021)
Prosthiostomoidea								
Prosthiostomidae	*Prosthiostomum amri*	MW376743	MW377496	MW376588	MW375900	MW392978	New South Wales, Australia	Rodríguez et al. (2021)
	Prosthiostomum siphunculus	MZ292816	MZ292836	MZ292864	MZ273080	PP856196	Almuñécar, Spain	Pérez-García et al. (2019)
	Prosthiostomum sp.	MZ292795	MZ292826	MZ292842	MZ273071	-	New South Wales, Australia	Rodriguez et al. (2021)
	Enchiridium magec	-	MK299349	MZ292844	-	PP856179	Canary Island, Spain	Cuadrado et al. (2021)
Pseudocerotoidea								

Family	Species	*18S*	*28S*	*16S*	*cox1*	*cytb*	Locality	Reference
Eurylepidae	*Eurylepta cornuta*	MZ292809	MZ292839	MZ292857	MF371139	PP856190	Galicia, Spain	Aguado et al. (2017)
	Eurylepta guayota	MZ292804	MK299372	MZ292850	-	PP856186	Martinique Island	Cuadrado et al. (2021)
	Prostheceraeus roseus	MZ292811	KY263688	MZ292859	MZ273078	PP856192	Galicia, Spain	Noreña et al. (2014)
Pseudocerotidae	*Phrikoceros sp.*	MZ292796	MZ292827	MZ292843	-	PP856178	Victoria, Australia	Rodriguez et al. (2021)
	Pseudoceros depiliktabub	MZ292813	MZ292837	MZ292861	-	PP856194	Lizard Island, Australia	Marquina et al. (2015b)
	Pseudoceros stimpsoni	MZ292812	MZ292838	MZ292860	MF371147	PP856193	Lizard Island, Australia	Aguado et al. (2017)
	Pseudoceros velutinus	MZ292798	MK299381	MZ292845	MZ273076	PP856181	Canary Island, Spain	Cuadrado et al. (2021)
	Pseudoceros rawlinsonae var. *galaxy*	-	MK299357	MZ292854	-	PP856188	Cape Verde Island	Cuadrado et al. (2021)
	Pseudobiceros flowersi	MZ292814	MZ292830	MZ292862	-	PP856195	Lizard Island, Australia	Marquina et al. (2015b)
	Pseudobiceros hymanae	MZ292815	MZ292831	MZ292863	-	-	Lizard Island, Australia	Marquina et al. (2015b)
	Pseudobiceros caribbensis	MZ292803	MK299378	MZ292849	MZ273077	PP856185	Martinique Island	Cuadrado et al. (2021)
	Thysanozoon alagoensis	MZ292802	MK299383	MZ292848	-	-	Martinique Island	Cuadrado et al. (2021)
	Thysanozoon brocchii	MW376738	MW377491	MW376583	-	MW392976	Victoria, Australia	Rodríguez et al. (2021)
	Yungia aurantiaca	-	MK299386	MZ292867	-	PP856199	Cádiz, Spain	Cuadrado et al. (2021)

(Castresana 2000) with relaxed parameters (smaller final blocks). This resulted in matrices of 521 bp (*cox1*), 500 bp (*16S rRNA*), 393 bp (*cytb*), 1047 bp (*28S rDNA*), and 859 bp (*18S rDNA*).

A supplementary entropy analysis was also performed with IQ-TREE version 1.6.12 (Trifinopoulos et al. 2016) to quantify the genetic variability across the length of the obtained sequences and assess the grade of conservation of each marker (entropy estimation by site).

The saturation rate of the substitutions of each genetic marker was quantified through a transition (Ti) and transversion (Tv) saturation graph using PAUP* Version 4.0a (Build 166) (Swofford 2003), as well as the distribution of variable sites and grade of genetic variability by site along the genes' matrices with an entropy analysis using DAMBE 5 (Xia 2013). Interspecific distances for each gene were calculated in Mega 6 (Tamura et al. 2013).

Maximum likelihood (ML) analysis was performed with IQ-TREE (Trifinopoulos et al. 2016). The optimal substitution model selected by the Bayesian information criterion (BIC) proposed by the ModelFinder (Kalyaanamoorthy et al. 2017) was GTR+F+I+G4 (*16S rDNA*, *cox1*), TIM+F+I+G4 (*cytb*), K2P+I (*18S rDNA*), and TIM3+F+I+G4 (*28S rDNA*). The consensus tree of 1000 standard bootstrap pseudo-replicates was selected and edited with iTOL version 4 (Letunic and Bork 2019). A node was considered well supported when the bootstrap value was 80% or greater. Phylogenies without outgroups have been analysed to avoid including inconsistencies since it was not possible to obtain a common outgroup for the five markers studied.

