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Molecular phylogeny and evolution of the Trypanorhyncha Diesing, 1863 (Platyhelminthes: Cestoda)

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ABSTRACT

Complete *ssrDNA* and partial *lsrDNA* (D1–D3) of 31 species, mainly from the Indo-Pacific region, were sequenced and added to 66 species of the marine cestode order Trypanorhyncha; thus 35% of the 277 known species were sampled. The resulting phylogenetic tree resolved two major clades that represent trypanorhynchs originally parasitizing rajiform (skate and ray) or galeoform hosts. The tree topology supports an earlier classification based on morphology that splits the order into the superfamily Eutetrarhynchoidea together with the Tentacularioidea, and the Gymnorhynchoidea together with the Lacistorhynchoidea and Obothrioidea. Three of the five recognized superfamilies are monophyletic (Tentacularioidea, Gymnorhynchoidea, Obothrioidea). Nodal support for the Eutetrarhynchidae and Lacistorhynchidae was poor and resulted in paraphyletic clades. Mapping of morphological characters showed the tentacular armature of the scolex to be highly variable within clades, demonstrating that armature patterns used traditionally in classification, are homoplasious. Similarly, the tetrabothriate scolex, currently utilized as a family-distinguishing character in traditional classifications, has developed independently in multiple groups. Synapomorphies for the higher taxa are detailed. Sequence data from duplicate taxa confirmed interoceanic distribution patterns and low intraspecific genetic divergence and host specificity for nine trypanorhynch species belonging to five families and four superfamilies. Four distinct lineages of trypanorhynchs can be recognized with one mainly infecting rajiform hosts whereas the others infecting both, sharks and rays. Eutetrarhynchoids and tentaculariooids have secondarily invaded shark hosts whilst utilising the marine food web involving teleost fishes as intermediate hosts. Three cases of host switching from sharks to rays can be inferred within the lacistorhynchoids, in one case caused by a switch from perciform to gadiform intermediate hosts. This likely enabled a radiation into the deep sea environment. Implications of the molecular phylogeny for the classification and evolutionary developments within the order are discussed.

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1. Introduction

Trypanorhynch cestodes are amongst the most common metazoan parasites of marine fish. Whilst the adults are typically found in the stomach and intestine of sharks and rays, larval forms infect a wide variety of marine invertebrates and teleosts. Though being widely distributed from brackish waters into the deep sea, the highest species diversity can be found in coastal tropical waters of the Indo-Australian region. Palm (2004) recorded over 20% (54) of the then known species from Indonesia, mainly from a single location on the southern Java coast. Morphological and recent

molecular evidence indicate that larval worms, especially, have low host specificity (Palm and Caira, 2008) and a wide zoogeographical, or even cosmopolitan, distribution (Palm, 2004; Palm et al., 2007). Palm (2007) and Palm and Klimpel (2007) used these cestodes as a model system to better understand the ecology and co-evolutionary history (cumulative evolution) of parasitic life cycles in the marine ecosystem. However, a detailed molecular phylogenetic analysis of the order including details of the host-parasite relationships is still missing.

The cestode order Trypanorhyncha Diesing, 1863 is characterized by a scolex bearing 2 or 4 bothria (Jones et al., 2004), and a tentacular apparatus, consisting of four retractile tentacles adorned with hooks as extensions of tentacle sheaths that are attached to four bulbs (Dollfus, 1942). Antagonistic bulbs and retractor muscles enable evagination and retraction of these structures that serve as holdfasts while the bothria are used for movement. This complex attachment apparatus is unique within the cestodes,

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and provides a strong synapomorphy that supports monophyly of this order. Palm (2004) recognized 254 different trypanorhynch species, and Beveridge and Campbell (2005, 2007), Friggens and Duszynski (2005), Beveridge and Justine (2006, 2007a–c), Campbell and Beveridge (2006a,b, 2007) and Beveridge (2008) have since added 23 more species to the order. Thus, a total of 277 trypanorhynch species can be considered valid, with additional species and genera being described every year.

Taxonomists originally considered scolex shape, number of bothria, and specific organs such as a double set of genitalia and bothrial pits as the most important characters for trypanorhynch systematics (e.g. Southwell, 1929; see Palm, 2004). Vaullegeard (1899), Dollfus (1942) and Campbell and Beveridge (1994) used the tentacular armature as a major character to classify the Trypanorhyncha. Dollfus (1942) also suggested the first classification based on an interpretation of phylogeny; a phylogenetic framework was not revised until Campbell and Beveridge (1994) provided their classification. The latter authors distinguished four different superfamilies with characteristic hook patterns, the Homeacanthoidea Dollfus, 1942, Heteracanthoidea Dollfus, 1942, Obothrioidea Dollfus, 1942 and Poecilacanthoidea Dollfus, 1942. Palm (1995, 1997) considered the tentacular armature as being of minor importance for defining the higher taxa such as superfamilies, and suggested an alternative classification. Special emphasis was given to two characters, the prebulbar organs and bothrial pits, as synapomorphies for the superfamilies Obothrioidea Dollfus, 1942 and Eutetrarhynchoidea Guiart, 1927, respectively. This morphologically based system, although presenting a possible phylogeny for the three recognized superfamilies and 12 families, lacked a detailed phylogenetic framework as emphasised within the earlier classification by Campbell and Beveridge (1994). Beveridge et al. (1999) carried out the first cladistic analysis of the order to test these initial contradictory classifications, but this provided conflicting evidence.

Palm (2004) revised the order Trypanorhyncha, introduced a new family and four new genera, and presented a new classification on the basis of his earlier assumptions. An amended nomenclature for the armature patterns and species descriptions for all then known taxa allowed a more detailed cladistic analysis, mainly supporting recognition of his five superfamilies and 15 families. With the Tentacularioidea Poche, 1926 and Eutetrarhynchoidea Guiart, 1927 forming a separate, earlier divergent clade from the more derived Gymnorhynchoidea Dollfus, 1935 and the modern Lacistorhynchoidea Guiart, 1927 together with the Obothrioidea Dollfus, 1942, the cladistic analysis clearly supported an earlier analysis of the complete small subunit (ssrDNA) and partial large subunit (lsrDNA) ribosomal RNA gene of 13 trypanorhynch genera belonging to eight different families by Olson et al. (2001). The most significant difference between the classification used by Palm (2004), was the position of the Sphyricephalidae together with the Gilquiniidae on a separate clade, distant to the Tentaculariidae.

New sequences herein represent one additional family, six genera and 20 species that have not been analyzed before. Further specimens represent duplicate taxa of trypanorhynchs from different locations; this was done in order to verify species diagnosis, especially of the larval forms, and to detect possible cosmopolitan distribution patterns (also see Palm et al., 2007). A new phylogenetic tree for the Trypanorhyncha including 35% (97) of the 277 known taxa is presented, making this order one of the phylogenetically best resolved and most densely sampled of the marine tapeworm groups, based on morphological and molecular evidence. A combined ssrDNA + lsrDNA dataset is presented in order to evaluate synapomorphic characters proposed in earlier studies; synapomorphies are mapped onto the resulting tree in order to identify the most suitable characters to be used for higher trypanorhynch

classification. Well supported clades are linked to the final elasmobranch hosts. Implications for the classification, zoogeography and evolution of trypanorhynch cestodes including their life cycles are discussed.

2. Materials and methods

2.1. Specimen collection and deposition

From January 2003 to August 2005, 22 trypanorhynch species from Indonesian coastal waters were isolated from teleost and elasmobranch hosts and were fixed and stored in 100% ethanol. The fishes were obtained at fish markets, either in Pelabuhan Ratu, southern coast of West Java (6°59'13 S and 106°32'38 E), or in Kedonganan, Bali (8°45'25 S and 115°10'05 E), Indonesia. Specimens of *Grillotiella exilis* (Linton, 1909) Palm, 2004 were obtained from the southern Sulawesi coast (Palm, 2008). Seven species were collected from Oahu, Hawaii, in October 2007. The examination of the teleosts followed a standard protocol (Palm et al., 1998a; Palm, 2004). Three additional species, *Obothrium cysticum* (Mayer, 1842), *Gilquinia squali* (Fabricius, 1794) and *Lacistorhynchus tenuis* (van Beneden, 1858) were collected from the North American coast off Massachusetts in June 2007 and in 1997 (*L. tenuis*). A specimen of *G. squali* ex *S. acanthias* was collected off the New Zealand coast. All samples were stored at 4 °C until subsequent analysis in the laboratory. A detailed list of taxa used in the study is shown in Tables 1 and 2.

Where possible, voucher specimens were designated for the sample used for gDNA extraction. In the case of adult worms, the scolex was stained in acetic carmine and prepared as whole mount specimens, with the strobila being used for molecular analyses. In the case of larger larval trypanorhynchs, the anterior portion of the worms, including the tentacular apparatus, was stained and mounted in Canada balsam. Otherwise, in the case of small specimens, whole-mounted specimens from the same collection were kept as voucher material. The species were identified by using the method of Palm (2004). A full list of taxa and their source is presented in Tables 1 and 2.

2.2. DNA amplification and sequencing

Total genomic DNA (gDNA) was extracted using DNeasy™ Tissue Kit (QIAGEN) following the standard manufacturer-recommended protocol. Two microliters gDNA (measured on a NanoDrop 1000; Thermo Scientific) were used as template in 25 µl reactions using Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech). Partial lsrDNA was amplified using primers ZX-1 (5'-ACCCGCTGAATTAAGCATAT-3'; modified from Van der Auwera et al., 1994) and 1500R (5'-GCTATCCTGAGGGAACTTCG-3'), using the following cycling conditions: denaturation for 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, 2 min at 72 °C; and 7 min extension at 72 °C. Complete ssrDNA rDNA was amplified using primers WormA (5'-GCCAATGGCTCATTAAATCAG-3') and WormB (5'-CTTGTTACGACTTTTACTTC-3') (Littlewood and Olson, 2001) using the following cycling conditions: denaturation for 2 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 54 °C, 2 min at 72 °C; and 7 min extension at 72 °C. PCR amplicons were either gel-excised using QIAquick™ Gel Extraction Kit (QIAGEN) or purified directly using QIAquick™ PCR Purification Kit (QIAGEN) following the standard manufacturer-recommended protocol, cycle-sequenced from both strands using ABI BigDye™ chemistry, alcohol-precipitated and run on an ABI 3730 DNA Analyzer (version 1.1). lsrDNA products were sequenced using the two PCR primers and internal primers 300F (5'-CAAGTACCGTGAGGGAAAGTTG-3'), ECD2 (5'-CTTGGTCCGTGTTCAAGACGGG-3'), 400R (5'-GCAGCTTGACTACCCCG-3') and 1090F (5'-TGAACACGGACCAAG-3').

Table 1

Taxonomic listing of trypanorhynch species, with information on sampling, voucher deposition and GenBank accession numbers. The classification follows Palm (2004). Abbreviations: adult (ad), larva (pl), rajiform (r), galeoform (g), teleost (t), body cavity (bc), gills (gi), head (he), mesentery (mes), musculature (m), spiral valve (sv), stomach (st), stomach wall (sw), viscera (v), Borneo (BO), Kedonganan, Bali (KD), Makassar, Sulawesi (SU), Pelabuhan Ratu, Java (PR), Gulf Coast Research Laboratory, Mississippi (GCLR), Lawrence R. Penner Collection (LRP), Natural History Museum Berlin (ZMB), Natural History Museum London (B), Queensland Museum (QM), South Australian Museum (SAM).

