
Molecular phylogeny of gill monogeneans (Platyhelminthes, Monogenea, Dactylogyridae) and colonization of Indo-West Pacific butterflyfish hosts (Perciformes, Chaetodontidae)

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We investigated the phylogenetic relationships among monogenean parasites of the Chaetodontidae (butterflyfishes) from the Indo-West Pacific Ocean. Molecular phylogenies of selected taxa within the Dactylogyridae, including the ancyrocephaline parasites of butterflyfishes, based on two nuclear and one mitochondrial gene fragments (complete 18S rDNA, partial 28S rDNA (D1-D3), and partial 16S rDNA) were reconstructed using parsimony, maximum likelihood and Bayesian inference methods. Our results show the non-monophyletic nature of the monogenean fauna of butterflyfishes. The group is divided into two independent lineages. The first clade contains species of the genera *Aliatrema* and *Eurybaliotrematoides*, which parasitize Chaetodontidae exclusively. The second contains species of *Haliotrema*, a generalist group of parasites. The positions of several other species of the Ancyrocephalinae, including freshwater species, at the base of the two clades, provide strong evidence that the monogenean fauna did not result from a single colonization event, but rather that they have colonized their butterflyfish hosts independently at least twice.

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Introduction

Butterflyfishes are one of the most distinctive, common and visually striking fishes to be found on coral reefs. Comprising a single family, the Chaetodontidae, they are represented by a total of 125 species belonging to nine genera. *Chaetodon* is the most diverse (89 spp.) (Froese & Pauly 2003). They are widely distributed throughout the Atlantic, Pacific and Indian Oceans.

Butterflyfishes are parasitized by gill monogeneans belonging to the family Dactylogyridae Bychowsky, 1933, with a total of 15 species described to date. Recent studies (Plaisance & Kritsky 2004; Plaisance *et al.* 2004) have revised the classification of the monogenean fauna of butterflyfishes in the Indo-West Pacific Ocean. Prior to this revision, two different genera were known to parasitize butterflyfishes: *Haliotrema* Johnston & Tiegs, 1922 and *Pseudobaliotrematoides*

Yamaguti, 1953. Using morphological criteria, Plaisance & Kritsky (2004) and Plaisance *et al.* (2004) divided the group of chaetodontid parasites into three different genera: *Haliotrema* Johnston & Tiegs, 1922, *Eurybaliotrematoides* Plaisance & Kritsky, 2004, and *Aliatrema* Plaisance & Kritsky, 2004.

Haliotrema is a highly diversified genus containing more than 100 species. It is found on a great range of hosts belonging to six orders of teleost fishes and is distributed throughout warm seas. Six species are known to parasitize butterflyfishes, although three have not been revised since their original description. Due to its great range of hosts and diverse morphology, *Haliotrema* has been suggested by a number of authors as representing a polyphyletic group (Klassen 1994a; Kritsky & Stephens 2001; Kritsky & Boeger 2002).

Eurybaliotrematoides is only found on fishes from the family Chaetodontidae. It has, to date, been recorded on

three different chaetodontid genera: *Chaetodon* L., 1758, *Forcipiger* Jordan & McGregor, 1898 and *Heniochus* Cuvier, 1816. It is the most species-rich genus, with seven species described.

Aliatrema is monotypic. Its single species, *A. cribbi* Plaisance & Kritsky, 2004, is differentiated from species belonging to *Euryhaliotrematoides* by the lack of an accessory piece in the male copulatory organ (Plaisance & Kritsky 2004).

The aim of this study is to build a molecular phylogeny of the monogenean parasites of Chaetodontidae in order to assess the monophyly of *Euryhaliotrematoides*, the relationships between the three genera, *Haliotrema*, *Euryhaliotrematoides* and *Aliatrema*, and their position within the Dactylogyridae.

Materials and methods

Taxon sampling

The 11 species of parasites from butterflyfishes reported from the Indo-West Pacific Ocean (Plaisance & Kritsky 2004; Plaisance *et al.* 2004) and belonging to the three genera parasitizing chaetodontids (*Haliotrema*, *Euryhaliotrematoides* and *Aliatrema*) were sampled. Sequences of the Dactylogyridae, together with sequences of other Polychaeta available from GenBank and chosen on the basis of the phylogeny of the Monogenea published by Olson & Littlewood (2002), were added to the analysis (See Table 1).

Collection and identification of specimens

Fish sampling was performed as described in Plaisance & Kritsky (2004). Parasite specimens were removed from the gills, placed on a slide in a drop of picrate ammonium glycerine (medium used for staining sclerotized structures) and observed under a light microscope. Identification of the species was based on the characters of the sclerotized parts (haptor and male copulatory organ). Images of their general morphology and sclerotized organs were captured for each individual using a digital camera and microscope with Visilog 5.2. (NorPix, Inc). Each identified parasite specimen was placed in a vial containing 1.5 mL of ethanol (80%) in order to wash the parasite from the picrate ammonium glycerine medium and to preserve the DNA.

DNA extraction, PCR amplification and sequencing

Specimens were removed from alcohol and allowed to air-dry. Total genomic DNA from one specimen was extracted using DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol. It was concentrated to a final volume of 15 µL using Microcon-100 columns (Millipore).

Three gene fragments were amplified and sequenced: the mitochondrial 16S rDNA and the nuclear 18S rDNA and 28S rDNA genes. The nuclear genes were chosen to cover a range of evolutionary rates and to complement existing data collected for monogenean phylogenetics. 16S rDNA was

added for parasites of butterflyfishes to provide additional phylogenetic resolution within this group.

Twenty-five µL primary PCR amplifications were performed with 2 µL of DNA extract, 10 pM of each PCR primer and Ready-To-Go PCR beads (Amersham Pharmacia Biotech), each containing 1.5 U Taq polymerase, 10 mM Tris-HCl at pH 9, 50 mM KCl, 1.5 mM MgCl₂, 200 µM each dNTP and stabilizers including bovine serum albumin.

Because of the low quantity of genomic DNA extracted from what are very small worms, ranging from 200 to 430 µm in length, and the relatively low yield obtained by primary PCR, we employed nested PCR using one internal primer and 1 µL of primary PCR product, following the same PCR protocol as described above.