Results

Entropy estimation by site

Entropy analysis revealed genetic variability across the length of the obtained sequences and assessed the grade of conservation of each marker. The variable positions of each studied gene presented a continuous distribution, with substitutions unequally distributed in the nuclear genes. *18S rDNA* presented 58 out of 859 (6.75% of the alignment) variable positions (37 parsimonies informative, PIs), while *28S rDNA* presented 388 out of 1047 (37.0%) variable positions (306 PIs). *16S rDNA* presented 322 out of 500 (64.4%) variable positions (286 PIs), while *cytb* presented 234 out of 393 (59.54%) variable positions (218 PIs), and *cox1* presented 293 out of 521 (56.2%) variable positions (280 PIs) (Table 3, Fig. 1A).

Table 3. Genetic variability of the analysed sequences.

Gene	Average distance (%)	Min distance (%)	Max distance (%)	S	Cs	PIs
18S rDNA	1.37	0.00	3.14	859	58 (6.75%)	37
28S rDNA	11.21	0.00	18.71	1047	388 (37.0%)	306
16S rRNA	22.06	0.28	32.77	500	322 (64.4%)	286
cytb	26.86	0.00	34.40	393	234 (59.5%)	218
cox1	24.86	0.22	34.44	521	293 (56.2%)	280

The minimum distance was calculated as the minimum divergence of all sequences; the maximum distance was calculated as the maximum divergence of all sequences; Cs: number of constant sites; S: total number of sites in the matrix; and PIs: number of parsimony informative sites.

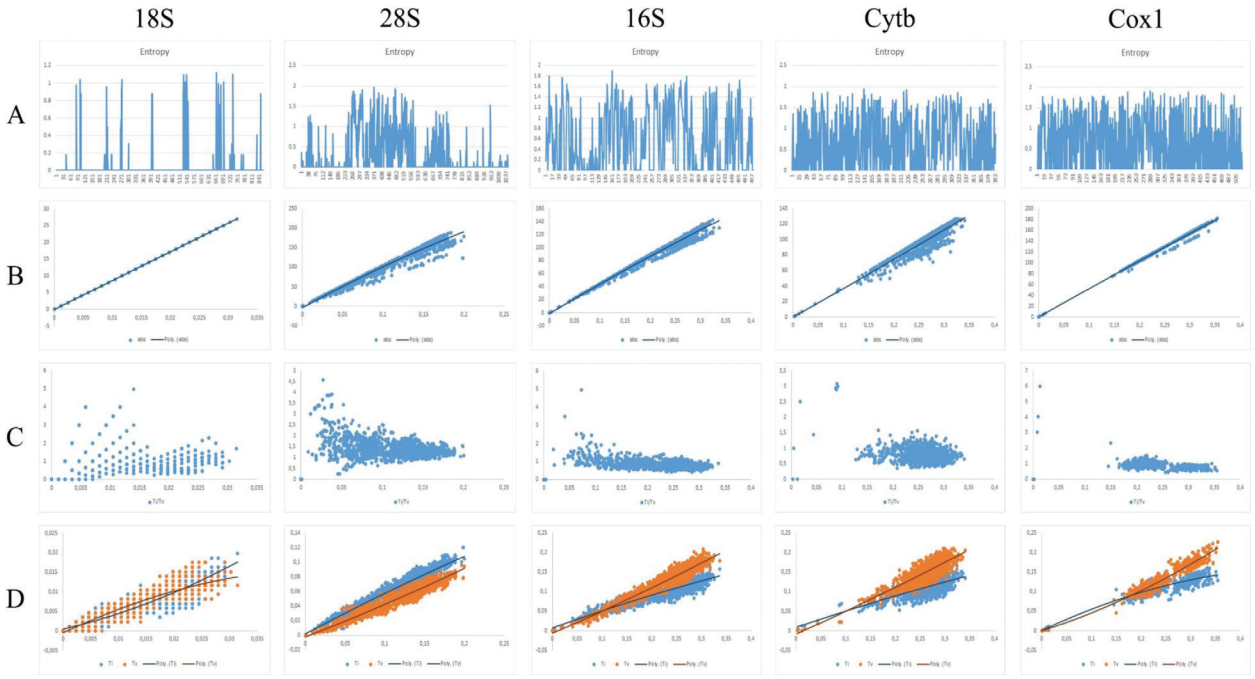

Figure 1. Genomic analysis of the studied genes. **A.** Entropy estimation by site: The X-axis indicates the number of sequenced positions, and the Y-axis indicates the number of variations of each position; **B.** Estimation of substitution rates in absolute values: The X-axis displays the pairwise genetic distance between sample pairs; the Y-axis indicates the number of mutations in absolute values; **C.** Estimation of Ti/Tv in pairwise sequence comparisons: The X-axis shows the pairwise genetic distance between sample pairs, and the Y-axis shows the Ti/Tv proportion; **D.** Estimation of transitions and transversions in pairwise sequence comparisons: The X-axis indicates the pairwise genetic distance between sample pairs, and the Y-axis indicates the proportion of transitions and transversions.