Taxon sequenced				Collection	Museum	GenBank	
(stage, host species, collection locality, site, date, voucher specimen, GenBank deposition no.)				Date	Voucher	SSU	LSU
Diphylloidea van Beneden in Carus, 1863 (Outgroup)							
Ditrachybothriidae Rees (1959)							
1 <i>Ditrachybothrium macrocephalum</i> (Dibm2, ad)	ex. <i>Apristurus laurussonii</i> (g)	NE Atlantic	sv	19.10.2002	B 2004.1.6.1-5	DQ642903	AY584864
Echinobothriidae Perrier (1897)							
2 <i>Echinobothrium chisholmae</i> (Eho, ad)	ex. <i>Rhinobatos typus</i> (r)	Australia, Qld	sv	July 1998	B 2000.8.3.4-7	AF286986	AF286922
3 <i>Echinobothrium harfordi</i> (Ehar, ad)	ex. <i>Rhinobatos naevus</i> (r)	North Sea, UK	sv	—	B 2001.1.23.4-7	AF286986	AF286922
4 <i>Echinobothrium</i> sp. (Echb, ad)	ex. <i>Raja</i> sp. (r)	NE Atlantic	sv	01.10.2002	B 2003.3.6.23-27	DQ642904	AY584862
Macrobothriidae Khalil and Abdul-Salam (1989)							
5 <i>Macrobothrium rhynchobati</i> (Mac, ad)	ex. <i>Rhinobatos typus</i> (r)	Australia, NT	sv	08.07.2001	B 2004.3.18.101	AF124463	AY584861
Trypanorhyncha Diesing (1863) (Ingroup)							
Tentacularioidea Poche (1926)							
Tentaculariidae Poche (1926)							
1 <i>Tentacularia coryphaenae</i> (Hp16, pl)	ex. <i>Katsuwonus pelamis</i> (t)	Indonesia, PR	bc	29.01.2003	see duplicate	FJ572890	EF095269
2 <i>Nybelinia cf aequidentata</i> (Nyb7, BO-83-02, ad)	ex. <i>Rhinoptera neglecta</i> (r)	Malaysia, BO	sv	26.06.2002	LRP	DQ642952	DQ642790
3 <i>Nybelinia cf africana</i> (Nyb5, BO-74-01, ad)	ex. <i>Lamiopsis temmincki</i> (g)	Malaysia, BO	sv	08-14.06.2002	LRP	DQ642948	DQ642786
4 <i>Nybelinia cf africana</i> (Hp23, pl)	ex. <i>Gempylus serpens</i> (t)	Indonesia, PR	sw	22.01.2004	—	FJ572892	FJ572928
5 <i>Nybelinia indica</i> (HP41, pl)	ex. <i>Heteropriacanthus cruenatus</i> (t)	Hawaii	sw	14.10.2007	ZMB 7434 a-b	FJ572894	FJ572930
6 <i>Nybelinia queenslandensis</i> (Nyq, ad)	ex. <i>Carcharhinus melanopterus</i> (g)	Australia, Qld	—	February 2002	QM G217521-31	AF287005	AF286975
7 <i>Nybelinia sphyrynae</i> (Nyb4, BO-69-01, ad)	ex. <i>Sphyryna lewini</i> (g)	Malaysia, BO	—	08-14.06.2002	LRP	DQ642953	DQ642791
8 <i>Nybelinia surmenicola</i> (Hp49, pl)	ex. <i>Pleurogrammus azonus</i> (t)	Japan	m	2007	—	FJ572893	FJ572929
9 <i>Heteronybelinia cf estigmene</i> (Nyb3, BO-58-01, ad)	ex. <i>Carcharhinus limbatus</i> (g)	Malaysia, BO	—	11.06.2002	LRP	DQ642951	DQ642789
10 <i>Heteronybelinia cf estigmene</i> (Hp21, pl)	ex. <i>Coryphaenae hippurus</i> (t)	Indonesia, KED	sw	04.07.2005	ZMB 7428	FJ572895	FJ572931
11 <i>Heteronybelinia yamagutii</i> (Hp11, pl)	ex. <i>Promethichthys prometheus</i> (t)	Indonesia, PR	sw	05.02.2005	ZMB 7372	FJ572896	FJ572932
12 <i>Mixonybelinia lepturi</i> (Hp32, pl)	ex. <i>Gempylus serpens</i> (t)	Indonesia, PR	sw	22.01.2004	see duplicate	see duplicate	FJ572933
13 <i>Kotorella pronosoma</i> (OVR-08, ad)	ex. <i>Dasyatis say</i> (r)	Gulf of Mexico	sw	19.04.1999	—	DQ642950	DQ642788
14 <i>Kotorella pronosoma</i> (Hp12, ad)	ex. <i>Dasyatis tethydis</i> (r)	Indonesia, PR	st	23.01.2004	ZMB 7373	FJ572899	FJ572935
15 <i>Kotorella</i> sp. nov. (Nyb8, BO-87-01, ad)	ex. <i>Taeniura lymma</i> (r)	Malaysia, BO	—	June 2002	LRP	DQ642949	DQ642787
Sphyriocephalidae Pintner (1913)							
16 <i>Sphyriocephalus viridis</i> (Hp2, pl)	ex. <i>Dalatias lichia</i> (g)	Indonesia, PR	st	03.02.2005	—	FJ572904	FJ572940
17 <i>Heterosphyriocephalus oheolumiae</i> (Hp13, pl)	ex. <i>Taractichthys steindachneri</i> (t)	Indonesia, PR	bc	03.02.2005	ZMB 7433	FJ572905	FJ572941
18 <i>Hepatoxylon trichiuri</i> (Hp17, pl)	ex. <i>Taractes rubescens</i> (t)	Indonesia, PR	st	01.02.2005	—	FJ572907	FJ572943
Gymnorhynchoidea Dollfus (1935)							
Aporhynchidae Poche (1926)							
19 <i>Aporhynchus tasmaniensis</i> (Apor, TE-157, ad)	ex. <i>Etmopterus spinax</i> (g)	Azores	sv	27.05.2006	LRP 4279	FJ572911	FJ572947
Gilquiniidae Dollfus (1942)							
20 <i>Gilquinia robertsoni</i> (HP10, ad)	ex. <i>Squalus megalops</i> white belly (g)	Indonesia, PR	sv	02.02.2005	—	FJ572908	FJ572944
21 <i>Gilquinia squali</i> (HP39, RDM-17, ad)	ex. <i>Squalus acanthias</i> (g)	Rhode Is., USA	sv	30.05.2007	—	FJ572909	FJ572945
22 <i>Saggitirhynchus aculeatus</i> (Gng1, ad)	ex. <i>Centrophorus</i> sp. (g)	New Caledonia	sv	31.01.2002	MNHN JN 70A	DQ642907	DQ642745
23 <i>Vittirhynchus squali</i> (Gng2, ad)	ex. <i>Squalus melanurus</i> (g)	New Caledonia	sv	30.01.2002	MNHN JN 73A	DQ642905	DQ642743
Rhopalothylacidae Guiart (1935)							
24 <i>Pintneriella muscolicola</i> (Hp1, ad)	ex. <i>Odontaspis ferox</i> (g)	Indonesia, PR	sv	01.02.2005	ZMB 7368	FJ572912	FJ572948
Gymnorhynchidae Dollfus (1935)							
25 <i>Molicola uncinatus</i> (Moli, pl)	ex. <i>Thyrstites atun</i> (t)	Australia, Vic	m	09.01.2000	B 2004.3.18.102	DQ642908	DQ642746
26 <i>Molicola</i> sp. (blastocyst, Hp5, pl)	ex. <i>Taractes rubescens</i> (t)	Indonesia, PR	m	01.02.2005	ZMB 7366 a-c	FJ572913	FJ572949
27 <i>Gymnorhynchus isuri</i> (Gymn, IF-4, ad)	ex. <i>Isurus oxyrinchus</i> (g)	New York, USA	sv	27.06.2003	LRP 3711	DQ642909	DQ642747
28 <i>Chimaerarhynchus rougetae</i> (Chir, ad)	ex. <i>Squalus cf megalops</i> (g)	New Caledonia	sv	26.01.2002	MNHN JN 4A	DQ642906	DQ642744
Lacistorhynchoidea Guiart (1927)							
Pterobothriidae Pintner (1931)							
29 <i>Pterobothrium lintoni</i> (Plin, pl)	ex. <i>Choerodon venustus</i> (t)	Australia, Qld	m	01.07.1998	SAM V4080	AJ287004	AF286973
30 <i>Pterobothrium</i> sp. (Ptpl1, NT-96, ad)	ex. <i>Himantura</i> sp. (r)	Australia, NT	sv	11-22.11.1999	LRP 3680-2	DQ642926	DQ642764
Lacistorhynchidae Guiart (1927)							
Grillotiinae Dollfus (1942)							
31 <i>Dasyrhynchus variouuncinnatus</i> (Hp19, pl)	ex. <i>Caranx sexfasciatus</i> (t)	Indonesia, PR	he	04.07.2006	see duplicate	FJ572914	FJ572950

(continued on next page)

Table 1 (continued)

