Evolution of the trypanorhynch tapeworms: Parasite phylogeny supports independent lineages of sharks and rays

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ABSTRACT

Trypanorhynch tapeworms (Platyhelminthes; Cestoda) are among the most diverse and abundant groups of metazoan parasites of elasmobranchs and are a ubiquitous part of the marine food webs that include these apex predators. Here we present a comprehensive analysis of their phylogeny, character evolution and host associations based on 10 years of sampling effort, including representatives of 12 of 15 and 44 of 66 currently recognized trypanorhynch families and genera, respectively. Using a combination of ssrDNA and lsrDNA (Domains 1–3) for 79 and 80 taxa, respectively, we maintain one-to-one correspondence between molecules and morphology by scoring 45 characters from the same specimens used for sequencing, and provide museum vouchers for this material. Host associations are examined through likelihood-based ancestral character state reconstructions (ACSRs) and by estimating dates of divergence using strict and relaxed molecular clock models in a Bayesian context. Maximum parsimony and Bayesian inference analyses of rDNA produced well-resolved and strongly supported trees in which the trypanorhynchns formed two primary lineages and were monophyletic with respect to the diphyllidean outgroup taxa. These lineages showed marked differences in their rates of divergence which in turn resulted in differing support and stability characteristics within the lineages. Mapping of morphological characters onto the tree resulting from combined analysis of rDNA showed most traits to be highly plastic, including some previously considered of key taxonomic importance such as underlying symmetries in tentacular armature. The resulting tree was found to be congruent with the most recent morphologically based superfamly designations in the order, providing support for four proposed superfamilies, but not for the Tentacularioidea and Eutetraharynchoida. ACSR based on the combined analysis of rDNA estimated the original hosts of the two primary parasite lineages to be alternatively rajiform batoids and carcharhiniform sharks. This fundamental split provides independent support for rejecting the notion that rays are derived sharks, and thus supports the most recent molecular phylogenies of the Neoselachii. Beyond the basal split between shark- and ray-inhabiting lineages, no pattern was found to suggest that the trypanorhynchns have closely tracked the evolutionary histories of these host lineages, but instead, it appears that host-switching has been common and that the subsequent evolution of the parasites has been ecologically driven primarily through overlap in the niches of their shark and ray hosts. Using a relaxed molecular clock model calibrated by means of host fossil data, the ray-inhabiting lineage is estimated to have diversified around the Jurassic–Cretaceous boundary, whereas the shark-inhabiting lineage is estimated to have diversified later, in the Middle Cretaceous. Although the large error associated with the estimated divergence dates prevents robust conclusions from being drawn, the dates are nevertheless found to be consistent in a relative sense with the origins of their major hosts groups. The erection and definition of the suborders Trypanobatoida and Trypanoselachoida, for the major clades of trypanorhynchns parasitizing primarily rays and sharks, respectively, is proposed for the two primary lineages recovered here.
The order Trypanorhyncha Diesing, 1863 (Platyhelminthes: Cestoda: Eucestoda) comprises one of the most ubiquitous and readily recognized groups of marine helminths. With four armed, retractable tentacles (Fig. 1), the often large-bodied larval stages of these tapeworms are commonly observed in the flesh of marine teleosts as well as a wide variety of invertebrate intermediate hosts, such as crustaceans which harbour the first larval stage of most tapeworm groups with aquatic life cycles (Palm and Caira, 2008). They do not parasitize humans, except through accidental infection (see Heinz, 1954; Kikuchi et al., 1981; Fripp and Mason, 1983), nor do they cause substantial economic loss to fisheries, although parasitized flesh can reduce commercial demand despite being harmless (indeed, some indigenous peoples actually prize parasitized flesh for its enhanced flavour; see Overstreet, 2003). As adults, they are almost exclusively enteric parasites of elasmobranchs and among tapeworms are second only to the Tetraphyllidea in terms of their species diversity in the marine realm (Caira and Reyda, 2005).