The variable sites of each codon position of the protein-codifying genes (*cytb* and *cox1*) were also assessed. The third codon position presented the highest values of interspecific maximum distances in both markers: 69.41% in *cytb* and 53.83% in *cox1*. On the other hand, the second codon position had the lowest values of maximum distances, with 16.66% in *cytb* and 13.42% in *cox1* (Table 4).

Table 4. Genetic variability of the analysed sequences of *cytb* and *cox1* by codon position.

Gene	Average distance (%)	Min distance (%)	Max distance (%)
cytb first codon position	20.38	0.00	30.58
cytb second codon position	8.88	0.00	16.66
cytb third codon position	51.71	0.00	69.41
cox1 first codon position	16.25	0.65	30.06
cox1 second codon position	5.51	0.00	13.42
cox1 third codon position	53.83	2.02	53.83

The minimum distance was calculated as the minimum divergence of all sequences; and the maximum distance was calculated as the maximum divergence of all sequences.

Estimate of substitution rate in absolute values

A total of 1485 (*18S rDNA*), 2556 (*28S rDNA*), 1653 (*16S rDNA*), 1176 (*cytb*), and 703 (*cox1*) pairwise comparisons from 43 (*18S rDNA*), 46 (*28S rDNA*), 45 (*16S rDNA*), 39 (*cytb*), and 30 (*cox1*) species were performed. Fig. 1B shows the number of substitutions in absolute values (abs) plotted against the pairwise distance between each sample. All cases presented a linear growth following these equations:

18S rDNA:

$$y = - 16.054x^2 + 858.37x – 8E-05$$

$$R^2 = 1.0000$$

28S rDNA:

$$y = - 760.97x^2 + 1123.5x – 3.5153$$

$$R^2 = 0.9743$$

16S rDNA:

$$y = - 114.54x^2 + 462.66x – 1.4178$$

$$R^2 = 0.9766$$

cytb:

$$y = 30.873x^2 + 366.5x + 0.3227$$

$$R^2 = 0.8436$$

cox1:

$$y = - 43.538x^2 + 524.81x + 0.1409$$

$$R^2 = 0.9901$$

The coefficient of determination (R^2) was close to 1 in most cases, indicating that all values were close to a linear progression except for the *cytb* mitochondrial gene ($R^2 = 0.84$).

Estimates of the transition/transversion ratio (Ti/Tv) in pairwise sequence comparisons

The estimated Ti/Tv ratios plotted against the estimated sequence distances showed the Ti/Tv ratio plotted against the pairwise distance between each sample (Fig. 1C). Two differentiated regions can be observed: the first was a region where the number of transitions and transversions randomly appeared with great variation. Due to the short distances between phylogenetic closely related species and the different numbers of transversions and transitions that each pair presented, the estimation showed disparate values depending on the selected samples, predominating the number of transitions, as they are the most probable among closely related species. As the distance between species pairs increased, a second region where the values stabilised around 1 (where 1 indicates the same number of transversions and transitions) appeared. While the value was slightly higher than 1 in most cases (indicating a greater number of transitions over transversions), starting from pairwise distances greater than 20%, the number of transversions increased compared to that of transitions in the case of the *16S rDNA* and *cox1* mitochondrial genes. Meanwhile, *28S rDNA* presented a Ti/Tv ratio between 1 and 2 at longer distances, indicating an overall higher number of transitions.

Estimates of transitions and transversions in pairwise sequence comparisons

Congruent with the results of the Ti/Tv ratio, the initial number of transitions was higher than that of transversions for all gene markers. However, the number of transversions was greater at higher distances across all markers, as observed in the graphs, except for *28S rDNA*, where transitions remained higher (Fig. 1D).

Differences among the three codon positions were evident (Fig. 2). The first codon position displayed maximum distances of 30.58% for *cytb* and 30.06% for *cox1*, compared to the maximum distances for the second codon position (16.66% and 13.42%, respectively) and those of the third codon position (69.41% and 70.94%).