Taxon sequenced (stage, host species, collection locality, site, date, voucher specimen, GenBank deposition no.)				Collection	Museum	GenBank		
				Date	Voucher	SSU	LSU	
32 <i>Pseudogilquinia microbothria</i> (Dmag1, NT-112, ad)	ex. <i>Sphyrna mokarran</i> (g)	Australia, NT	sv	21.11.1999	LRP 3706-8	DQ642928	DQ642766	
33 <i>Pseudogilquinia pillersi</i> (Das, pl)	ex. <i>Lethrinus atkinsoni</i> (t)	Australia, Qld	bc	11.01.1998	B 2004.3.18.98-99	AJ287496	AF286964	
34 <i>Grillotiella exile</i> (Hp30, pl)	ex. <i>Scomberomorus commerson</i> (t)	Indonesia, SU	gi	January 2004	ZMB 7431	FJ572917	FJ572953	
35 <i>Protogrillotia</i> sp. (NT-113A, ad)	ex. <i>Carcharhinus amboinensis</i> (g)	Australia, NT	sv	11-22.11.1999	SAM 28646	DQ642929	DQ642767	
36 <i>Grillotia erinaceus</i> (Geri, ad)	ex. <i>Raja radiata</i> (r)	North Sea, Scot	sp	21.05.1990	–	AJ228781	AF286967	
37 <i>Grillotia pristiophori</i> (GripA, ad)	ex. <i>Pristiophorus nudipinnis</i> (g)	Australia, Vic	sv	01.01.2001	SAM 28386	DQ642925	DQ642763	
38 <i>Grillotia rowei</i> (1393536, pl)	ex. <i>Coryphaenoides armatus</i> (t)	North Atlantic	v	15.04.2001	B 2003.3.7.1-15	DQ642927	DQ642765	
39 <i>Grillotia yuniariae</i> (Hp7, pl)	ex. unid. Ophiidae (t)	Indonesia, PR	bc	23.01.2004	–	FJ572916	FJ572952	
40 <i>Paragrillotia similis</i> (Te 51, ad)	ex. <i>Ginglyostoma cirratum</i> (g)	Florida, USA	sv	05.02.1993	LRP 4280	FJ572918	FJ572954	
Lacistorhynchinae Dollfus (1942)								
41 <i>Pseudolacistorhynchus heronensis</i> (Ghe, pl)	ex. <i>Plectropomus leopardus</i> (t)	Australia, Qld	bc	19.01.1998	QM G217515-7	AJ287519	AF286968	
42 <i>Lacistorhynchus dollfus</i> (Ldol, ad)	ex. <i>Mustelus antarcticus</i> (g)	Australia, Vic	sv	15.01.2000	B 2001.1.23.8-13	DQ642923	DQ642761	
43 <i>Lacistorhynchus tenuis</i> (Hp38, VJ-4, ad)	ex. <i>Mustelus canis</i> (g)	NE Atlantic, USA	sv	13.01-06.02.1997	–	FJ572919	FJ572955	
44 <i>Hornelliella annandalei</i> (Hp4, ad)	ex. <i>Stegostoma fasciatum</i> (g)	Indonesia, PR	sv	15.04.2004	ZMB 7367 a-b	FJ572920	FJ572956	
45 <i>Callitetrarhynchus gracilis</i> (Hp15, pl)	ex. <i>Scomberomorus commerson</i> (t)	Indonesia, KED	bc	12.01.2005	–	FJ572921	FJ572957	
46 <i>Callitetrarhynchus gracilis</i> (Ctg, ad)	ex. <i>Carcharhinus melanopterus</i> (g)	Australia, Qld	sv	January 1998	QM G217612	AJ287487	AF286970	
47 <i>Floriceps minacanthus</i> (Flo, pl)	ex. <i>Euthynnus affinis</i> (t)	Australia, Qld	bc	January 1998	QM G217612	AF287003	AF286971	
48 <i>Floriceps saccatus</i> (Hp3, pl)	ex. <i>Lepidocybium flavobrunneum</i> (t)	Indonesia, PR	bc	02.08.2005	–	FJ572922	FJ572958	
49 <i>Diesingium lomentaceum</i> (Dies, SE-99, ad)	ex. <i>Mustelus mustelus</i> (g)	Senegal	sv	10-17.01.2003	LRP 3713	DQ642922	DQ642760	
Otobothrioidea Dollfus (1942)								
Pseudotobothriidae Palm (1995)								
50 <i>Parotobothrium balli</i> (Hp8, pl)	ex. <i>Caranx sexfasciatus</i> (t)	Indonesia, PR	sw	04.02.2005	ZMB 7370	FJ572923	FJ572959	
51 <i>Parotobothrium balli</i> (Paran, pl)	ex. <i>Chaetodon lunula</i> (t)	Australia, West	v	01.07.2003	B 2008.5.21.5	DQ642918	DQ642756	
52 <i>Pseudotobothrium arii</i> (Poto, BO-74-02, ad)	ex. <i>Lamioptis temminckii</i> (g)	Malaysia, BO	sp	08-14.06.2002	LRP 4277	DQ642910	DQ642748	
53 <i>Pseudotobothrium dipsacum</i> (Otb3, pl)	ex. <i>Choerodon venustus</i> (t)	Australia, Qld	bc	01.01.1998	–	AJ287552	AF286972	
Otobothriidae Dollfus (1942)								
54 <i>Symbothrorhynchus tigaminacantha</i> (HP14, pl)	ex. <i>Uranoscopus</i> sp. II (t)	Indonesia, PR	bc	16.04.2004	ZMB 7427	FJ572924	FJ572960	
55 <i>Proemotobothrium linstowi</i> (Prol, NT-49, ad)	ex. <i>Rhynchobatus djiddensis</i> (r)	Australia, NT	sv	11-22.11.1999	LRP 3669-72	DQ642917	DQ642755	
56 <i>Proemotobothrium</i> sp. (Proe, NT-33, ad)	ex. <i>Himantura jenkinsii</i> (r)	Australia, NT	sv	11-22.11.1999	LRP 4282	DQ642915	DQ642753	
57 <i>Otobothrium carcharidis</i> (Ocar1, NT-53A, ad)	ex. <i>Carcharhinus dussumieri</i> (g)	Australia, NT	sv	11-22.11.1999	LRP 3675-7	DQ642911	DQ642749	
58 <i>Otobothrium cysticum</i> (Hp37, pl)	ex. <i>Peprilus triacanthus</i> (t)	Rhode Is., USA	m	30.05.2007	–	FJ572926	FJ572962	
59 <i>Otobothrium mugilis</i> (NT-99A, ad)	ex. <i>Sphyrna mokarran</i> (g)	Australia, NT	sv	11-22.11.1999	LRP 4276	DQ642912	DQ642750	
60 <i>Otobothrium penetrans</i> (Hp6, pl)	ex. <i>Tylosurus crocodiles</i> (t)	Indonesia, PR	my	02.02.2005	ZMB 7369 a-b	FJ572925	FJ572961	
61 <i>Otobothrium propeycysticum</i> (Ontsp, NT-112E, ad)	ex. <i>Sphyrna mokarran</i> (g)	Australia, NT	sv	11-22.11.1999	SAM 29101	DQ642913	DQ642751	
62 <i>Fossobothrium perplexum</i> (Onsp1A, NT-65A, ad)	ex. <i>Anoxypristis scipadate</i> (r)	Australia, NT	sv	11-22.11.1999	LRP 3714	DQ642914	DQ642752	
63 <i>lobobothrium elegans</i> (NT-33A, ad)	ex. <i>Himantura jenkinsii</i> (r)	Australia, NT	sv	11-22.11.1999	SAM 28634	DQ642916	DQ642754	
Eutetrarhynchoidea Guiart (1927)								
Rhinoptericolidae Carvajal and Campbell (1975)								
64 <i>Shirleyrhynchus aetobatis</i> (Shir, BO-82-01, ad)	ex. <i>Himantura uarnak</i> (r)	Malaysia, BO	sv	26.06.2002	LRP 4275	DQ642938	DQ642776	
65 <i>Rhinoptericola megacantha</i> (DNA-01-100L, ad)	ex. <i>Rhinoptera bonasus</i> (r)	Gulf of Mexico	sv	13.03.2001	B 2008.5.21.1	DQ642954	DQ642792	
66 Unidentified gen. nov. sp. nov. (Hp 47, pl)	ex. <i>Heteropriacanthus cruenatus</i> (t)	Hawaii	sv	25.10.2007	ZMB 7439	FJ572903	FJ572939	
Mixodigmatidae Dailey and Vogelbein (1982)								
67 <i>Halysiorhynchus macrocephalus</i> (Haly2, NT-44, ad)	ex. <i>Pastinachus sephen</i> (r)	Australia, NT	sv	11-22.11.1999	LRP 3663-6, 8	DQ642940	DQ642778	
68 <i>Trygonicola macroporus</i> (Tryg, BO-23-02, ad)	ex. <i>Himantura gerrardi</i> (r)	Malaysia, BO	sv	02-04.06.2002	LRP 4274	DQ642941	DQ642779	
Eutetrarhynchidae Guiart (1927)								
69 <i>Tetrarhynchobothrium</i> sp. (Hp9, ad)	ex. <i>Pastinachus sephen</i> (r)	Indonesia, PR	sv	02.02.2005	ZMB 7371	FJ572900	FJ572936	
70 <i>Tetrarhynchobothrium</i> sp. (Trhy, BO-23-01, ad)	ex. <i>Himantura gerrardi</i> (r)	Malaysia, BO	sv	03.06.2002	LRP 4273	DQ642960	DQ642798	
71 <i>Tetrarhynchobothrium</i> sp. (Ttm, ad)	ex. <i>Carcharhinus melanopterus</i> (g)	Australia, Qld	sv	14.01.1998	B 2001.1.26.1	AF287002	AF286965	
72 <i>Dollfusiella geraschmidti</i> (Dfsp, ad)	ex. <i>Urolophus paucimaculatus</i> (r)	Australia, Vic	sv	25.02.1999	B 2001.1.25.6-7	DQ642955	DQ642793	
73 <i>Dollfusiella martini</i> (Dolm, ad)	ex. <i>Trygonorhina fasciata</i> (r)	Australia, Vic	sv	16-25.02.1999	B 2001.1.25.2-4	DQ642964	DQ642802	
74 <i>Dollfusiella michiae</i> (Dmic1, NT-103A, ad)	ex. <i>Rhina ancylostoma</i> (r)	Australia, NT	sv	11-22.11.1999	LRP 3683-4	DQ642966	DQ642804	
75 <i>Dollfusiella spinulifera</i> (Doll, ad)	ex. <i>Rhinobatus typus</i> (r)	Australia, Qld	sv	January 1996	B 1999.9.16.1-2	DQ642965	DQ642803	
76 <i>Dollfusiella tenuispinis</i> (DNA-00-88, ad)	ex. <i>Dasyatis sabina</i> (r)	Gulf of Mexico	sv	–	B 2008.5.21.2	DQ642958	DQ642796	
77 <i>Dollfusiella</i> sp. (Doca1-2, NT-33C, ad)	ex. <i>Himantura jenkinsii</i> (r)	Australia, NT	sv	11-22.11.1999	LRP 3661-2	DQ642961	DQ642799	
78 <i>Dollfusiella</i> sp. (Dolb, BO-24-01, ad)	ex. <i>Himantura fava</i> (r)	Malaysia, BO	sv	02-04.06.2002	LRP 4269	DQ642959	DQ642797	
79 <i>Dollfusiella</i> sp. (Dolb3, BO-76-02, ad)	ex. <i>Himantura pastinacoides</i> (r)	Malaysia, BO	sv	21.06.2002	LRP 4270	DQ642962	DQ642800	

Table 3

Comparison of *ssrDNA* and *lsrDNA* sequences of conspecific trypanorhynch cestodes from different hosts or/and different regions (adult, ad; plerocercus/oid, pl). Indian, Pacific and Atlantic refer to respective oceans.