Comprehensive taxonomic accounts of the group have been given by Dollfus (1942), Campbell and Beveridge (1994) and Palm (2004). Diagnosis and classification have been based chiefly on larval type and features of their rhyncheal apparatus (Fig. 2) that consists of a system of hydrostatic eversion by bulbs within the scolex and retraction by muscles within the tentacles (cf. the simple tentacles of the diphyllobothrid tapeworm *Haplobothrium*; Kuchta et al., 2008), and may be characterized by what appears to be four basic underlying symmetries in the arrangement of their armature (Dollfus, 1942; Campbell and Beveridge, 1994). This unique apparatus makes specific identification in the larval form possible. In contrast, the larval forms of most other marine tapeworm groups typically lack any species-specific characters and can only be identified with certainty through genetic analysis. Similarly, whereas all other recognized tapeworm orders are defined by unique combinations of characters (see Khalil et al., 1994), the complex try-

![Fig. 1. Scanning electron micrographs of scoleces of trypanorhynchs representing 11 of the 15 families. (A) Eutetrarhynchidae (*Puronomegas araya*). (B) Mixodigitmatidae (*Halsiorhynchus macrocephalus*). (C) Rhinoptericolidae (*Rhinoptericola megacantha*). (D) Aporhynchidae (*Aporhynchus* sp.). (E) Gilquinidae (*Gilquinia squali*). (F) Gymnorhynchidae (*Gymnorhynchus isuri*). (G) Lacistorhynchidae: Lacistorhynchiinae (*Dasyrhynchus* sp.). (H) Lacistorhynchidae: Grillotiinae (*Hornelliella annandelei*). (I) Otobothriidae (*Otobothrium mugilis*). (J) Pseudotobothriidae (*Pseudotobothrium aritii*). (K) Sphyriocephalidae (*Hepatoxylon* sp.). (L) Tentaculariidae (*Tentacularia* sp.). Scale bars: A–E, I, J, 200 μm; F, G, L, 500 μm; H, 1 mm; K, 2 mm.]

Panorhynch rhyncheal apparatus is considered a synapomorphy that underpins the naturalness of the group. The fact that preliminary molecular data have supported the existence of two independent trypanorhynch lineages (i.e. paraphyly; see Olson and Tkach, 2005 for a review) is likely to be a result of the disparate rates of evolution between these lineages (i.e. among-lineage rate variation) rather than the non-natural nature of the order. Moreover, where monophyly has not been supported, the two primary lineages indicated by molecular analyses tend to be placed either in immediate succession to one another (e.g. Olson et al., 2001) or are grouped in a clade together with their most likely sister group (see below), the Diphyllidea (e.g. Waeschenbach et al., 2007; Olson et al., 2008). For these reasons, here we assume monophyly of the trypanorhynchs, and focus instead on resolving its
internal structure and examining the historical associations formed with their hosts.

Morphologically-based views of trypanorhynch evolution continue to differ with respect to the traits considered and the relative importance given to them. This has resulted in conflicting ideas on the plasticity of various characters and host associations, as well as competing classifications and ideas as to their evolution. The preliminary cladistic analysis by Beveridge et al. (1999), for example, resulted in clades that largely reflected the nature of their tentacular armature and differed significantly in its systematic implications from the arguments of Palm (1997, 2004) who considered the armature of less importance for establishing higher taxa. Until recently, molecular data from trypanorhynchns had been applied only to the two extremes of the taxonomic spectrum: to represent the group using a small number of exemplar taxa in higher (i.e. ordinal) level analyses of the Cestoda (e.g. Olson and Cairra, 1999; Olson et al., 2001) and to examine levels of divergence within a species (e.g. Palm et al., 2007). Here we provide a comprehensive analysis of trypanorhynch interrelationships based on a combination of ssrDNA and lsrDNA data which have proven to be informative for estimating interrelationships within other tapeworm groups, e.g. Proteocephalidea (de Chambrier et al., 2004) and Rhinobothriidea (Healy et al., 2009); see also Olson and Tkach (2005).