Thermal cycling was performed with an initial denaturation for 3 min at 94 °C, followed by 40 cycles for primary PCR and 35 cycles for nested PCR (30 s at 94 °C, 30 s at a gene specific-annealing temperature, 2 min at 72 °C, with a final extension of 10 min at 72 °C). Annealing temperatures were as follows: 52 °C for primary 28S rDNA and 18S rDNA; 58 °C for nested 28S rDNA and 18S rDNA; 44 °C for primary 16S rDNA and 46 °C for nested 16S rDNA.

18S rDNA was amplified in primary PCR in one section using the primer combination WormA + WormB. Two overlapping sections were amplified in nested PCR using the combinations WormA + 1270R and 930F + WormB. The two other genes were amplified in a single section from (1) primary PCR, using combinations Ancy55F + LSU 1200R for 28S rDNA and 16SF1 + 16SR1 for 16S rDNA, and (2) nested PCR, using combinations Ancy55F + Ancy1200R for 28S rDNA and 16SF1 + 16SR2 for 16S rDNA (see Table 2).

Successful PCRs, where a single fragment was amplified, were purified using the QIAquick PCR Purification Kit (Qiagen). When several fragments were obtained, the PCR product was run on an agarose gel (1%) containing ethidium bromide and the target fragment excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen).

Automated sequencing was performed directly on purified PCR products using ABI BigDye chemistry following the manufacturer's protocols for cycle sequencing. Several more internal primers for 18S (600R, 600F, 1200R, 1200F) and 28S rDNA (L300F, ECD2) were used to obtain the full sequence on both strands (see Table 2). Sequence reactions were alcohol precipitated and run on an ABI prism 377 automated sequencer. Sequences were assembled and edited using Sequencher v. 3.1.1 (Gene Codes, Ann Arbor, MI).

Alignment

Each data set was aligned separately using Clustal X (Thompson *et al.* 1994, 1997) and adjustments were made by eye using MacClade (Maddison & Maddison 2000).

Table 1 List of monogenean species analysed, their hosts and GenBank sequence accession numbers. *New sequences.

Taxon	Host	Locality	18S	28S	16S
Capsalidae					
<i>Benedenia</i> sp.	Unspecified percid fish		AJ228774	AF382052	
<i>Capsala martinieri</i> Bosc, 1811	<i>Mola mola</i>	UK	AJ276423	AF382053	
<i>Encotyllabe chironemi</i> Robinson, 1961	<i>Chironemus marmoratus</i>	Australia	AJ228780	AF382054	
Monocotylidae					
<i>Calicotyle affinis</i> Scot, 1910	<i>Chimaera monstrosa</i>	Norway	AJ228777	AF382061	
<i>Dictyocotyle coeliaca</i> Nybelin, 1941	<i>Raja radiata</i>	UK	AJ228778	AF382062	
<i>Troglocephalus rhinobatidis</i> Young, 1967	<i>Rhinobatos typus</i>	Australia	AJ228795	AF026110	
Gyrodactylidae					
<i>Gyrodactylus salaris</i> (Malmberg, 1957)			Z26942	AJ132549	
<i>Gyrodactylus rhodei</i> Zitnan, 1964	<i>Rhodeus sericeus</i>	Czech Republic	AJ567670	AJ407933	
<i>Macroglyrodactylus polypteri</i> Malmberg, 1957			AJ567671		
Microbothriidae					
<i>Leptocotyle minor</i> (Monticelli, 1888)	<i>Scyliorhinus canicula</i>	UK	AJ228784	AF382063	
Anoplodiscidae					
<i>Anoplodiscus cirruspiralis</i> Roubal, Armitage & Rohde, 1983	<i>Sparus aurata</i>	Australia	AJ287475	AF382060	
Sundanonchidae					
<i>Sundanonchus micropeltis</i> Lim & Furtado, 1985	<i>Channa micropeltis</i>	Malaysia	AJ287579	AF218122	
Diplectanidae					
<i>Acleotrema</i> sp.	<i>Xyphosus vaigienis</i>	Australia		AF026118	
<i>Diplectanum aequans</i> (Wagener, 1857)	<i>Dicentrarchus labrax</i>	France	AJ276439		
<i>Furnestinia echeneis</i> (Wagener, 1857)	<i>Sparus aurata</i>	France	AF294953	AF131711	
<i>Lamelloglydus elegans</i> Bychowsky, 1957	<i>Diplodus sargus</i>	France	AF294956		
<i>Lamelloglydus erythrini</i> Euzet & Oliver, 1967	<i>Pagellus erythrinus</i>	France	AJ276440		
Pseudomurraytrematidae					
<i>Pseudomurraytrema</i> sp.	<i>Catastomus ardens</i>	USA	AJ228793	AF382059	
Pseudodactylogyridae					
<i>Pseudodactylogyroides apogonis</i> (Yamaguti, 1940)	<i>Apogon semilineatus</i>	Japan	AB065115		
<i>Pseudodactylogyrus anguillae</i> (Yin & Sproston, 1948)			AB060591		
<i>Pseudodactylogyrus bini</i> (Kikuchi, 1929)	<i>Anguilla japonica</i>	Japan	AB065113		
<i>Pseudodactylogyrus haze</i> (Ogawa, 1984)	<i>Acanthogobius flavimanus</i>	Japan	AB065114		
<i>Pseudodactylogyrus</i> sp.	<i>Anguilla</i> sp.	UK	AJ287567	AF382057	
Dactylogyridae					
<i>Aliotrema cribbi</i> Plaisance & Kritsky, 2004	<i>Chaetodon citrinellus</i>	French Polynesia	AY820601*	AY820612*	AY820590*
<i>Ancyrocephalus percae</i> Ergens, 1966	<i>Perca fluviatilis</i>	Finland	AJ490166		
<i>Cichlidogyrus</i> sp.	<i>Tilapia</i> sp.	Malaysia		AF218124	
<i>Cleidodiscus pricei</i> Mueller, 1936	<i>Ictalurus nebulosus</i>	Czech Republic	AJ490168		
<i>Dactylogyrus anchoratus</i> (Dujardin, 1845)	<i>Cyprinus carpio</i>	Czech Republic	AJ490161		
<i>Dactylogyrus difformis</i> Wagener, 1857	<i>Scardinius erythrophthalmus</i>	Czech Republic	AJ490160		
<i>Dactylogyrus lamellatus</i> Achmerov, 1952	<i>Ctenopharyngodon idellus</i>	China	AJ564141	AY307019	
<i>Euryhaliotrematoides annulocirrus</i> (Yamaguti, 1968)	<i>Chaetodon vagabundus</i>	Australia	AY820602*	AY820613*	AY820591*
<i>Euryhaliotrematoides aspistis</i> Plaisance & Kritsky, 2004	<i>Chaetodon vagabundus</i>	Australia	AY820603*	AY820614*	AY820592*
<i>Euryhaliotrematoides berenguelae</i> Plaisance & Kritsky, 2004	<i>Chaetodon citrinellus</i>	French Polynesia	AY820604*	AY820615*	AY820593*
<i>Euryhaliotrematoides grandis</i> (Mizelle & Kritsky, 1969)	<i>Chaetodon vagabundus</i>	Palau	AY820605*	AY820616*	AY820594*
<i>Euryhaliotrematoides microphallus</i> (Yamaguti, 1968)	<i>Heniochus chrysostomus</i>	Palau	AY820606*	AY820617*	AY820595*
<i>Euryhaliotrematoides pirulum</i> Plaisance & Kritsky, 2004	<i>Chaetodon lunula</i>	French Polynesia	AY820607*	AY820618*	AY820596*
<i>Euryhaliotrematoides triangulovagina</i> (Yamaguti, 1968)	<i>Chaetodon kleinii</i>	Palau	AY820608*	AY820619*	AY820597*
<i>Haliotrema angelopterum</i> Plaisance, Bouamer & Morand, 2004	<i>Chaetodon kleinii</i>	Palau	AY820609*	AY820620*	AY820598*
<i>Haliotrema aurigae</i> (Yamaguti, 1968)	<i>Chaetodon auriga</i>	Australia	AY820610*	AY820621*	AY820599*
<i>Haliotrema scyphovagina</i> Yamaguti, 1968	<i>Forcipiger flavissimus</i>	French Polynesia	AY820611*	AY820622*	AY820600*
<i>Euryhaliotrema chrysotaeniae</i> Young, 1968	<i>Lutjanus carponotatus</i>	Australia		AF026115	
<i>Ligophorus mugilinus</i> (Hargis, 1955)	<i>Mugil cephalus</i>	France		AF131710	
<i>Pseudohaliotrema sphincteroporos</i> Yamaguti, 1953	<i>Siganus doliatus</i>	Australia	AJ287568	AF382058	
<i>Tetrancistrum</i> sp.	<i>Siganus fuscescens</i>	Australia		AF026114	
<i>Thaparocleidus siluri</i> (Zandt, 1924)	<i>Silurus glanis</i>	Czech Republic	AJ490164		
<i>Thaparocleidus vistulensis</i> (Sivak, 1932)	<i>Silurus glanis</i>	Czech Republic	AJ490165		
<i>Thylacocleidus</i> sp.	<i>Tetraodon fluviatilis</i>	South-East Asia	AJ490169		
<i>Urocleidus similis</i> (Mueller, 1936)	<i>Lepomis gibbosus</i>	Austria	AJ490167		