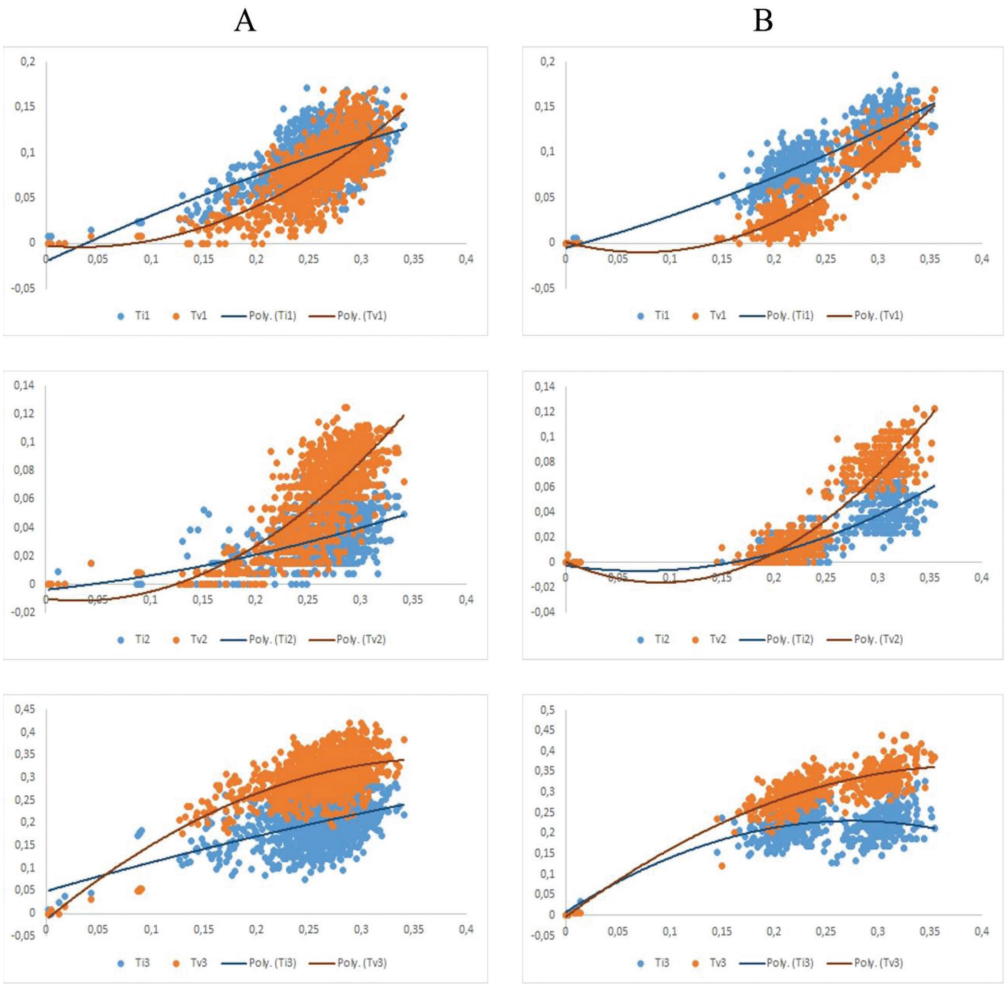

Figure 2. Estimation of transitions and transversions for each codon position (from top to bottom: first (1), second (2), and third (3) codon positions) in *cytb* (**A**) and *cox1* (**B**). The X-axis indicates the pairwise genetic distance between sample pairs, and the Y-axis indicates the proportion of transitions and transversions.

In both markers, the overall number of transversions was higher than that of transitions, apart from the first codon position, where the number of transitions was always higher than that of transversions. The second codon position displayed a lower mutation rate at shorter distances. Lastly, the third codon position presented a higher number of overall mutations (both transitions and transversions), with a higher proportion of transversions in both markers; however, a decrease in transition in the *cox1* gene was observed at pairwise distances higher than 25%.

Maximum-likelihood phylogenetic analyses

The matrices employed to analyse substitution ratios provided the following phylogenetic results through a maximum-likelihood analysis performed for each gene (Figs 3, 4). The results obtained for each marker are:

18S rDNA (Fig. 3): This marker showed the separation of the two suborders of Polycladida (Cotylea and Acotylea) with a bootstrap support (BS) of 97.