Taxon	ad/pl	<i>ssrDNA</i>			<i>lsrDNA</i> (D1–D3)		
		Length (bp)	Differences		Length (bp)	Differences	
			Total	Indels		Total	Indels
Eutetrarhynchoidea							
Eutetrarhynchidae							
<i>Prochristianella</i> sp. (Gulf of Mexico, ex <i>D. sabina</i>)	ad						
<i>Prochristianella</i> sp. (Gulf of Mexico, ex <i>D. sabina</i>)	ad	1841	0	0	1057	0	0
<i>Oncomegoides celatus</i> (Indian/Pacific, ex <i>D. microps</i>)	ad						
<i>Oncomegoides celatus</i> (Indian/Pacific, ex <i>H. jenkinsii</i>)	ad	1924	0	0	1105	0	0
Tentacularioidea							
Tentaculariidae							
<i>Tentacularia coryphaenae</i> (Indian, ex <i>K. pelamys</i>)	pl						
<i>T. coryphaenae</i> (Atlantic, ex <i>P. glauca</i>)	ad	1980	18*	10	856	1	0
<i>T. coryphaenae</i> (Indian, ex <i>C. hippurus</i>)	pl	—	—	—	856	0	0
<i>T. coryphaenae</i> (Indian, ex <i>L. flavobrunneum</i>)	pl	—	—	—	856	0	0
<i>T. coryphaenae</i> (Indian, ex <i>P. prometheus</i>)	pl	—	—	—	856	0	0
<i>T. coryphaenae</i> (Indian, ex <i>T. lepturus</i>)	pl	—	—	—	856	0	0
<i>T. coryphaenae</i> (Pacific, ex <i>S. crumenophthalmus</i>)	pl	1980	0	0	856	0	0
<i>Mixonybelinia lepturi</i> (Indian, ex <i>G. serpens</i>)	pl						
<i>M. lepturi</i> (Pacific, ex <i>H. cruenatus</i>)	pl	1967	0	0	1499	0	0
Gymnorhynchoidea							
Gilquiniidae							
<i>Gilquinia squali</i> (NW Atlantic, ex <i>S. acanthias</i>)	ad						
<i>G. squali</i> (NE Atlantic, ex <i>S. acanthias</i>)	ad	1979	1	1	1248	1	1
<i>G. squali</i> (Pacific, ex <i>S. acanthias</i>)	ad	1979	4	0	1248	0	0
Sphyriocephalidae							
<i>Sphyriocephalus viridis</i> (Indian, ex <i>D. lichia</i>)	ad						
<i>S. viridis</i> (Atlantic, ex <i>D. lichia</i>)	ad	2052	0	0	1565	0	0
<i>Heterosphyriocephalus oheolumiae</i> (Indian, ex <i>T. steindachneri</i>)	pl						
<i>H. oheolumiae</i> (Indian, ex <i>T. rubescens</i>)	pl	2027	0	0	1553	0	0
<i>Hepatoxylon trichiuri</i> (Indian, ex <i>T. rubescens</i>)	pl						
<i>H. trichiuri</i> (Atlantic, ex <i>P. glauca</i>)	ad	[1994	39*	26]	1237	0	0
Lacistorhynchoidea							
Lacistorhynchidae							
<i>Callitetrarhynchus gracilis</i> (Indian, ex <i>S. commerson</i>)	pl						
<i>C. gracilis</i> (Indian/Pacific, ex <i>C. amboiensis</i>)	ad	1951	0	0	1177	4	4
<i>C. gracilis</i> (Indian, ex <i>L. nebulosus</i>)	pl	1951	0	0	1177	3	3
<i>C. gracilis</i> (Pacific, ex <i>C. melanopterus</i>)	ad	[1951	40*	4]	1177	3	3
<i>Floriceps saccatus</i> (Indian, ex <i>L. flavobrunneum</i>)	pl						
<i>F. saccatus</i> (Pacific, ex <i>D. hystrix</i>)	pl	1932	0	0	1307	0	0
<i>Dasyrhynchus variouncinatus</i> (Indian, ex <i>C. sexfasciatus</i>)	pl						
<i>D. variouncinatus</i> (Pacific, ex <i>C. melampygyus</i>)	pl	2015	0	0	1532	0	0
<i>Hormelliella annandalei</i> (Indian, ex <i>S. fasciatum</i>)	ad						
<i>H. annandalei</i> (Indian/Pacific, ex <i>S. fasciatum</i>)	ad	1963	0	0	1305	21*	3

* Discrepancy between *ssrDNA* and *lsrDNA* likely caused by sequencing errors; *ssrDNA* results in square brackets indicate older sequences determined using less accurate technology and should therefore be viewed with caution, or disregarded, in comparisons within species.

3.3. Molecular data and phylogenetic analyses

ssrDNA sequence length ranged from 1860 to 2127 bp and *lsrDNA* sequence length ranged from 1065 to 1611 bp. The concatenated sequence alignment consisted of the following partitions: *ssrDNA*—comprising 2200 positions of which 446 were excluded (20.3%), *lsrDNA*—comprising 1685 positions of which 635 were excluded (37.7%). Of the remaining unambiguously alignable positions, 446 (25.4%) for *ssrDNA* and 603 (57.4%) for *lsrDNA* were variable, and 359 (20.5%) and 488 (46.5%) were informative under the principles of parsimony.

ML and BI analyses of individual gene partitions gave almost identical trees (not shown). However, there were differences in tree topologies between *ssrDNA* and *lsrDNA* data partitions (not shown). Interrelationships within and between 'eutetrarhynchoid' and tentacularioid taxa were largely in agreement for both *ssrDNA* and *lsrDNA*. Interrelationships within gymnorhynchoid, 'lacistorhynchoid' and otobothrioid taxa were generally congruent between data partitions but interrelation-

ships between these major groupings differed. Amongst the nodes that differed between estimates of phylogeny from *ssrDNA* and *lsrDNA*, most were poorly supported in one or both gene partitions. These results from individual gene analyses were not unexpected, as we have previously shown that *ssrDNA* and *lsrDNA* offer complementary support in estimating relationships amongst many groups of Platyhelminthes (Olson and Littlewood, 2002; Olson et al., 2003; see also review by Olson and Tkach, 2005), and in particular cestodes (Olson et al., 2001; Waeschenbach et al., 2007). The data sets for *ssrDNA* and *lsrDNA* were shown to pass a partition homogeneity (incongruence length difference) test in PAUP* (heuristic search under parsimony, 1000 replicates, tree-bisection–reconnection branch swapping; $P = 0.99$), justifying combining these two genes under the principles of conditional combination (Huelsenbeck et al., 1996). As such, we discuss in detail, the combined evidence estimates of phylogeny from the concatenated data sets, as each individual data partition represents congruent, but in regions of each tree, relatively poorly supported estimates.

3.4. *ssrDNA + lsrDNA combined*

The combined analysis of *ssrDNA* and *lsrDNA* was almost identical between ML and BI; the BI tree is shown in Fig. 1 with nodal support from posterior probabilities and ML bootstrapping. The combined data set provided reasonably robust estimates of interrelationships, as indicated by high nodal support. Branch lengths were generally longer amongst 'eutetrarhynchoid' and tentacularioid taxa, and nodal support was low at deeper nodes within these clades. In contrast, branch lengths were considerably shorter amongst taxa of the remaining subfamilies, and nodal support was higher; 52% of nodes in the 'eutetrarhynchoid' + tentacularioid clade were supported by 100% posterior probabilities, in contrast to 68% for the second clade. Generally, the combined gene analysis provides a phylogenetic estimate with a well-supported structure.

Molecular phylogenetic analyses revealed strong nodal support for two major lineages of trypanorhynch. The first clade includes the superfamilies 'Eutetrarhynchoidea' (inverted commas indicating apparent paraphyly) and Tentacularioidea, excluding the Sphyricephalidae. At the family level, strong nodal support is given for the monophyly of the Tentaculariidae, being a sister taxon of *Rhinoptericola megacantha* and the new genus from Hawaii, the former being the type species for the family Rhinoptericolidae. The generic relationships within the tentaculariids are poorly resolved. The genus *Tentacularia* is the sister taxon to the four other existing genera within this family; these, in turn, form a polyphyletic assemblage. The species *Nybelinia cf. africana* and *Mixonybelinia lepturi*, both recorded from the same host, *Alopias superciliosus*, in Indonesia, form a derived cluster. There is significant morphological variability within the family Tentaculariidae.

The genus rich family Eutetrarhynchidae appears to be paraphyletic, consisting of three larger clades and the genus *Oncomegas* being sister to the *Rhinoptericola*/Tentaculariidae albeit with poor nodal support. High divergence rates resulted in long branch lengths and poor nodal support for species currently assigned into the same or related genera. The earliest divergent clade includes species belonging to *Prochristianella* and *Parachristianella* (both Eutetrarhynchidae) and species of two further families, the Mixodigmatidae and Rhinoptericolidae. The second clade includes species of *Prochristianella* combined with other eutetrarhynchid genera, *Mecistobothrium*, *Oncomegoides* and *Tetrarhynchobothrium*. The third clade includes a strongly supported monophyletic group of *Dollfusiella* species (with the only known freshwater trypanorhynch *Paroncomegas araya*) and another *Tetrarhynchobothrium* species, emerging within this clade but difficult to place with any confidence. The genus *Oncomegas* is, together with *Dollfusiella geraschmidti*, sister to *Rhinoptericola megacantha*, the new genus from Hawaii and the tentaculariids. Nodal support is low for the Eutetrarhynchoidea, its major family Eutetrarhynchidae and the three clades of eutetrarhynchids.

The second major lineage of trypanorhynch cestodes is monophyletic, with high nodal support for most emergent lineages. The earliest divergent monophyletic sister group to the remaining taxa consists of the families Aporhynchidae, Gilquiniidae, Rhopalothyllacidae and Gymnorhynchidae, all representing the superfamily Gymnorhynchoidea. The family Sphyricephalidae, formerly Tentacularioidea (see above), is resolved as sister group to the Gymnorhynchidae, however, with weak nodal support concerning its exact position within this clade. The sister group of the Gymnorhynchoidea is formed by the superfamilies 'Lacistorhynchoidea' and Obothrioidea. Whilst the latter superfamily forms the most derived monophyletic assemblage, the former consists of three different paraphyletic clades that are separated from each other with weak nodal support.

The sister group to all other 'lacistorhynchoid' genera consists of the family Pterobothriidae together with the genus *Grillotia*,

however, with low nodal support. These are separated with strong nodal support from *Hornelliella annandalei*, *Paragrillotia similis* and *Pseudolacistorhynchus heroniensis*. The second well supported monophyletic clade consists of the genera *Pseudogilquinia*, *Grillotia*, *Protogrillotia* and *Dasyrhynchus*. These taxa form the sister group to the monophyletic genera *Lacistorhynchus*, *Callitetrarhynchus*, *Floriceps*, *Diesingium* and the Obothrioidea. Branch lengths within the 'lacistorhynchoids' are relatively short, and the exact interrelationships of the three different 'lacistorhynchoid' clades cannot be resolved with the present analyses.

The Obothrioidea represent a well supported monophyletic group consisting of three clades. However, support for the interrelationships of the major clades within this group is poor. The earliest divergent clade includes species of the genera *Obothrium* and *Symbothriorhynchus*, and is sister group to the other obothriids. The next emerging clade consists of the genera *Proemotobothrium*, *Lobothrium* and *Fossobothrium*, being sister to the remainder obothrioids. The Pseudotobothriidae fail to be resolved as monophyletic because of the placement of *Obothrium cysticum* within it.

4. Discussion

This study presents a large scale molecular phylogenetic analysis of the elasmobranch tapeworms Trypanorhyncha. Complete *ssrDNA* in combination with partial *lsrDNA* was used to resolve the interrelationships within the order, and to assess conflicting hypotheses on the phylogeny and classification of these cestodes. According to the ordinal level relationships of tapeworms by *Waeschenbach et al. (2007)*, the trypanorhynch are monophyletic in all model-based analyses of complete *lsrDNA* + *ssrDNA*, forming the sister group to the Diphyllidea. Together with unequivocal morphological evidence, including the presence of a highly complex unique tentacular armature system, we consider the order Trypanorhyncha as a monophyletic group. Separate *ssrDNA* and *lsrDNA* analyses revealed slightly different but congruent topologies, each consistent with the resulting combined analysis trees. *lsrDNA* contributed a greater proportion of phylogenetically informative positions than the *ssrDNA*, and, as previously shown (see *Olson and Tkach, 2005*), provided resolution among more recently divergent clades; *ssrDNA* provided signal amongst the deeper nodes. This is consistent with the findings of *Waeschenbach et al. (2007)*, and the results of the first molecular phylogenetic analysis of 13 trypanorhynch genera by *Olson et al. (2001)*. Consequently, the combined information of both data partitions was used.