Table 2 PCR and sequencing primers.

Gene/Primer	Sequence 5'–3'	Source
18S rDNA		
WormA	GCGAATGGCTCATTAATCAG	Littlewood & Olson (2001)
WormB	CTGTGACGACTTTACTTCC	Littlewood & Olson (2001)
1270R	CCGTCAATCCTTTAAGT	Littlewood & Olson (2001)
930F	GCATGGAATAATGGAATAGG	Littlewood & Olson (2001)
600R	ACCGCGGCKGCTGGCACC	Littlewood & Olson (2001)
600F	GGTGCCAGCMGCCGCGGT	Littlewood & Olson (2001)
1200R	GGGCATCACAGACTTG	Littlewood & Olson (2001)
1200F	CAGGTCTGTGATGCC	Littlewood & Olson (2001)
28S rDNA		
Ancy55F	GAGATTAGCCCATCACGAAG	Designed from aligned dactylogyrid sequences
LSU1200R	GCATAGTTCACCATCTTTCGG	Littlewood <i>et al.</i> (2000)
Ancy1200R	CACCATCTTTCGGTCTCAACC	Designed from aligned dactylogyrid sequences
L300F	CAAGTACCGTGAGGAAAAGTTG	Littlewood <i>et al.</i> (2000)
ECD2	CCTTGGTCCGTGTTCAAGACGGG	Littlewood <i>et al.</i> (2000)
16S rDNA		
16S F1	CTATGGTTATAGGGATAC	Designed from aligned dactylogyrid sequences
16S R1	CAGCTTGCTTCGAAAAC	Designed from aligned dactylogyrid sequences
16S R2	CATCGAGGTAGCAACTAAG	Designed from aligned dactylogyrid sequences

The 18S, 28S and 16S rDNA were concatenated in MacClade and regions in which positional homology could not be determined unambiguously were excluded from the analyses. Data partition, character exclusion and taxa sets were defined for 18S and 28S rDNA and the combined data set from 18S, 28S and 16S rDNA.

Phylogenetic analyses

Two studies were conducted with these data. The first used the 18S rDNA data set in an analysis containing 31 ingroup taxa from the Dactylogyrynea and 12 outgroup taxa belonging to the Polyonchoinea (families Anoplodiscidae, Capsalidae, Gyrodactylidae, Microbothriidae, Monocotylidae, Sundanonchidae). The second included individual analyses of the 18S rDNA (31 taxa) and 28S rDNA (22 taxa) data sets and a combined analysis (18S, 28S and 16S rDNA; 37 taxa) of the Dactylogyrynea using the most basal taxa identified from the first analysis (members of the Pseudomurraytremitidae and the Diplectanidae) as a functional outgroup (Watrout & Wheeler 1981).

In the second analysis, representatives of the Polyonchoinea previously used as outgroups were excluded, thus allowing for the alignment and inclusion of additional unambiguous regions (see Table 3).