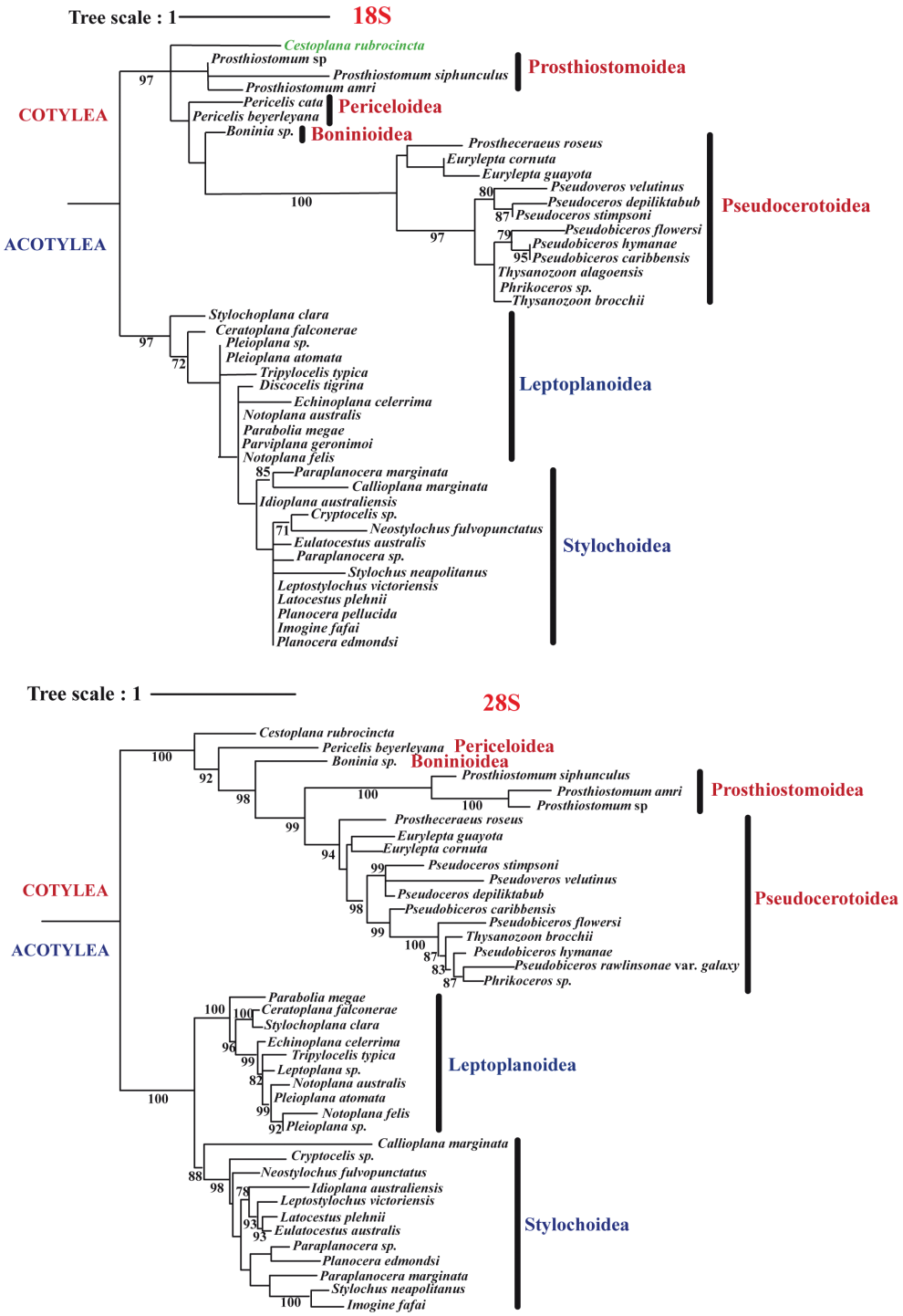

Figure 3. Maximum-likelihood phylogenetic analysis of nuclear gene markers (*18S* and *28S*).

The superfamily Pseudocerotidae (Cotylea) was highly supported (BS = 100), as were the genera *Pseudoceros* (BS = 80) and *Pseudobiceros* (BS = 79). The superfamilies and families of Acotylea appeared without strong support (BS < 70).

28S rDNA (Fig. 3): In this study, the two suborders were well supported (BS = 100). Within Cotylea, the four analysed superfamilies were well delimited and held: Periceloidea and Boninioidea produced two independent lineages, and Pseuderotoidea (BS = 94) and Prosthiostomoidea (BS = 100) were highly supported. Within the last superfamily Pseudocerotidae (including the genera *Pseudoceros*, *Pseudobiceros*, *Thysanozoon*, and *Phrikoceros*; BS = 98), an independent cluster for the family Eurylepitidae was seen. Similarly, within the Acotylea suborder, the different superfamilies Leptoplanoidea and Stylochoidea showed high support values (BS = 100 and 88, respectively). Families such as Styochoplanidae (BS = 100), Leptoplanidae (BS = 99), Latocestidae (BS = 93), and Stylochidae (BS = 100) were also well supported.