4.1. General tree topology and trypanorhynch armature

Molecular evidence demonstrates that the trypanorhynch consist of two well supported major lineages (Fig. 1). Fig. 2 shows a schematic version of Fig. 1 with important morphological synapomorphies mapped onto it. In addition, the distribution of the four characteristic armature patterns that were used in traditional trypanorhynch classification (see *Dollfus, 1942*; *Campbell and Beveridge, 1994*) is given; illustrated as symbols. Explanations of the characters discussed are given in Figs. 3 and 4, and Appendix A. The tree topology demonstrates that the armature patterns occur in different clades over the entire tree, in some cases having different armatures mapped onto the same branch. It can be concluded that the tentacular armature is highly variable, demonstrating that the armature patterns as used in earlier trypanorhynch classifications (homeo-, poecilo-, heteroacanth typical or atypical) can be considered homoplasious and misleading for nomenclature. Instead, the tree topology seems to be very similar to the results of a cladistic analysis of the trypanorhynch by *Palm (2004)* that

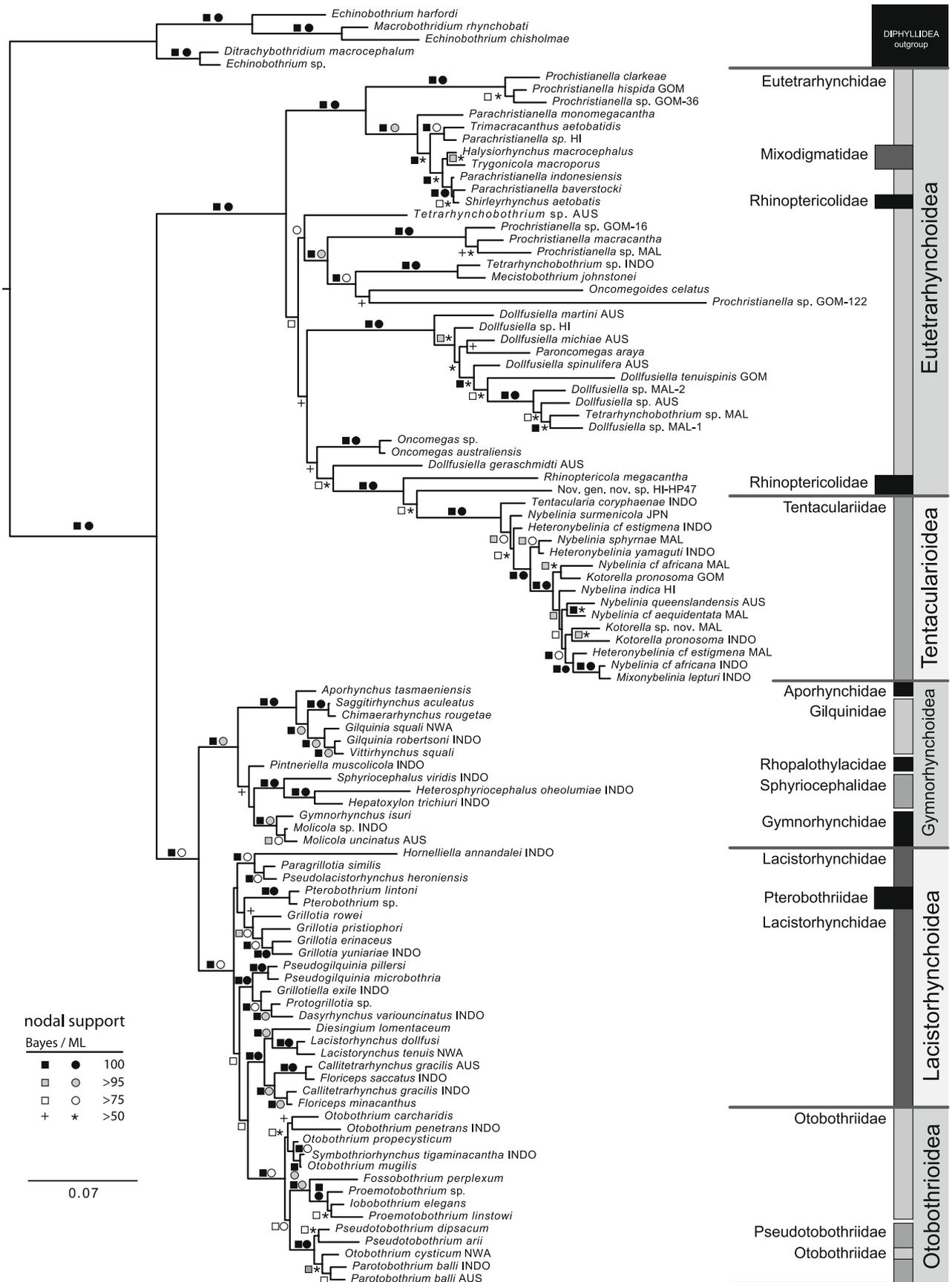


Fig. 1. Bayesian consensus phylogram based on analysis of combined *lsr*DNA and *ssr*DNA data partitions. Major families and superfamilies are indicated. Nodal support is indicated for BI (posterior probabilities) and ML (bootstrap, $n = 100$). The branch length scale is number of substitutions per site.

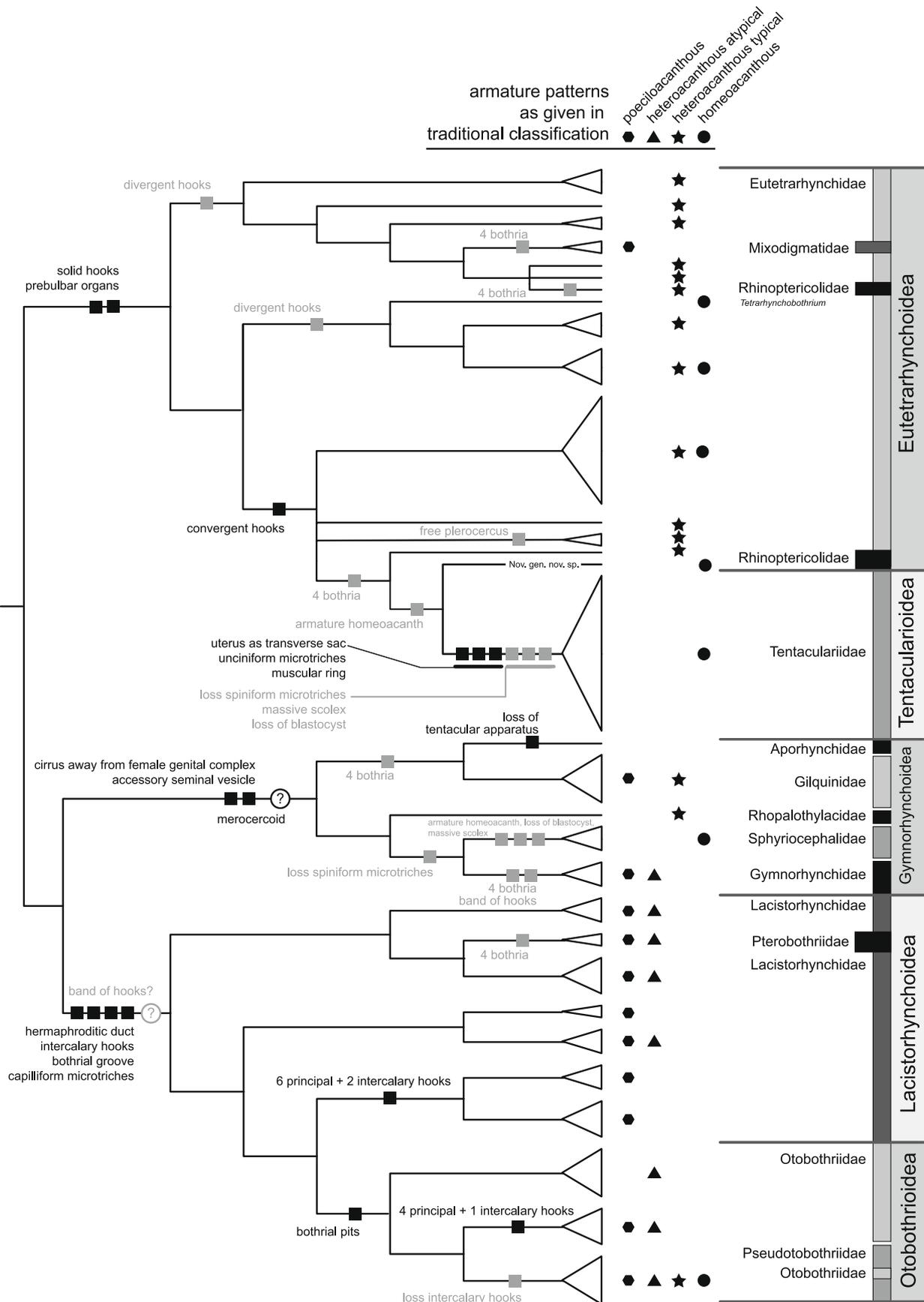


Fig. 2. Cartoon representing major clades and interrelationships of trypanorhynch flatworms, estimated from molecular data, with apomorphies and character changes indicated. Symbols show the distribution of scolex armature as used traditionally in trypanorhynch classification. Black boxes indicate likely synapomorphies, solid grey boxes indicate homoplastic characters; 4 bothria refers to the acquisition of the tetrabothriate scolex condition. Most of the mapped characters are drawn from Palm (2004) and other literature; see text for details. Additional terms and definitions are given in Appendix A.