A majority of the sequence data collected for the present study were made publicly available in 2007 and were used recently by Palm et al. (2009), together with additional taxa representing the Indo-Pacific region, in order to address specific family and genus-level questions. Their work focused on the implications of these data with respect to the classification system and ideas of trypanorhynch evolution proposed by Palm (2004) and discussed their morphological evolution by way of annotating 13 putatively synapomorphic characters taken from the literature onto their phylogenetic estimate. Here we present a comprehensive suite of analyses in which character evolution is inferred by way of explicit coding of 45 traits observed, interpreted and scored from the actual specimens sequenced, and employ parsimony to arbitrate character state changes across our molecular-based topology. In this way we maintained one-to-one correspondence between information from molecules and morphology and thus avoid the need to make assumptions or generalizations with regard to character state consistency within supra-specific taxa (i.e. genera, families, etc.). Full collection data for the 66 trypanorhynch species in common between the two studies are also presented here for the first time. We formally revise the classification of trypanorhynchs, the aim of moving away from the retention of paraphyletic taxa (e.g. Eutetrarhynchoidea of Palm et al., 2009) to a system in which all major groups are monophyletic. We then present an in-depth analysis of their host associations through statistically-based ancestral host reconstructions and estimates of divergence times with respect to the geologic time scale and discuss the implications of these results in light of elasmobranch evolution (see below). In addition, we take the opportunity to re-evaluate and contrast some of the conclusions drawn by Palm et al. (2009).

A long standing question in elasmobranch evolution has concerned the monophyly of sharks relative to rays, with the early, pre-cladistic idea consisting of a basal split of the Neoselachii into sharks and rays (e.g. Bigelow and Schroeder, 1948, 1953; Compagno, 1973). This notion was later challenged by a cladistic-based hypothesis of Shirai (1992, 1996) who united the shark groups Chlamydoselachiformes, Hexanchiformes, Squaliformes, Squatiniformes and Pristichorhnomiformes, and the true rays, or ‘batoïds’; the latter four dorsal–ventrally flattened groups being collectively termed ‘hypnosqualeans’ (Shirai, 1996). Based on this hypothesis, rays were considered to have resulted from an evolutionary trajectory towards increasingly dorso-ventrally flattened bauplans, with hypothesized intermediate forms such as angel sharks (Squatini-
Palm (2004). Thus the representation is nearly comprehensive at the level of family and includes over 60% of known genera, of which 40% are represented herein by multiple species. In a few cases, the same species collected from different hosts were included to test the conspecificity of the parasites (i.e. Callitetrarhynchus gracilis, Oncomegoides celatus and Prochristianella sp.).

2.2. Specimen voucher deposition

To anchor sequence data to actual specimens and enhance taxonomic collections, stained, whole-mounted vouchers were deposited in well-curated, publicly available collections for the majority of the newly collected material. Where possible, molecular vouchers represent the actual specimens from which tissue was obtained for the extraction of genomic DNA (gDNA) (i.e. ‘hologenophores’ sensu Pleijel et al., 2008), and generally comprise the anterior scolex plus posterior portion of the strobila. Additional whole-mounted specimens representing the same collections (i.e. collected from the same host individuals; ‘paragenophores’ sensu Pleijel et al., 2008) were also deposited when available as members of a series. Accession numbers and museums in which vouchers have been deposited are given in Table 1.