16S rRNA (Fig. 4): This marker provided robust support for the suborders Cotylea and Acotylea and good resolution for the Cotylean superfamilies Periceloidea (BS = 100), Prosthiostomoidea (BS = 99), and Pseudocerotoidea (BS = 98). The two largest Cotylean superfamilies (Prosthiostomoidea and Pseudocerotoidea) were grouped in a clade with a bootstrap support of 95. Within Acotylea, the superfamily Stylochoidea was not supported (BS = 75), and the superfamily Leptoplanoidea did not form a monophyletic assemblage. As a result, at the family and genus levels, *16S rRNA* did not yield clear groups within the leptoplanoids.

cox1 (Fig. 4): This marker is considered the molecular "barcode" for the majority of species. In this study, support varied depending on the taxonomic level. At the suborder level, the support values were lower than those from other genes (BS = 77). At the next level, the mainly Cotylean and Acotylean superfamilies were recovered. The majority of families in both suborders did not form monophyletic clusters.

cytb (Fig. 4): Regarding the last of the studied markers, *cytb* separated the two suborders Cotylea and Acotylea (BS = 100). It also displayed high support for the Cotylean and Acotylean superfamilies. At family level, *cytb* provided good support in both suborders (Colylea: Eurylepitidae BS = 86 and Pseudocerotidae BS = 99; Acotylea: Leptoplanidae BS = 98, Planoceridae BS = 77, Latocestidae BS = 82, and Stylochidae BS = 80), but the majority of Cotylean and Acotylean superfamilies were not recovered (Fig. 4).

All assessed markers placed *Cestoplana* within or as the sister lineage of Cotylea, but none showed an unequivocal phylogenetic or kinship relationship between *Cestoplana rubrocincta* and the other taxa.

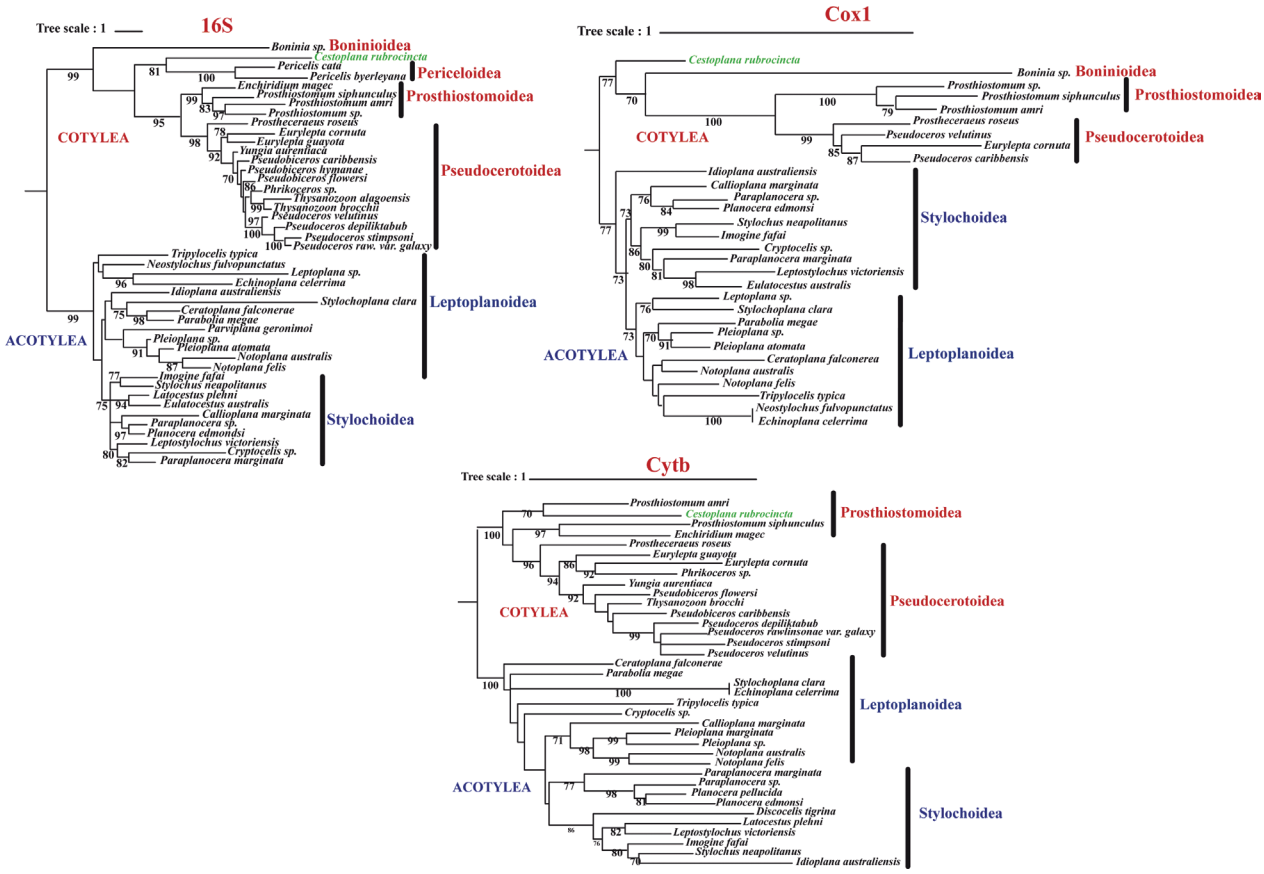

Figure 4. Maximum-likelihood phylogenetic analysis of mitochondrial gene markers (*16S*, *cox1*, and *cytb*).