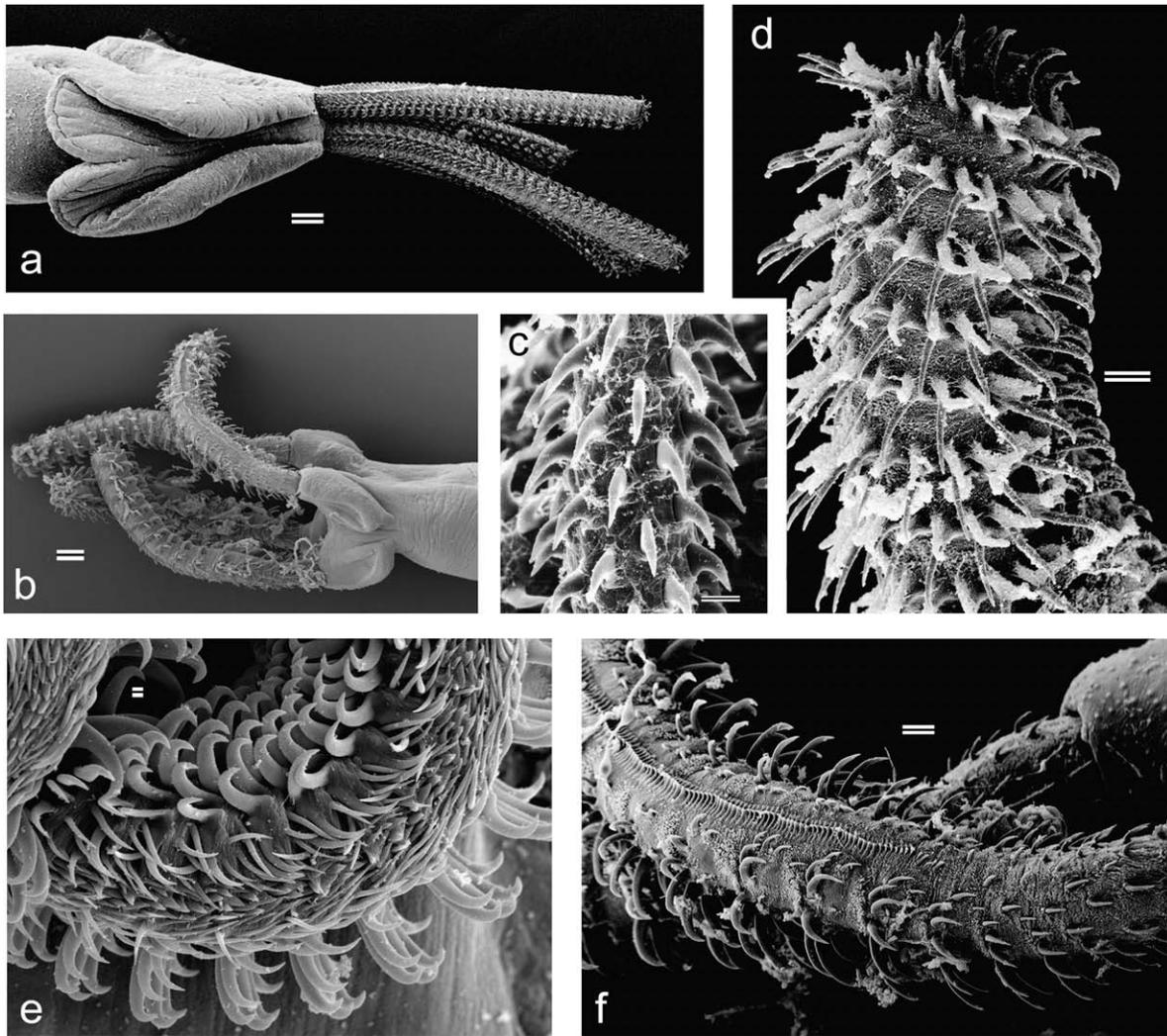


Fig. 3. SEM-micrographs of bothria and tentacular armature. (a) Dibothriate scolex of *Floriceps saccatus*. (b) Tetrabothriate scolex of *Halysiorhynchus macrocephalus*. (c) Homeoacanth armature of *Nybelinia indica* ('homeoacanthous homeomorphous' according to Palm, 2004). (d) Heteroacanthous typical armature of *Pintneriella musclicola* ('heteroacanthous typical heteromorphous'). (e) Heteroacanthous atypical armature of *Grillotiella exilis* ('heteroacanthous multiatypical'). (f) Poecilacanthous armature of *H. macrocephalus* ('poecilacanthous multiatypical'). Scale bars: a, b, 100 μ m; c, 10 μ m; d, 20 μ m; e, 2 μ m; f, 30 μ m.

splits the group into two major clades, the superfamily 'Eutetrarhynchoidea' together with the Tentacularioidea and the Gymnorhynchoidea together with the 'Lacistorhynchoidea' and Otobothrioidea. This author (HWP) coded an emended armature nomenclature to distinguish among the different taxa.

The molecular analyses suggest that the 'Eutetrarhynchoidea' consist of three major clades, however, two of them with low nodal support. These low supported clades represent species with a 'heteroacanthous typical heteromorphous' armature of divergent hooks (glide reflection symmetry). They also include tetrabothriate taxa (4 bothria) with a poecilacanthous armature and also the dibothriate (2 bothria) genus *Tetrarhynchobothrium* with a homeoacanthous armature. Neither the number of bothria nor the armature patterns characterize definite groups within these clades. A better supported clade includes species with a 'heteroacanthous typical homeomorphous' armature of convergent hooks. It consists of the dibothriate genera *Dollfusiella*, *Paronomegas* and *Oncomegas* and a monophyletic clade of tetrabothriate species, the Tentaculariidae, Tentacularioidea. The new species and genus from Hawaiian waters, with an elongated scolex with four bothria, a prebulbar organ and a homeoacanthous armature, is the sister taxon of the tentaculariids, occupying an intermediate position between the dibothriate

'eutetrarhynchoids' and the tetrabothriate tentacularioids; a formal description may highlight morphological apomorphies that support this placement. This latter superfamily now includes the two families Tentaculariidae and Paranybeliniidae (see Palm, 2008), with the Sphyriocephalidae moved into the Gymnorhynchoidea (see below).

The second monophyletic clade consists of the Gymnorhynchoidea, again having a variety of armature patterns but being characterized by unique segment morphology (see below). Within this clade, the Gilquiniidae have maintained the 'heteroacanthous typical' armature, with the exception of *Chimaerarhynchus rougetae* being poecilacanthous. Aporhynchidae have lost the tentacular armature, suggesting that the loss of the entire complex structure is possible and can be achieved rapidly, in evolutionary terms. The newly included Sphyriocephalidae are homeoacanthous and the Gymnorhynchidae 'heteroacanthous typical' (cf. Palm, 2004) or poecilacanthous. However, all these species have in common hollow hooks, with two dibothriate and three tetrabothriate families being included within this superfamily.

The possession of intercalary hooks, most probably combined with a band of hooks (character state 'heteroacanthous multiatypical', according to Palm, 2004), is considered synapomorphic for the remaining trypanorhynchs (see below), the 'Lacistorhynchoidea'

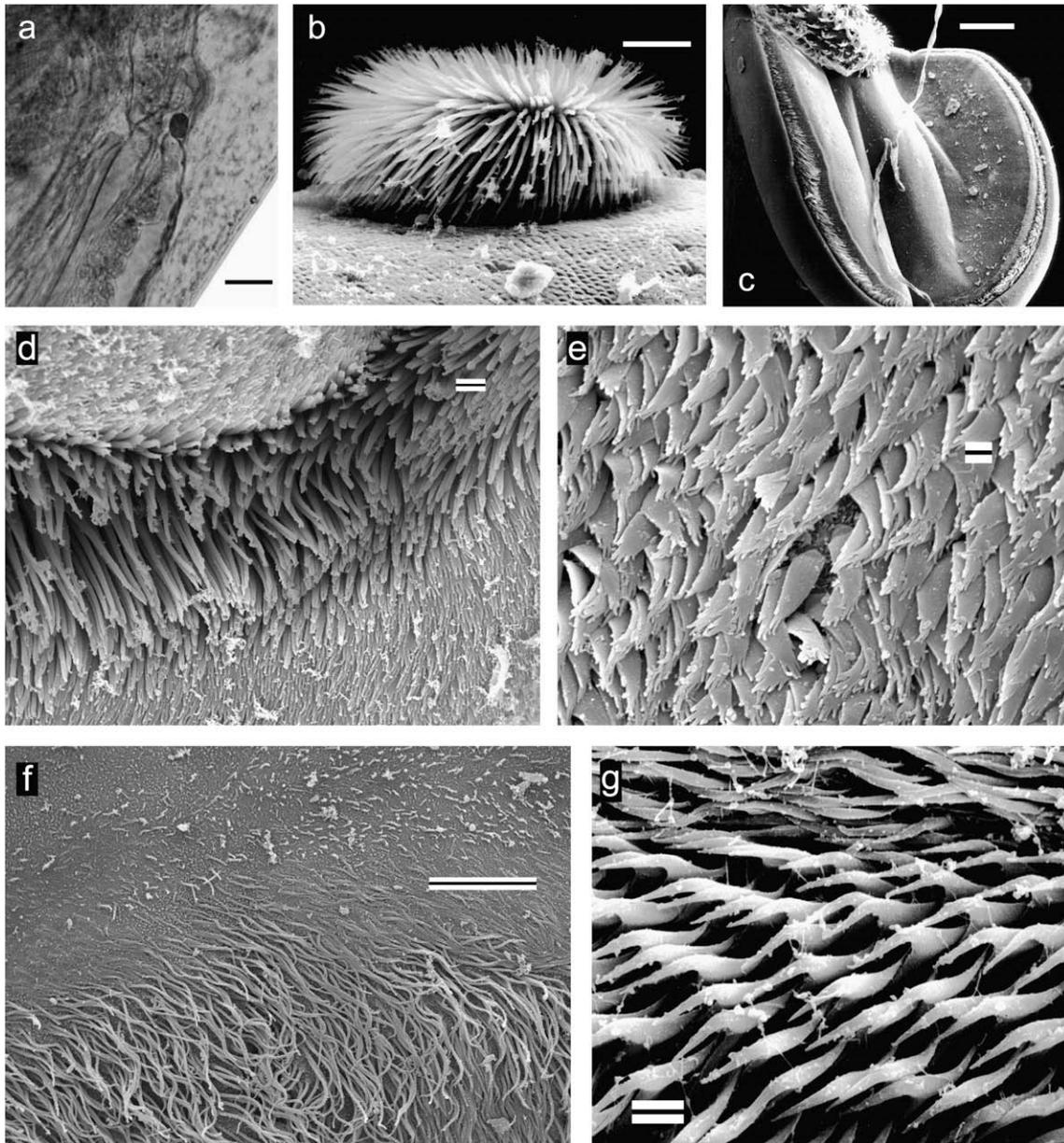


Fig. 4. Trypanorhynch ultrastructure. (a) Prebulbar organ of *Eutetrarhynchus platycephali* with darkly stained core in the wall of the tentacle sheath. (b) Everted bothrial pit of *Parotobothrium balli*. (c) Bothrial groove (Sinneskante) with characteristic microtriches of *Callitetrarhynchus gracilis*. (d) Microtriches that adorn the bothrial groove (Sinneskante) of *Floriceps saccatus*. (e) Spiniform palmate microtriches on the pars bulbosa cover filiform microtriches of the scolex peduncle of *F. saccatus*. (f) Capilliform microtriches at the terminal end of *Grillotiella exilis*. (g) Unciniform microtriches along the bothrial margins of *Nybelinia indica*. Scale bars: a, 75 μ m; b, f, 10 μ m; c, 100 μ m; d, g, 2 μ m; e, 1 μ m.

and Obothrioidea. Within all major clades ‘heteroacanthous atypical’ or poeciloacanthous species occur, demonstrating that the poeciloacanthous state (with the development of a chainette; see Fig. 3 and Appendix A) is the most derived within the order (see Dollfus, 1942; Campbell and Beveridge, 1994) but homoplastic (Palm, 1995, 1997, 2004). Beveridge and Campbell (1989) tentatively, and Beveridge et al. (1999) using cladistic analysis, suggested that the transition from typical heteroacanthous to poeciloacanthous has occurred independently in several trypanorhynch lineages. The lacistorhynchoids consist of three paraphyletic clades with less nodal support, represented by hetero- and ‘poeciloacanthous multiatypical’ (e.g. *Grillotia*, *Protogrillotia* and *Dasyrhynchus*) and ‘poeciloacanthous atypical’ (e.g. *Lacistorhynchus*) species, respectively. The latter clade represents the sister taxon to the monophyletic Obothrioidea with bothrial pits (see Palm et al., 2000; Palm, 2008) and the highest range of differ-

ent armature patterns within a single clade. The family Pterobothriidae with four pedicellate bothria, synapomorphic according to Campbell and Beveridge (1994), is included within the ‘Lacistorhynchoidea’, represented by species with heteroacanthous or poeciloacanthous armatures.