Individual and combined analyses were conducted using the methods of maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). MP and ML analyses were performed using PAUP* v.4.0b10 (Swofford 2002); BI of phylogeny was estimated using MrBayes (Huelsenbeck & Ronquist 2001). The substitution model for each data partition was evaluated independently using Modeltest v. 3.06 (Posada & Crandall 1998).

Parsimony analyses were performed using a heuristic search (1000 search replicates), random addition sequence and tree-bisection reconnection (TBR) branch swapping. All characters were run unordered with equal weights and gaps were treated as missing data. Nodal support was estimated by bootstrap analysis (100 pseudoreplicates, with 10 random sequence additions each).

ML analyses were performed using a heuristic search with a starting tree (found with Neighbour joining) and tree-bisection reconnection (TBR) branch swapping. Nodal support was estimated by bootstrap analysis (100 pseudoreplicates, NNI swapping using the same parameters as the heuristic search).

In the Bayesian analysis, the substitution model, estimated with ModelTest, differed depending on the data set analysed, but in each case base frequencies were estimated, four chains were used (default temperature) and the analysis was run for 2 million generations with a sampling frequency of 100. Each analysis was repeated twice in order to check the similarity of the likelihood plateau and confirm they represent 'real' optima. Trees from the 'burn-in' generations were discarded and a final 50% majority-rule consensus tree constructed from the remaining trees. Numbers at the interior branches of the majority-rule consensus tree represent posterior probabilities (PP) (Huelsenbeck & Ronquist 2001).

Results

As the topologies obtained from the different analysis methods were similar, we present only the results of the Bayesian analysis and discuss the differences with the ML and MP trees. Data partition and tree statistics for each analysis are shown in Table 3.

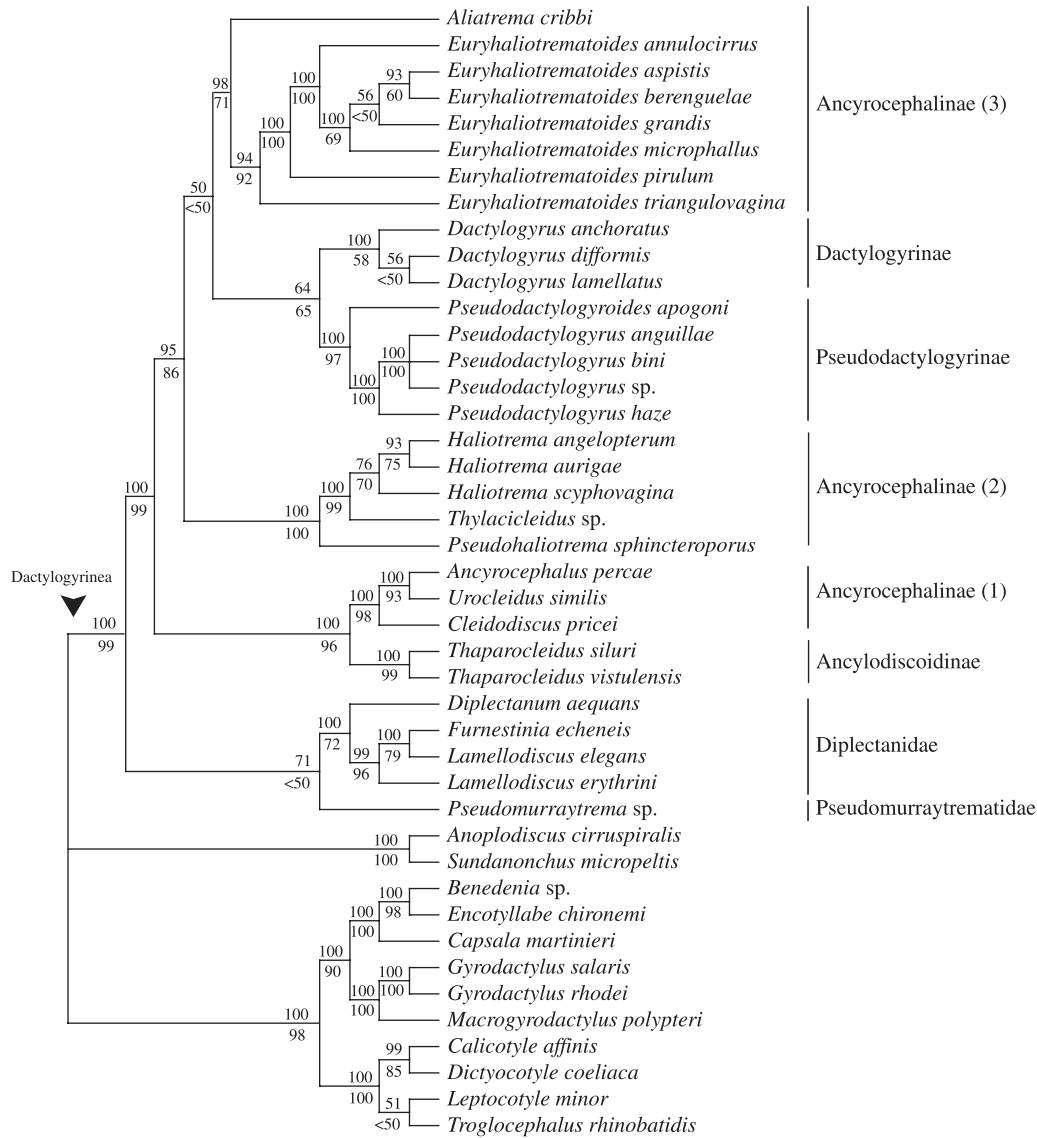


Fig. 1 Molecular phylogeny of the Dactylogyrinea (with outgroup from the families Anoplodiscidae, Capsalidae, Gyrodactylidae, Microbothriidae, Monocotylidae, Sundanonchidae) estimated by Bayesian analysis of gene sequence data of the 18S rDNA. Nodal support shown by posterior probabilities (above) and bootstrap percentages from the parsimony analysis (below).

General phylogenetic analysis of the Dactylogyrinea using 18S rDNA

Figure 1 depicts the result of the Bayesian analysis for the 18S rDNA data set. The different methods used gave a very similar topology, differing mainly by the position of a clade comprising the Dactylogyrinae and Pseudodactylogyrinae. This was sister either to the *Aliatrema/Euryhaliotrematoides* group in the ML and BI analyses or to the *Haliotrema* group in the parsimony analysis. However, neither of these two positions was well supported.