Discussion

This study compares, for the first time, the substitutions of mitochondrial and nuclear molecular markers at the order level for polyclad flatworms, including representatives of all superfamilies within the suborders Cotylea and Acotylea.

Regarding entropy values, it is worth noting the small proportion of variable sites in the *18S rDNA* nuclear gene that denote low phylogenetic values in our analyses of the order compared to *28S rDNA*, which presented regions with clear variability alternating with conserved regions (Fig. 1A). This difference is made more apparent when compared to the studied mitochondrial markers (*16S rDNA*, *cox1*, and *cytb*), which all presented high variability and substitution rates. In addition, in the three mitochondrial markers, variability was present throughout the entire DNA sequence, which is possibly one of the reasons for the difficulty in creating generic primers for these species, especially in the case of *cox1*. In most invertebrate taxa, *cox1* sequencing is possible using universal primers such as those designed by Folmer and co-workers (Folmer et al. 1994) or, more recently, Lobo and colleagues (Lobo et al. 2013). For some taxonomic groups, however, these markers do not hybridise, and this appears to be the case for most Rhabditophora (Platyhelminthes), including those in the order Polycladida (mainly in the suborder Cotylea). In this situation, specific primers are frequently required (Aguado et al. 2017; Oya et al. 2019; Cuadrado et al. 2021).

The absolute values of substitution rates observed in our research reflect a linear increase in variability in all cases. A decrease in the absolute mutation rate was only observed in *cytb*, which may have been caused by a certain saturation in the signal of sequence substitution due to multiple recurrent changes since more than 80% of each sequence displayed variability. This saturation trend could lead to underestimating the variation in determinate terminal taxa. Therefore, it would be more advisable to use this marker for conducting phylogenetic analyses of closer groups, such as families or superfamilies.

The Ti/Tv ratio remained relatively stable for most cases, except for *28S rDNA*, which presented a higher number of transitions at all distances, and *cytb*, which displayed a higher number of transversions. In the case of the *28S* ribosomal gene, the elevated number of transitions is most likely due to a lack of conservation of the secondary structure of the RNA molecule (Rivas 2021), which may be required to preserve its function, with stem structures forming at transitions where needed.

In contrast, the overall increase in transversions in mitochondrial genes, particularly in *cytb*, could be the accumulation of substitutions when comparing variable sequences very distant from each other. All four types of transitions, as opposed to eight types of transversions, need to be considered in such situations. Previous studies have suggested that, compared with non-synonymous transversions, non-synonymous transitions are less deleterious because they tend not to cause radical changes in amino acid physicochemical properties such as charge, polarity, and size (e.g., Zhang 2000). However, our research shows a higher proportion of transitions were observed for the *28S rDNA* nuclear gene in comparison to the mitochondrial markers. Meanwhile, *18S rDNA* presented so few changes that it could hardly indicate a tendency in the analyses. Our results appear to validate the proposition made by Zou and Zhang (2021), who stated that the Ti/Tv ratio can be more or less than 1 (i.e., transversions or transitions being more prevalent) depending on the group studied. They attributed variations between interchangeable amino acids in protein-coding genes as a possible cause for this (e.g., variations in the genetic code of different taxa, differences in the functionality of generated proteins, etc.). In the case of the phylum Platyhelminthes, it is important to point out that the flatworm mitochondrial genetic code possesses four variations compared to the standard invertebrate mitochondrial code. For example, AAA codifies for asparagine (Asn) in flatworms, while in the standard mitochondrial code only AAT and AAC codify for this amino acid, which leads to fixating a transversion in this group. Likewise, the codons AGA and AGG translate to serine (Ser) in the flatworm mitochondrial genetic code instead of Arg, fixating two additional transversions, and UGA codifies for Trp rather than being a stop codon (Telford et al. 2000).

Different patterns of substitutions were also observed for the results of the *28S rDNA* nuclear gene (Fig. 1B) and those present in the mitochondrial genes when comparing transition and transversion rates. The number of transitions surpasses the number of transversions in the *28S rDNA*, while the mitochondrial markers show more transversions than transitions. The variations in the mitochondrial genetic code of flatworms mentioned earlier could lead to a higher chance of fixating transversions. Because of this, we suggest a more exhaustive study on this increase in transversions in the mitochondrial DNA of polyclads and its implications for Platyhelminthes more generally.