2.3. Molecular phylogenetic analyses

2.3.1. Outgroup choice

The precise phylogenetic position of the Trypanorhyncha within the Eucestoda, and thus the identities of its nearest relatives, remain problematic. From a traditional viewpoint (see review in Hoberg et al., 1997), the group most often linked to trypanorhynchs is the Diphyllidea, members of which are also restricted to elasmobranchs (primarily rays) and are similarly ‘bothriate’ (i.e. possess a scolex consisting of weakly muscular bothria). In molecular phylogenetic analyses of the Cestoda, these two orders are also generally found in close proximity, although poor nodal support and a lack of resolution among the bothriate and monofossate tapeworm
<table>
<thead>
<tr>
<th>Taxon ex Host species (common name), Host accession number, collection locality</th>
<th>18S ssrDNA/28S lsrDNA sequence accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dolfinella truncipes ex Dasyatis sabina (Atlantic stingray), Gulf of Mexico, Mississippi, USA [BMNH 2008.5.21.2]</td>
<td>DQ642958/DQ642796 (DNA–00–88)</td>
</tr>
<tr>
<td>Dolfinella sp. ex Himantura fava (honeycomb whipray), BO-24, BO-25</td>
<td>DQ642965/DQ642803 (D1–D6) (Doll)</td>
</tr>
<tr>
<td>Dolfinella sp. ex Himantura pastinacoides (Round whip ray), BO-76, Sulu Sea off Kampung Tetabuan, Sabah, Malaysia [LRP 4270]</td>
<td>DQ642962/DQ642800 (Doll)</td>
</tr>
<tr>
<td>Dolfinella germandisci ex Urolophus paucimaculatus (Sparingly-spotted stingaree), Queenseille, Victoria, Australia [BMNH 2001.12.5.6–7]</td>
<td>DQ642855/DQ642793 (Dolb)</td>
</tr>
<tr>
<td>Dolfinella martini ex Trygymorhina fasciata (Southern fiddler), Queenseille, Victoria, Australia [BMNH 2001.1.25.2–4]</td>
<td>DQ642964/DQ642802 (Doll)</td>
</tr>
<tr>
<td>Dolfinella michae ex Rhinoptera australiensis (Bomownouth guitarfish), NT-103, east of Wessel Islands, Arafura Sea, Northern Territory, Australia [LRP 3683]</td>
<td>DQ642966/DQ642804 (Dellm1)</td>
</tr>
<tr>
<td>Dolfinella ocallaghani ex Himantura jenkinsii (Pointed-nose stingray), NT-33, east of Wessel Islands, Arafura Sea, Northern Territory, Australia [LRP 3661]</td>
<td>DQ642961/DQ642799 (Doca1)</td>
</tr>
<tr>
<td>Dolfinella spinifera ex Dasyurus coburgicus (Giant shovel-nose ray), Coral Sea, Heron Island, Queensland, Australia [BMNH 1999.9.16.1–2]</td>
<td>GM217372</td>
</tr>
<tr>
<td>Mecistobothrium brevispinus ex Rhinoptera coburgica (Cow nose ray), MS05–49, off Ship Island, Gulf of Mexico, Mississippi, USA [LRP 4278] (no 18S)</td>
<td>FJ881110 (M505–49–10)</td>
</tr>
<tr>
<td>Mecistobothrium johnstonae ex Portinanus cf. sephen (cownose stingray), NT-44, east of Wessel Islands, Arafura Sea, Northern Territory, Australia [LRP 3667]</td>
<td>DQ642936/DQ642774 (NT44)</td>
</tr>
<tr>
<td>Oncomaeus australiensis ex Aetobatus cf. narini (spotted eagle ray), NT-76, east of Wessel Islands, Arafura Sea, Northern Territory, Australia [LRP 3678]</td>
<td>DQ642957/DQ642795 (Onco)</td>
</tr>
<tr>
<td>Oncomaeus ex Aetobatus cf. narini (spotted eagle ray), NT-76, east of Wessel Islands, Arafura Sea, Northern Territory, Australia [LRP 3679]</td>
<td>DQ642956/DQ642794 (Omeq1)</td>
</tr>
<tr>
<td>Oncomaeus celatus ex Dasyatis microps (Small eye stingaree), NT-108, east of Wessel Islands, Arafura Sea, Northern Territory, Australia [LRP 3698]</td>
<td>DQ642934/DQ642772 (NT108A)</td>
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<td>Oncomaeus celatus ex Himantura jenkinsii (Pointed-nose stingray), NT-33, east of Wessel Islands, Arafura Sea, Northern Territory, Australia [LRP 3662]</td>
<td>DQ642935/DQ642773 (NT33D)</td>
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<td>Parachristianella bowenstoki ex Portinanus cf. sephen (cownose stingray), NT-44, east of Wessel Islands, Arafura Sea, Northern Territory, Australia [LRP 3669]</td>
<td>DQ642937/DQ642775 (Pbav1)</td>
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<td>Parachristianella indonesiensis ex Rhynchosubus coburgicus (Australian wedgefish), NT-49, east of Wessel Islands, Arafura Sea, Northern Territory, Australia [LRP 3673]</td>