The Dactylogyrinea represents a monophyletic group. The basal clade of this suborder comprises the monophyletic Diplectanidae (PP 100%, bootstrap: 72% MP, 89% ML) and Pseudomurraytrematidae. However, the position of the latter as sister to the former was not well supported (71% BI, < 50% ML and MP). In contrast, the Dactylogyrinae was well supported (100% PP, 91% ML, 99% MP) and within the family, three subfamilies are circumscribed (Ancylodiscoidinae, Dactylogyrinae and Pseudodactylogyrinae). The Ancyrocephalinae is a polyphyletic group represented by three subgroups: the first is sister to the

	18S rDNA	18S rDNA restricted	28S rDNA	Combined analysis
No. characters	2179	2000	807	3466
No. included	1659	1846	524	2929
PARSIMONY				
No. informative sites	437	349	208	725
No. trees	3	2	2	108
Length	1496	1169	753	2454
CI	0.482	0.531	0.529	0.532
RI	0.740	0.713	0.623	0.650
SUBSTITUTION MODEL				
	GTR + I + G	TN + I + G	TN + G	GTR + I + G
MAXIMUM LIKELIHOOD				
Log likelihood (–Ln L)	9864.50	8280.15	4039.65	15554.11
BAYESIAN INFERENCE				
No. trees retained	19800	19850	19950	19800
Mean of Ln L	–9919.5058	–8302.6084	–4044.0243	–15598.4000
Variance	60.3823	43.3584	26.5894	49.8956

Table 3 Data partitions and analysis metrics. Abbreviations for nucleotide substitution models: I + G, estimate of the proportion of invariable sites + gamma distributed among site rate variation; GTR, general time reversible; TN, Tamura–Nei.

Ancylo-discoidinae, the second contains *Haliotrema* species from butterflyfishes together with *Pseudohaliotrema sphincteroporos* and *Thylacicleidus* sp., and the last comprises species of the genera *Aliatrema* and *Euryhaliotrematoides*. Each group was well supported (PP 100%, 100% and 98%, respectively).

Phylogenetic analysis of the Dactylogyridae

Figure 2 depicts Bayesian trees obtained from the separate analyses of 18S rDNA (reduced taxon set) and 28S rDNA. Results of each are described separately below.

Analysis of 18S rDNA using a reduced taxon set. Monogeneans from butterflyfishes did not form a monophyletic lineage and were shown to form two independent lineages. The first contains species from *Euryhaliotrematoides* and *Aliatrema*, and is strongly supported (100% BI, ML, MP). The second group, also very well supported as indicated by the PP of 100%, contains species of *Haliotrema* with, at their base, *Thylacicleidus* sp. and sister to *Pseudohaliotrema sphincteroporos*.

Euryhaliotrematoides does not form a monophyletic group, owing to the position of *Aliatrema cribbi* as sister group to *E. triangulovagina*. However, this relationship is weakly supported (PP 53%) and was not supported by parsimony. The Dactylogyrinae and the Pseudodactylogyrinae represent well-supported monophyletic subfamilies, but their relationships with other Dactylogyridae were not resolved. At the base of these taxa, a monophyletic clade comprising the Ancylo-discoidinae and a subgroup of the Ancyrocephalinae is present.

Analysis of 28S rDNA. As with 18S rDNA, monogeneans from butterflyfishes did not form a monophyletic clade and the same two subgroups are present. The first comprises species of *Aliatrema* and *Euryhaliotrematoides*, which represent a derived group inside a grade containing *Euryhaliotrema chrysoaenia*, *Ligophorus mugilinus* and *Cichlidogyrus* sp. The entire

clade was very well supported and the same relationships are present in the ML and MP trees. In this analysis, *Euryhaliotrematoides* is resolved as a strongly supported monophyletic genus, with *A. cribbi* at its base.

The second group of monogeneans from butterflyfishes contains species of *Haliotrema* sister to a clade containing *Pseudohaliotrema sphincteroporos* (the same relationship was found with the 18S rDNA data) and *Tetrancistrum* sp. This clade is highly supported. The two groups containing parasites from butterflyfishes are sister to the Dactylogyrinae and more basal to the Pseudodactylogyrinae. However, the position of the latter was poorly supported.

Analysis of the combined data. Fig. 3 shows the results of Bayesian analysis of the combined data. Results were congruent with those obtained from each data set analysed separately for relationships between main groups, whereas minor differences were found within some clades.

At the base of the Dactylogyridae, a clade containing the Ancylo-discoidinae, sister to a subgroup of Ancyrocephalinae (*Ancyrocephalus percae*, *Urocleidus similis*, and *Cleidodiscus pricei*) is present. The Dactylogyrinae/Pseudodactylogyrinae form a clade in BI and ML, but this relationship is not resolved in the parsimony analysis. This group is at the base of the other Ancyrocephalinae that form a single clade which is not strongly supported (79% BI, > 50% ML, MP).

Dactylogyrid parasites of butterflyfishes are distributed between two independent lineages. The first contains *Euryhaliotrematoides* and *Aliatrema* and the relationships are congruent with those obtained from the 28S rDNA data set. *Euryhaliotrematoides* is monophyletic. *E. triangulovagina* is the most basal taxon of this genus, sister to a clade containing *E. pirulum* and two diversified clades (*E. annulocirrus* (*E. berenguelae*, *E. microphallus*) (*E. grandis*, *E. aspistis*)). *Aliatrema* is resolved as the sister group to *Euryhaliotrematoides*, with