Conspicuous differences were observed when comparing all codon positions of each of the studied protein-codifying genes (*cytb* and *cox1*). Saturation of the transversions was observed in the third codon position of *cox1*. Such saturation has been reported previously for other taxonomic groups such as triclads (Alvarez-Presas et al. 2008), protists (Liu and Zhang 2019), insects (Schrider et al. 2013), plants (Ossowski et al. 2010), and nematodes (Denver et al. 2009). It is possible that the decrease in the signal of the number of transitions at distances higher than 25% observed in our research could lead to errors in phylogenetic analyses of polyclad flatworms when using the *cox1* genetic marker. A plausible solution to reduce this effect during future phylogenetic analyses would be to delete the third codon position from the alignment. The effectiveness of this, however, is beyond the scope of this study.

Based on the results obtained in the ML analysis (Figs 3, 4), the markers with the best clade support and agree-

ment with morphological relationships by histological analyses (Faubel 1983, 1984) were *28S rDNA* (nuclear) and the mitochondrial markers *16S rDNA* and *cytb*. *18S rDNA* did not offer strong support at any taxonomic level studied. Moreover, substantial differences in support existed within the effective and resolving markers: *28S rDNA* (nuclear) and *16S rDNA*/*cytb* (mitochondrial). The differences observed between mitochondrial and nuclear markers, along with their potential incongruences in phylogenetic analyses, have previously been documented in other taxa, including Anthozoa, Insecta, and mammals (Zadra et al. 2021; Fedorov et al. 2022; Quattrini et al. 2023).

28S rDNA resolved the majority of nodes well for the systematics of suborder, superfamily, family, and, in some cases, at the genus and species level in Cotylea. Nevertheless, the *28S rDNA* proved less effective in resolving deep nodes within Acotylea, resulting in the formation of paraphyletic nodes.

Within the mitochondrial markers, the best resolution level (>75 bootstrap support), compared to current phylogeny (Goodheart et al. 2023), was observed at the genus and species level. Both *16S rDNA* and *cytb* strengthened and delimited the genera, resolving specific clusters within Cotylea and Acotylea. The specific combinations presented in our analyses revealed differences, such as the relationship between *Cestoplana* and *Pericelis* within Cotylea or *Echinoplana* with *Leptoplana* in Acotylea, although these relationships were not recovered by *cytb*. This may be caused by the increased substitution rate present among distantly related taxa within the phylogenetic tree (resulting in decreased linear progression of R^2) and a more complex evolutionary history for these genes or taxa.

Conclusion

Among the tested markers, *cytb* presented a higher rate of variability and did not show saturation of transitions for any codon position. Moreover, this marker presented the highest range of distances (0% to 34.40%), with an average distance of 26.86% compared to that of *cox1* (highest range of distances: 0.22% to 34.44%, average distance: 24.86%).

The use of a common marker for the order Polycladida would allow direct phylogenetic comparison across studies. General primers for these mitochondrial genes often fail to hybridise, so we also recommend designing *de novo cox1*-specific primers for families within the suborder Cotylea and *cytb*-specific primers for those within Acotylea, taking into consideration third base positions. The *de novo* design markers will allow amplification of *cox1* and *cytb* sequences for certain groups of polyclad flatworms that previously could not be analysed due to the high number of substitutions across the whole sequence and the lack of conserved regions.

Thus, for polyclad flatworms, we conclude that for future studies at the order level, we encourage the use of mitochondrial genes *cytb* and *16S rDNA* and nuclear ribosomal genes *28S rDNA*. We also encourage the use of the *cox1* gene with the caution of analysing the third codon position to avoid errors in the analyses and resolution of deep nodes at a generic or specific level. Certainly, the most crucial aspect is to determine the specific research inquiry and taxonomic level (such as order, family, or genus) and consequently select the appropriate genes to better address the study. In the present study, we analysed five markers currently used in the resolution of phylogenies, kinship analysis, delimitation of species, etc. We look forward to future polyclad studies using our suggested approach so that we can continue advancing the systematics and origin of this taxon on a global scale. New sequencing techniques offer the possibility of incorporating additional molecular information if the selected genes accurately represent the evolutionary history of the species. Concatenating data from different suitable markers will further bolster support for the analysed clusters.

Our case study highlights the need to evaluate how well nuclear and mitochondrial genes perform within a specific taxonomic group level. We propose that the use of transition bias is a useful tool for distinguishing which markers may be more effective for any taxon and could help streamline success for future systematic studies. It would also make cross-study evaluation within a taxonomic group more effective. A more globally collaborative approach to molecular systematics would certainly facilitate the use of this approach.

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