<td>DQ642939/DQ642777 (Pbav5)</td>
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<td>Parachristianella monomaculanta ex Himantura draco (Dragon stingray), NT-106, east of Wessel Islands, Arafura Sea, Northern Territory, Australia [LRP 3694]</td>
<td>DQ642943/DQ642781 (Pmon2)</td>
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<tr>
<td>Paracomaeus araya ex Potamotrygon motoro (Ocellate river stingray), Rio coastline, Santa Fe, Argentina [BMNH 2003.3.31.23-2004.3.18.100]</td>
<td>DQ642963/DQ642801 (Pmex2)</td>
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<tr>
<td>Prochristianella clarksae ex Rhynchosubus australiensis (australian wedgefish), NT-7, Gove Harbor off Nhulunbuy-Gove, Gulf of Carpentaria, Northern Territory, Australia [BMNH 2003.3.31.23]</td>
<td>DQ642947/DQ642785 (Pc1a)</td>
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<td>Prochristianella histida ex Dasyatis say (Blunt nose stingray), Gulf of Mexico, Mississippi, USA</td>
<td>DQ642946/DQ642784 (DNA–01–97)</td>
</tr>
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<td>Prochristianella macracantha ex Torniru trilobus (Bluespotted ribbontail ray), BO-80, Celebes Sea off Kunak, Sabah, Malaysia [LRP 4271]</td>
<td>DQ642932/DQ642770 (Proch2)</td>
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<tr>
<td>Prochristianella sp. ex Dasyatis sabina (Atlantic stingray), Gulf of Mexico, Mississippi, USA [BMNH 2008.5.21.4]</td>
<td>DQ642945/DQ642783 (DNA–00–90)</td>
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<tr>
<td>Prochristianella sp. ex Himantura pastinacoides (Round whip ray), BO-76, Sulu Sea off Kampung Tetabuan, Sabah, Malaysia [LRP 4272]</td>
<td>DQ642933/DQ642771 (Proch)</td>
</tr>
<tr>
<td>Prochristianella sp. ex Sphyra tiburo (Bonnethead), M-00–2502, Gulf of Mexico, Mississippi, USA</td>
<td>DQ642931/DQ642769 (DNA–00–16)</td>
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<td>Prochristianella sp. ex Dasyatis sabina (Atlantic stingray), Gulf of Mexico, Mississippi, USA</td>
<td>DQ642930/DQ642768 (DNA–01–122A)</td>
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<tr>
<td>Prochristianella sp. ex Dasyatis sabina (Atlantic stingray), Gulf of Mexico, Mississippi, USA</td>
<td>DQ642944/DQ642762 (DVr-36b)</td>
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<td>Tetranychobothrium sp. ex Charchinus melanopterus (Blacktip reef shark), Coral Sea, Heron Island, Queensland, Australia [BMNH 2001.1.26.1]</td>
<td>DQ642792 (Trhy)</td>
</tr>
<tr>
<td>Tetranychobothrium sp. ex Himantura cf. gerardi (sharppnose stingray), BO-23, South China Sea off Sematan, Sarawak, Malaysia [LRP 4273]</td>
<td>DQ642960/DQ642798 (Trhy)</td>
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<td>Tricymactacanthus aequatus ex Trygonorhina fasciata (Southern fiddler), Queenseille, Victoria, Australia [BMNH 2001.1.25.1]</td>
<td>DQ642942/DQ642780 (Tria)</td>
</tr>
<tr>
<td>Mixidicmatidae Dailey &amp; Vogelbein, 1982</td>
<td>DQ642940/DQ642778 (Haly2)</td>
</tr>
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<td>Halysiorhynchus macrocephalus ex Portinanus cf. sephen (cownose stingray), NT-44, east of Wessel Islands, Arafura Sea, Northern Territory, Australia [LRP 3664]</td>
<td>DQ642940/DQ642778 (Haly2)</td>
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<tr>
<td>Trygonocidae ex Himantura cf. gerardi (sharppnose stingray), BO-23, South China Sea off Sematan, Sarawak, East Malaysia [LRP 4274]</td>
<td>DQ642941/DQ642779 (Trhy)</td>
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<tr>
<td>Rhinopteroclidae Carvajal &amp; Campbell, 1975</td>
<td>DQ642954/DQ642792 (DNA–01–14)</td>
</tr>
<tr>
<td>Shirleyhynchus aequatus ex Himantura cf. uarnak (honeycomb stingaree), BO-82, Celebes Sea off Semporna, Sabah, Malaysia [LRP 4275]</td>
<td>DQ642938/DQ642776 (Shir)</td>
</tr>
</tbody>
</table>
GYMNORHYNCHOIDEA Dollfus, 1935