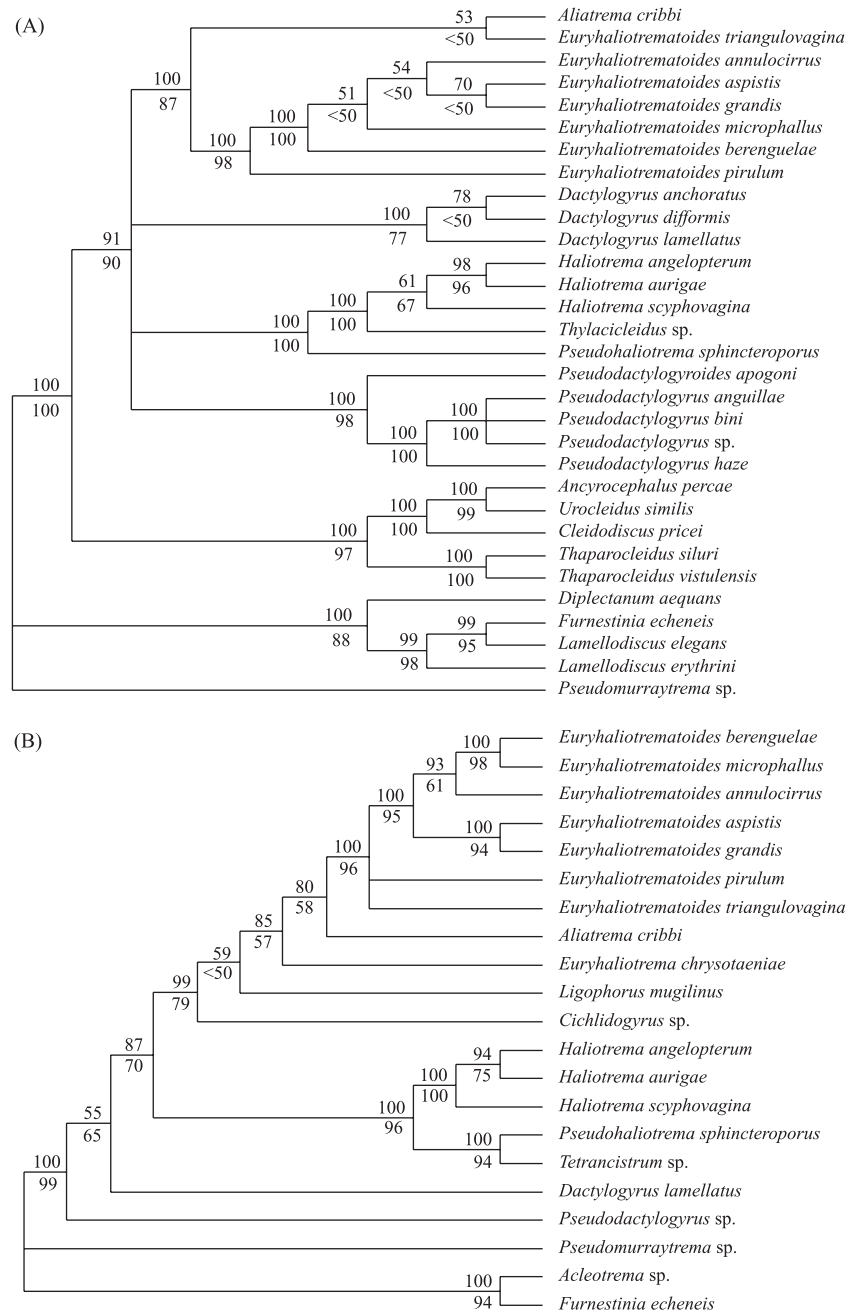


Fig. 2 A, B. Molecular phylogeny of the Dactylogyridae (with the families Diplectanidae and Pseudomurraytrematidae designated as a functional outgroup) estimated by Bayesian analysis. —A. 18S rDNA restricted taxon data set (see text). —B. 28S rDNA. Nodal support shown by posterior probabilities (above) and bootstrap percentages from the parsimony analysis (below).

reasonable support (BI 97%, 52% MP, > 50% ML). Sister to this clade is a grade containing *Euryhaliotrema chrysotaeniae*, *Ligophorus mugilinus* and, at the base, *Cichlidogyrus* sp.

The second group is composed of the monophyletic, but weakly supported, *Haliotrema* species. *Haliotrema* and *Thylacicleidus* are resolved as sister genera, reflecting the result obtained from 18S rDNA data alone. These in turn are resolved as sister to a well supported clade comprising *Pseudohaliotrema sphincteroporos* and *Tetrancistrum* sp., reflecting the 28S rDNA results.

Discussion

Polyphyly of the Ancyrocephalinae

Simková *et al.* (2003) showed, when analysing the phylogenetic relationships of the Dactylogyridae using molecular data, that the Ancyrocephalinae do not form a monophyletic group; they thus confirmed the morphological study of Kritsky & Boeger (1989). Simková *et al.* (2003) identified two subgroups within the Ancyrocephalinae, the first containing *Ancyrocephalus*, *Urocleidus* and *Cleidodiscus* and the second *Pseudohaliotrema* and *Thylacicleidus*. They hypothesized that



Fig. 3 Molecular phylogeny of the Dactylogyrina (with the families Diplectanidae and Pseudomurraytrema designated as a functional outgroup) estimated by Bayesian analysis of the combined sequence data, 18S, 28S and 16S rDNA. Nodal support shown by posterior probabilities (above) and bootstrap percentages from the parsimony analysis (below).

the Ancyrocephalinae should be revised with special emphasis on host distribution, as the first subgroup is present in fresh water from the Northern Hemisphere, and the latter in tropical and subtropical waters.

In our study, the Ancyrocephalinae also represents a polyphyletic subfamily. The first analysis (18S rDNA) showed three subgroups within the subfamily, while the combined analysis showed two. *Ligophorus mugilinus*, a parasite of European freshwater fishes, lies at the base of the tropical marine subgroup. Its position is well supported and the separation of the subgroups corresponding to geographical area is therefore not clear. The Ancyrocephalinae include diverse parasite genera and a revision of this subfamily is needed as it is itself clearly polyphyletic (Kritsky & Boeger 1989; Simková *et al.* 2003). A more comprehensive molecular phylogeny of the Ancyrocephalinae is needed to help circumscribe monophyletic clades within the subfamily.

Phylogeny of butterflyfish monogeneans

The primary aim of the present study was to build a molecular phylogeny of the monogenean species parasitizing chaetodontid fishes. The general conclusion that can be drawn based on the trees obtained is these parasites do not form a monophyletic group. Two independent lineages can be identified, the first containing species of *Aliatrema* and *Euryhaliotrematoides*, the second species of *Haliotrema*.