4.2. Morphological characters and classification

The lack of support in classifying trypanorhynchids according to their tentacular armature necessitates a search for characters that can be considered synapomorphic for the respective clades; see Fig. 2. The prebulbar organ (Fig. 4a) and solid hooks are considered the main synapomorphies for the ‘Eutetrarhynchoidea’ and Tentacularioidea. Solid hooks were recognized important as diagnostic features by Dollfus (1942) and as synapomorphic by Palm (2008), and prebulbar organs as synapomorphic by Palm (1995, 1997,

2004, 2008). Within this clade, species with divergent armatures ('heteroacanthous typical', *Prochristianella* and *Parachristianella* as main taxa, 'Eutetrarhynchoidea') are separate from those with convergent hooks or rotational symmetric armatures. The importance of armature symmetry was discussed in detail by Campbell and Beveridge (1994). We can infer that the tetrabothriate species (Tentacularioidea) split from the dibothriate *Dollfusiella* clade ('Eutetrarhynchoidea'), developing a muscular ring at the entrance to the bulbs (also see Palm et al., 1997; Palm, 1999, 2004) and secondarily reducing the prebulbar organ. The tentacularioids possess robust, muscular scolices (labeled 'massive' in Fig. 2), the uterus shaped as a transverse sac and without a blastocyst in the larval form. The scolex has unciniform microtriches with characteristic internal ultrastructure (see Palm, 2000; Palm et al., 2000) along the bothrial margins; these characters may be considered as synapomorphic (see Fig. 2). Unciniform microtriches (Fig. 4g) have already been suggested as a synapomorphy for the family Tentaculariidae and superfamily Tentacularioidea by Palm et al. (2000) and Palm (2008), respectively, and the shape of the uterus by Campbell and Beveridge (1994). Another possible synapomorphic development within the 'eutetrarhynchooids'/tentacularioids is the recognition of free plerocerci (see Palm, 2004) in the teleost intermediate host, as known from the genus *Oncomegas* (also in *Progrillotiidae*, a missing family within these analyses).

The Gymnorhynchoidea are characterized by a specific segment anatomy, where the cirrus pouch is widely separate from the posterior female genital complex close to the anterior end of the segment, a condition found to be synapomorphic by Palm (2004). Beveridge and Campbell (1989) and Beveridge et al. (1999) suggested an accessory seminal vesicle as a synapomorphy for this group. The most significant change within this clade is the complete loss of the tentacular apparatus that was considered autapomorphic for the family Aporhynchidae (Poche, 1926; Palm, 1995, 1997). The Sphyricephalidae, formerly member of the Tentacularioidea, have a massive scolex, a homeoacanthous armature of hollow hooks and plerocercoids without a blastocyst; all considered synapomorphies for the family. The Gymnorhynchidae have a band of hooks without intercalary hooks and merocercoids (Chervy, 2002) as larval forms. The exact origin of the merocercoid stage in the life cycle of trypanorhynchids, however, cannot be determined before confirmation of the larval stage morphology in *Gilquinia* (see discussion in Palm, 2004, p. 15) and *Pintneriella*. Both, the Sphyricephalidae and Gymnorhynchidae have lost spiniform microtriches entirely.

The 'Lacistorhynchoidea' and Obothrioidea are characterized by the presence of intercalary hooks and probably a band of hooks, that were considered synapomorphic by Palm (1995, 1997, character state 'rows of hooks partly reduced'). They also possess spiniform palmate microtriches (Fig. 4e) with a characteristic internal ultrastructure (see Palm et al., 1998a, 1998b, 2000). The bothrial groove (originally described as a 'Sinneskante', Fig. 4c and d), suggested as being homologous to the bothrial pits (Fig. 4b) by Palm et al. (2000), the presence of capilliform microtriches (Fig. 4f) at the terminal end of the scolex (probably resulting from a similar larval development/plerocercus stage) and the presence of a hermaphroditic duct, as suggested by Campbell and Beveridge (1994) are considered the main synapomorphies for the 'lacistorhynchoid'/otobothrioid clade. The Obothrioidea are characterized by bothrial pits that were considered a unique feature by Linton (1890) and Southwell (1929) and synapomorphic by Palm (1995, 1997, 2004). The secondary loss of intercalary hooks is considered synapomorphic within the Pseudotobothriidae (Palm, 2004). The sister clade of the Pseudotobothriidae can be characterized by the presence of four principal and an intercalary hook, while the 'lacistorhynchoid' sister group of the Obothrioidea has a poeciloacanthous armature of six to eight principal and one

or two intercalary hooks. Important morphological characters of trypanorhynch cestodes as discussed above are summarized in Figs. 3 and 4.

The current analysis has implications for our estimate of the ancestral character set of trypanorhynchids. With reference to character states of the outgroup, the stem group trypanorhynch was likely dibothriate, with an elongated scolex and with spiniform microtriches. The tentacular apparatus is unique within the Trypanorhyncha, and common features include a 'heteroacanthous typical' arrangement of hooks, and retractor muscles attached at the base of the bulbs. Additionally, we infer the postequatorial genital pore, a cirrus pouch nearby the female genital complex and the presence of a plerocercus-like stage as plesiomorphic, while Beveridge (2001) suggested non-operculate eggs and other life cycle characteristics (polylecithal and tanned eggs, a hexacanth with protonephridia, a proceroid with a cercomer and a metacestode without primary lacuna and apical organ). A range of characters have already been utilized in different combinations according to the then known species in an attempt to classify the trypanorhynchids (e.g. Dollfus, 1942; Wardle and McLeod, 1952; Yamaguti, 1959; Schmidt, 1970, 1986; Wardle et al., 1974; Campbell and Beveridge, 1994; Palm, 2004), e.g. the presence of two/four bothria (de Blainville, 1828; Southwell, 1929; Fig. 3a and b), the loss of the blastocyst (Rudolphi, 1819; Diesing, 1850, 1863; Vaullegeard, 1899; Poche, 1926; Guiart, 1931), a stout and muscular scolex morphology (Lönnberg, 1889; Southwell, 1929), a double set of genitalia (Lönnberg, 1889), a homeoacanthous/heteroacanthous (Vaullegeard, 1899; Dollfus, 1942) or poeciloacanthous (Dollfus, 1942) armature (Fig. 3c–f), a craspedote scolex (Pintner, 1913) and the loss and arrangement of microtriches (Palm, 1995, 2004; Fig. 4d–g). The present phylogenetic analyses illustrate that these characters are not synapomorphic for the higher trypanorhynch taxa but represent cases of parallel evolution within different lineages. As distinct and easily recognisable characters, however, they remain suitable to distinguish trypanorhynchids at family (only in part) and lower genus levels. It has to be kept in mind that in these cases, a traditional classification (as based on morphology) does not necessarily reflect monophyletic entities that result from the current molecular phylogenetic tree.

4.3. Evolutionary radiation of trypanorhynchids in relation to hosts

An analysis of the parasite–host records of this globally distributed group of cestodes necessitates confirmation of the host identification as given in the keys and parasite–host list by Palm (2004). Palm et al. (2007) recorded between 0% and 0.01% (14–21 bp) difference in the *lscDNA* and *mtDNA* (partial *cox1*, complete *trnT* and partial 16S ribosomal RNA) of *Tentacularia coryphaenae*, respectively, from five different hosts of different water depths and from the Atlantic and Indian Ocean. Within the present study, the *lscDNA* of a further specimen from another host from the Hawaiian Islands, Central Pacific, was identical to the above material. This provides molecular evidence that *T. coryphaenae* (Tentaculariidae, Tentacularioidea) is cosmopolitan, and confirms its low host specificity (Palm et al., 2007; Palm and Caira, 2008). For the first time, interoceanic distribution patterns based on molecular data are recorded for *Mixonybelinia lepturi* (Indian-Pacific, Tentaculariidae, Tentacularioidea), *Gilquinia squali* (Atlantic-Pacific, Gilquiniidae, Gymnorhynchoidea), *Sphyricephalus viridis* (Atlantic-Indian) and *Hepatoxylon trichiuri* (Atlantic-Indian, Gilquiniidae and Sphyricephalidae, both Gymnorhynchoidea), and *Callitetrarhynchus gracilis*, *Dasyrhynchus varioucinatus*, *Floriceps saccatus* and *Hornelliella annandalei* (all Indian-Pacific, Lacistorhynchidae, Lacistorhynchoidea); Table 3. Similarly, different hosts are infected by the same trypanorhynch, in the larval and adult stage, indicating relaxed host specificity for this group of tapeworms (see

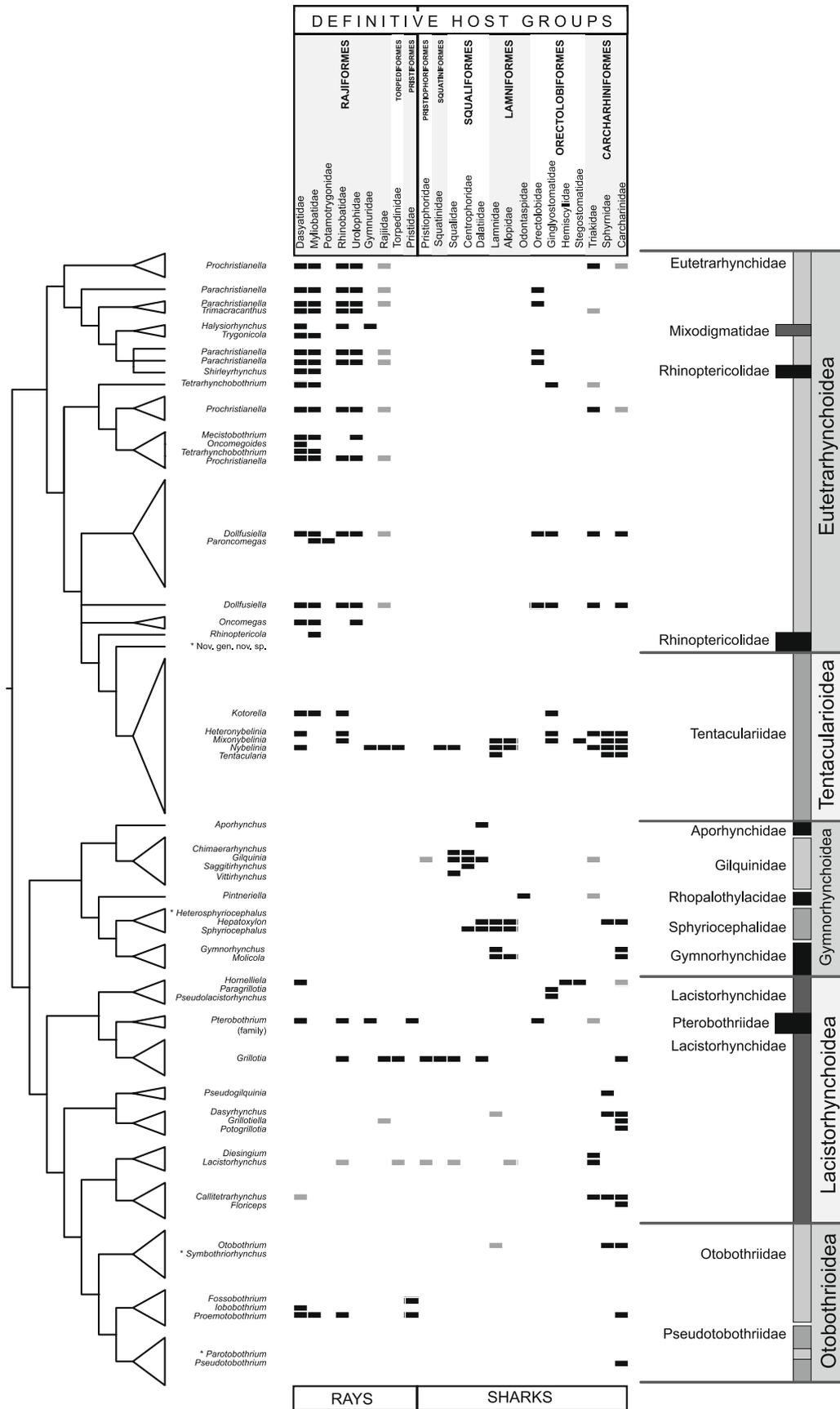


Fig. 5. Cartoon representing major clades of trypanorhynch parasites and their use of sharks and rays as definitive hosts. Host records represent known associations for genera of parasites; most data are from Palm (2004). Black boxes are confirmed hosts found commonly in the literature; grey boxes indicate unusual, or less frequent, occurrences found in the literature and may represent misidentified parasites or simply accidental host use. *—Definitive host association unknown (known only from teleost second intermediate hosts).