APORYNCHIDAE Poche, 1926

Aporhynchus sp. ex Etmopterus spinax (Velvet belly lantern shark), AZ-8, Horta, Faial, Azores, Portugal [LRP 4279] FJ572911/FJ572947 (Apor/TE-157)

GILQUINIIIDAE Dollfus (1942)

Giliquia sp. ex Squids acanthus (Piked dogfish), North Sea, UK AJ287516/AF286966 (Gsq)

Squatitritrychiaceae ex Centrophorus sp. (gulper shark), L'ile des Pins, New Caledonia [MNHN JN70A] DQ642907/DQ642745 (Gnp1)

Vittitritrychiaceae ex Squids melanurus (Blacktailed spurdog), Coetlogon, New Caledonia [MNHN JN73A] DQ642905/DQ642743 (Gen2)

GYMNORHYNCHOIDEA Dollfus (1935)

Chimarrhynchoidea rorugare ex Squids cf. megatops (spurdog), Baie de Santal, Lifou, New Caledonia [MNHN JN4A] DQ642906/DQ642744 (Chir)

Gymnorhynchiidae ex Isurus oxyrinchus (Shortfin mako), Montauban, Longue, New York, USA [LRP 3711] DQ642909/DQ642747 (Gymn)

Molusc uncinatex ex Thyrsites atun (Snook), Apollo Bay, Victoria, Australia [BMNH 2004.3.18.102] DQ642908/DQ642746 (Mol)

LACISTORHYNCHOIDEA Guiart (1927)

LACISTORHYNCHIDAE Guiart (1927)

Paraotobothrium balli

Molicola uncinatus

Gymnorhynchus isuri

Chimaerarhynchus rougetae

Gilquinia squali

sp. ex

Nybelinia aequidentata

Pseudotobothrium arii

Pterobothrium lintoni

Proemotobothrium linstowi

sp. ex

Proemotobothrium

Callitetrarhynchus gracilis

sp. ex

Protogrillotia

Sphyriocephalus viridis

Kotorella pronosoma

Grillotia erinaceus

Grillotia pristiophori

Pseudogilquinia microbothria

Floriceps minacanthus

Fossobothrium perplexum

Otobothrium mugilis

Otobothrium propecysticum

Poecilancistrium caryophyllum

Otobothrium carcharidis

Coryphaenoides armatus

ex

Amblyraja radiata

(Sphynx sawshark), San Remo, Victoria, Australia [SAMA 28386] DQ642925/DQ642763 (Calg1)

DQ642920/DQ642758 (Calg1)

DQ642764 (Ptpl1)

DQ642767 (NT113A)

DQ642916/DQ642754 (NT33A)

DQ642914/DQ642752 (Onsp1A)

DQ642750 (NT99A)

DQ642913/DQ642751 (Otsp)

(continued on next page)
orders has left their relative positions open to question (for a review see Olson and Tkach, 2005). Although it might appear desirable to include a large number of candidate sister lineages of the Trypanorhyncha as outgroup taxa, sequence divergence among such groups would require the consideration of more positions due to the lack of recognizable homology (see Appendix 1 in Olson and Caira, 1999). Moreover, previous rDNA-based studies have already demonstrated rDNA data to be insufficient in strongly arbitrating on this question, whilst nevertheless supporting a close association (often as sister group) between the Diphylloidea and the Trypanorhyncha (e.g. Waeschenbach et al., 2007; Olson et al., 2008). For these reasons, only members of the Diphylloidea were included as outgroups.