At the base of the first group is *Euryhaliotrema chrysotaeniae*, a parasite of the Spanish flag snapper, *Lutjanus carponatus* from Australia. This species was originally described in the genus *Haliotrema* by Young (1968) but recently Kritsky & Boeger (2002) changed its status and placed it in *Euryhaliotrema* based on the morphology of the male copulatory organ. The phylogenetic position of *E. chrysotaeniae* (closer to *Euryhaliotrematoides* than *Haliotrema*) validates this revision. The two most basal taxa of the clade are represented by parasites

of freshwater fishes from Europe (*Ligophorus mugilinus*) and Malaysia (*Ciclidogyrus* sp.).

The sister taxon of *Haliotrema* is a species of *Thylacicleidus*, a parasite of an estuarine fish belonging to the Tetraodontidae. At the base of this clade is a monophyletic group comprising *Pseudobaliootrema sphincteroporos* and *Tetrancistrum* sp. Both parasites infest reef-associated perciforms.

The two groups of Ancyrocephalinae are separated from each other by several species, including freshwater parasites. It appears that the monogenean fauna of butterflyfishes did not arise from a single colonization event followed by diversification on the host group. Instead, at least two independent colonization events corresponding to the two parasite clades may explain the actual parasite diversity on chaetodontid fishes.

As *Eurybaliootrema* is specific to the Chaetodontidae, we may conclude that the seven species presently described radiated on butterflyfishes from a single ancestor. In contrast, *Haliotrema* is found on a great range of hosts belonging to 33 families of fishes representing six orders (Kritsky & Stephens 2001); the three species found on butterflyfishes form a monophyletic group, suggesting a single colonization event. However, *Haliotrema* is clearly able to adapt readily to new hosts and the position of *H. scyphovagina* at the base of the genus is not well supported. Moreover, some species have been reported on different families. For example, *H. parabaliotrema* (Mizelle & Price, 1964) is found on fishes from the Zanclidae as well as the Acanthuridae.

The presence of this genus on chaetodontids may be due to a single or several invasions. Only a phylogeny based on greater sampling, especially of the species with hosts close to the Chaetodontidae (e.g. Pomacanthidae, considered to be the sister group to Chaetodontidae; Burgess 1978; Motta 1989), will show whether *H. scyphovagina*, *H. angelopterum* and *H. aurigae* share a common ancestor. If this were confirmed, we may conclude that these taxa diversified on chaetodontids from a single colonization event.

However, if *Haliotrema* spp. found on chaetodontids did not form a monophyletic group it would suggest that they have colonized the family several times. *Aliatrema* – a monotypic genus specific to the Chaetodontidae – was resolved as the sister taxon to *Eurybaliootrematoides*. However, its position remains weakly supported and it is thus difficult to say whether or not it originated from the same colonization event as that giving rise to its sister taxon.

Klassen (1994a,b) tested the monophyly of monogenean parasites of box fishes from the family Ostraciidae based on a morphological phylogeny. His results, like ours, showed that the monogenean community of the fish host family did not form a monophyletic group, but comprised three independent lineages.

Klassen (1994a) also proposed a morphological phylogeny of the species from *Haliotrema* where he included several species from butterflyfishes. *Pseudobaliootrematoides microphallus*, *P. triangulovagina* (both species now belonging to *Eurybaliootrematoides*; Plaisance & Kritsky 2004) and *P. aurigae* (which

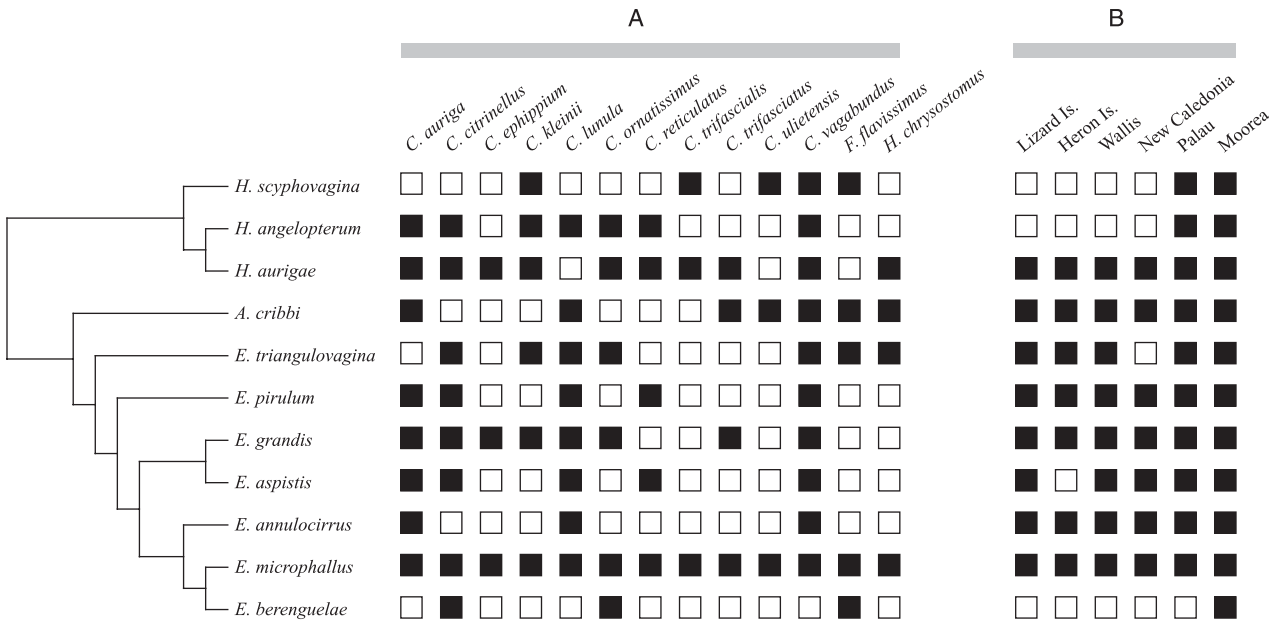


Fig. 4 Structure of the parasite assemblage with regard to (A) host species (C = *Chaetodon*, F = *Forcipiger*, H = *Heniobchus*), and (B) locality. Black squares indicate the presence of the parasite species.