also Palm and Caira, 2008). This makes it difficult to demonstrate close host–parasite co-evolution at the species level. We note that two published *ssrDNA* sequences from our group (AF124462: *Hepatoxylon trichiuri*, and AJ287487: *Callitetrarhynchus gracilis*) likely include a number of sequencing errors, since many polymorphisms occur in highly conserved regions of the gene. As such, comparisons with these two sequences give a false impression of intraspecific variability within *ssrDNA* and should best be ignored; squared brackets in Table 3.

In order to reveal patterns of host use and the evolutionary radiation of trypanorhynchs, the known main definitive hosts (at the family level) were mapped onto the resulting phylogenetic tree (Fig. 5). According to the final hosts, the two major branches consist of clades mainly parasitizing rajiforms (especially ‘eutetrarhynchoidea’) and galeoforms (gymnorhynchoidea, ‘lacistorhynchoidea’ and otobothrioidea). Within the eutetrarhynchoidea, the first two clades mainly parasitize dasyatid, myliobatid and rhinobatid rays (e.g. *Prochristianella*, *Parachristianella*, *Shirleyrhynchus*). The third clade (*Dollfusiella*) occurs in the same rajiforms but also infects carcharhiniforms and orectolobiforms as definitive hosts. The tentacularioidea (*Tentacularia*, *Nybelinia*) likewise infect rays and sharks. Within the shark-parasitizing clade, the gymnorhynchoidea split into a squaliform, deep water (Aporhynchidae, Gilquiniidae) and a lamniform, oceanic clade (Rhopalothylacidae, Sphyricephalidae and Gymnorhynchidae). The ‘lacistorhynchoidea’ often infect carcharhiniforms, regularly both Carcharhinidae and Sphyrnidae within the same clade. However, the *Lacistorhynchus* and *Diesingium* clade infects the Triakidae. The earliest divergent clade of the lacistorhynchoidea are parasites of rajiforms and pristiforms together with squatiniform, squaliform, orectolobiform and carcharhiniform sharks. The otobothrioidea are parasites of carcharhiniforms within the earliest divergent clade (*Otobothrium*), however, with the second other otobothriid clade infecting rajiform, pristiform and also carcharhiniform hosts (Fig. 5).

Final host ranges of the trypanorhynchs allow comments to be made on the current match of molecular phylogenetic and morphological information. Considering the wide taxonomic spread of host groups, covering the rajiform, carcharhiniform and orectolobiform within three major groupings (clades), there seem to be four evolutionarily independent groups of trypanorhynchs. Phylogenetically and morphologically best resolved, is the monophyletic and mainly shark parasitic gymnorhynchoid, ‘lacistorhynchoid’ and otobothrioid clade, with well understood armature patterns, morphology and a high number of host records also from teleosts. The phylogeny suggests these parasites were originally in sharks and have evolved into one squaliform, one lamniform and one carcharhiniform clade, with host switches back from the carcharhiniforms into the rajiforms (*Pterobothrium*, *Grillotia* and *Proemotobothrium* clades; see Fig. 5). These host switches appear possible through the involvement of different second intermediate hosts (perciform and gadiform in the case of *Grillotia*; host list in Palm, 2004), also allowing the exploration of the deep sea (e.g. *Grillotia rowei*, host switch from Gadidae into Macrouridae as second intermediate hosts, both Gadiformes). The second group are monophyletic tentacularioidea that secondarily have infected the sharks from a clade originally parasitic in rays (the sister taxa mainly infect rajiforms). The adult worms have explored and diversified within most available elasmobranch host orders, including a diversity of sharks. We infer that this was possible through the development of the robust plerocercoid larval stage that can be transferred through the marine food web into a wide range of invertebrate and vertebrate hosts. The development of the plerocercoid (in Tentaculariidae, Sphyricephalidae) from the original plerocercus-like stage (see above) was suggested being an adaptation to the stomach instead of the spiral valve as the main site of infection in the final host (see Palm, 1995; Palm et al., 1997). The morphology

within the tentacularioidea is highly plastic even between phylogenetically closely related species within the same final host (e.g. *Nybelinia* cf. *africana* and *Mixonybelinia lepturi* in *Alopias superciliosus*, Alopiidae; Fig. 1). Due to difficulties with accurate larval identification, the interrelationships within this group are neither phylogenetically nor morphologically well understood, and would benefit from further studies.

The two other groups are clades of the ‘Eutetrarhynchoidea’. Adults of the genus *Dollfusiella* and related species have been recorded as adults from rajiforms, carcharhiniforms and orectolobiforms. The paraphyly of this genus is caused by the position of *D. geraschmidti* from urolophid rays, the genus *Tetrarhynchobothrium* in the related eutetrarhynchoid clade and *Oncomegas* close to the tentacularioidea. The plerocerci of *Dollfusiella* have been recorded from decapod shrimps, while free plerocerci of *Oncomegas* typically infect fish, possibly explaining the position close to the fish parasitic tentacularioidea (Palm, 2004). However, in general very little is known about the life cycle within this group. Species identification that traditionally uses the tentacular armature is particularly difficult, caused by the often small size and enormous number of small hooks that adorn the tentacles. Thus, neither the present molecular analyses nor the morphological data so far distinguish monophyletic clades. A similar situation exists in the *Prochristianella/Parachristianella* group, consisting of two separate clades; it also includes paraphyletic tetrabothriate species. In this group of trypanorhynchs, morphology can unequivocally place the specimens into their respective genera. However, the molecular analysis fails to support monophyly; a more in-depth analysis is warranted. These trypanorhynchs typically infest penaeid shrimps as second intermediate hosts and a wide range of rajiforms, especially Dasyatidae, Myliobatidae and Rhinobatidae, as final hosts (Palm, 2004). The group seems to radiate entirely within rajiform hosts.

In summary, with over 30% of known taxa analyzed here, the trypanorhynchs belong to the phylogenetically best studied marine tapeworm groups based on molecular evidence. Strong nodal support for some superfamilies, families and genera as recognized in the recent classification by Palm (2004), based on morphology and cladistics, will provide greater systematic stability within the order. This represents a major advance considering this group has been one of the most chaotic and confusing tapeworm groups until now (cited in Wardle and McLeod, 1952; Campbell and Beveridge, 1994; Palm, 1995, 1997, 2004). We demonstrated that, three of the five recognized superfamilies are monophyletic (Tentacularioidea, Gymnorhynchoidea, Otobothrioidea), and the ‘Eutetrarhynchoidea’ and ‘Lacistorhynchoidea’ are paraphyletic. This may be caused by insufficient taxon sampling, species descriptions or the lack of resolution offered by *ssrDNA* and partial *lssrDNA* genes alone, but requires additional gene and taxon sampling to provide greater resolution. Waeschenbach et al. (2007) added significant resolution to the ordinal tapeworm relationships by analysing the complete *ssr+lssrDNA* genes, adding 47.5% of the total alignable sites or 34.8% of parsimony informative positions. A better resolution of the relationships especially among the ‘eutetrarhynchoid’ trypanorhynchs remains a challenge for future studies. The unique possibility to identify trypanorhynch cestode larvae in the second intermediate and paratenic hosts as well as the adults in the elasmobranch final hosts based on morphology enables future comprehensive host–parasite lists and a detailed documentation of the host species and zoogeographical distribution of these parasites. This may turn the trypanorhynchs, a widely neglected order of tapeworms so far, into model organisms to study the zoogeography and evolution of fish parasitic helminths in the world oceans (Palm and Klimpel, 2007). With the use of molecular tools for diagnosing species and larva identity also in other cestodes, the entire class of parasites has much to offer for ecological and evolutionary disciplines.

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Appendix A. Terminology of morphological characters in trypanorhynch cestodes (see Sections 4.1 and 4.2)

A.1. Terms and definitions

A.1.1. Larval morphology

Free plerocercus: larval stage, where the scolex is not withdrawn but still attached to its blastocyst. It can move freely in the intestine of its host.

Merocercoid: larval stage with a retracted scolex within a blastocyst, with the anterior part of the scolex additionally being invaginated.

Plerocercoid: larval stage with an external scolex and no blastocyst. It can move freely inside the host intestine.

Plerocercus: larval stage with a retracted scolex within a blastocyst.

A.1.2. Scolex

Bothrial pit: pit with characteristic microtriches at the bothrial margin (Fig. 4b).

Bothrial groove: groove along the distal bothrial margins with characteristic microtriches (Fig. 4c and d).

Capilliform microtriches (Fig. 4f): typical hair-like shaped microtriches at the terminal end of scolex.

Prebulbar organ: organ of the attachment apparatus (Fig. 4a).

Spiniform microtriches (Fig. 4e): microtriches on the surface of the bothria and scolex peduncle, characteristic internal ultrastructure (see Palm et al., 1998a, 1998b, 2000).

Unciniform microtriches (Fig. 4g): microtriches at the bothrial margins, characteristic internal ultrastructure (see Palm, 2000; Palm et al., 2000).

A.1.3. Scolex armature

Atypical: with intercalary hooks (Fig. 3e).

Convergent: no distinct space the beginning of the rows of principal hooks.

Divergent: distinct space between the beginning of the rows of principal hooks.

Heteroacanth: hooks arranged in half spiral rows around the tentacle (Fig. 3d).

Homeoacanth: hooks arranged in complete spirals surrounding the tentacle (Fig. 3c).

Heteromorph: hooks of different shape (Fig. 3d).

Homeomorph: hooks of similar shape (Fig. 3c).

Intercalary hook: interpolated microhooks between rows of principal hooks (Fig. 3e).

Principal hook: enlarged hooks arranged in half spiral rows around the tentacle (Fig. 3d).

Poeciloacanth: one tentacle surface bears characteristic hooks arranged in 1–3 longitudinal files along the tentacle, forming a chain of hooks that differs in form and/or size from principal and intercalary hooks (Fig. 3f).

Typical: without intercalary hooks (Fig. 3d).

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.01.019.

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