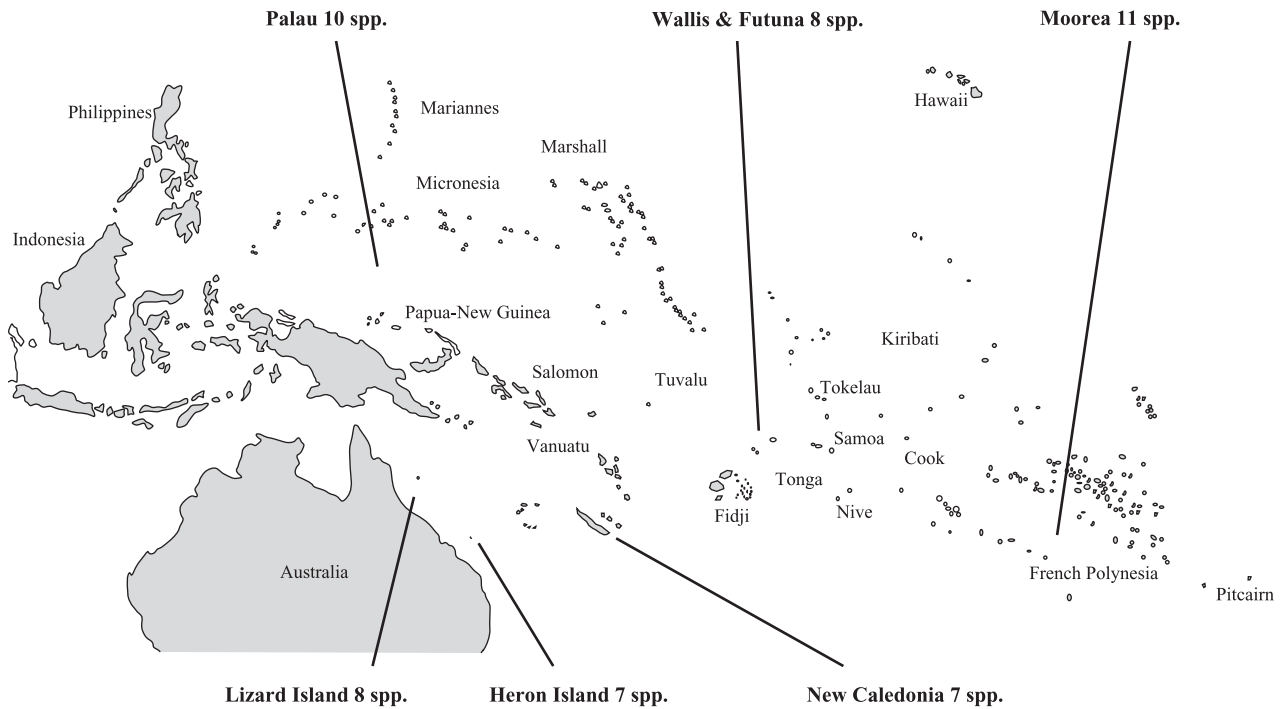


Fig. 5 Indo-West Pacific map showing sampling sites (Moorea, Palau, New Caledonia, Wallis, Lizard Island, Heron Island) and corresponding number of monogenean species.

now belongs to *Haliotrema*; Plaisance *et al.* 2004) were used as outgroups. He included in the ingroup *Haliotrema annulocirrus* (now *Euryhaliotrematoides annulocirrus*; Plaisance & Kritsky 2004), *H. scyphovagina* and *H. flagellatum*, the latter now considered conspecific with *H. scyphovagina* (Plaisance *et al.* 2004). No close relationships between parasites of butterflyfishes were obtained, although his findings may have been due to the limited availability of morphological characters and high levels of homoplasy amongst them. The molecular evidence appears to provide sufficient information to resolve relationships at the generic level.

Origin and diversification of the parasite fauna of butterflyfishes

Diversification of marine parasite faunas has been seldom studied and we know relatively little about the assemblages of parasite faunas with regard to their host and geographical ranges (see Hoberg & Klassen 2002 for a review).

The phylogeny produced in this study provides the foundation from which to examine the historical biogeography of these monogenean parasites. Fig. 4A shows the range of hosts and illustrates the complexity of the host–parasite relationship. These parasites exhibit a low level of specificity, with between three and 13 different host species per parasite. They also have the ability to infect host species of different genera. Cospeciation is considered likely to occur where

monogeneans exhibit relatively high host-specificity (Noble *et al.* 1989). However, Morand *et al.* (2002) showed that they may not be randomly associated with their hosts even when we cannot observe any obvious pattern of coevolution.

It remains very difficult to resolve the origin of the parasite group on chaetodontids because of the complexity of the host–parasite relationship and the numerous host-switching events that may have occurred. A wider range of sampling from other species of Chaetodontidae, and a reliable species-level phylogeny for the fish hosts would provide the basis for accurate cophylogeny mapping.

If we consider the geographical range of the parasite assemblage (Fig. 4B), almost all the species have colonized the six localities intensively, although they are separated by distances of several thousand kilometers. Moorea is the locality where the diversity of the parasite fauna is the highest of the Indo-West Pacific (IWP), with 11 species described from the other IWP islands (Fig. 5). A decrease of the parasite richness from East to West may be observed. This result contrasts with what has been observed for endoparasitic helminths of serranid fishes, which decrease from the Great Barrier Reef toward the Central Pacific (Rigby *et al.* 1997).

This trend parallels the decrease in the diversity of fish and other reef organisms along the same axis. Richness of monogenean parasites of butterflyfishes does not follow the same longitudinal gradient. A second observation is that the parasite

fauna of Moorea is more similar to that of Palau than to that of Wallis, even though the same host species are available in each locality and Wallis is closer to Moorea than the other localities sampled.

The processes of colonization across large expanses of ocean, and to remote islands, are still unclear. We know that monogeneans are not taken with pelagic fish larvae from island to island because chaetodontid larvae are not parasitized (Cribb *et al.* 2000). However, the broad distribution of several parasite species throughout the IWP shows their ability to travel large distances from their centre of origin.

Even though there is no clear biogeographical pattern, hypotheses concerning the geographical origin and routes of colonization of the monogenean fauna of butterflyfishes could be tested at the intraspecific level with some widely distributed species using phylogeography. This could test whether Moorea is a centre of diversification and further resolve the colonization of the Great Barrier Reef through Palau.

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