2.3.2. DNA amplification and sequencing

The complete 18S ssrRNA (minus the distal 5' and 3' priming regions) and partial (Domains 1–3) 28S ssrRNA genes were characterized for all trypanorhynch taxa and for diphylleidean outgroup taxa where such sequences were not already available from GenBank based on the works of Olson and Caira (1999), Olson et al. (2001) and Bray and Olson (2004). In total, 63 new 18S and 65 new 28S sequences were characterized. Tissue samples from ethanol-preserved specimens were soaked overnight in Tris–EDTA buffer and the total gDNA extracted using the Qiagen® DNeasy® tissue kit following manufacturer-recommended protocols, with the exceptions that the incubation period with proteinase-K was extended to overnight in a rotating incubator and the final elution volume was reduced to 200 µl. One to three microliters gDNA were used as a template in 25 µl reactions using Ready-To-Go™ (Amersham Pharmacia Biotech) PCR beads and the following thermocycling profile: 3 min denaturation at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 52 °C, 2 min at 72 °C; and 7 min extension at 72 °C. The complete 18S gene was amplified using primers Worm-A and Worm-B (see Littlewood and Olson, 2001 for a complete list of 18S primer definitions) and the D1–D3 domains of the 28S amplified using primers LSU-5 and 1200R (see Olson et al., 2003 for 28S primer definitions). PCR amplifications were either gel-excised or purified directly using Qiagen Qiaquick® columns, cycle-sequenced from both strands using ABI BigDye® chemistry, alcohol-precipitated, and run on an ABI Prism 377™ automated sequencer. The products were sequenced bidirectionally using the PCR primers and a variety of internal primers following Farris (2004). Trypanorhynch classification follows Farris (2004): Trypanorhyncha classification follows Farris (2004).

2.3.3. Sequence alignment and data partitions

The molecular data sets consisted of 79 trypanorhynch (80 for 28S which included a second species of Mecistobothrium; i.e. Mecistobothrium brevispine) and five diphylleidean taxa. A concatenated alignment of the two genes was constructed initially using MUSCLE (Edgar, 2004) with default parameters and subsequently examined by eye using MacClade (Maddison, D.R., Maddison, W.P., 2005. MacClade 4: Analysis of phylogeny and character evolution, version 4.08. Sinauer, Sunderland, MA, USA) using a heuristic search strategy with 1000 search replicates, random-addition taxon sampling and tree-bisection reconnection branch-swapping, with all characters run unordered with equal weights and gaps treated as missing data. Nodal support was assessed by bootstrap analysis based on 1000 replicates with three full heuristic searches/replicate. Bayesian inference was conducted using MrBayes (Ronquist and Huelsenbeck, 2003; version 3.2.1). Models of nucleotide substitution were evaluated for both data partitions independently using MrModelTest (Nylander, J.A.A., 2004. MrModelTest, program distributed by the author. Evolutionary Biology, Upsala University, Sweden, version 2), and for both the most parameter-rich model (i.e. general-time-reversible including estimates of invariant sites and gamma distribution among-site rate variation) was found to provide the best fit to the data based on the Akaike information criterion. The following model parameters were thus used in MrBayes: nst = 6, rates = invgamma, ngamma = 4, and samplefreq = 100 with default prior probabilities. Two separate runs were performed (default behaviour in MrBayes 3.2.1) and convergence between them examined using the sumt command. Consensus trees with mean branch lengths were constructed using the contype = allcompat option and ignoring the topologies saved during the initial n-generations before log-likelihood values and substitution parameters reached stationarity (i.e. ‘burn-in’). Burn-in was estimated by plotting the log probabilities against generation and visualizing the plateau in parameter values. Burn-in values were 2000 for 28S and 800 for both the 18S and combined data sets (representing the first 200,000 and 80,000 of a total
of 2,000,000 generations in each analysis). Nodal support was estimated as the mean posterior probabilities (Huelsnbeck et al., 2001) as calculated by the sumt command.

2.3.5. Stability analyses

In order to identify relatively unstable taxa whose inclusion can diminish support across the tree (see Wilkinson, 2003), we ranked taxa by their leaf stabilities (Thorley and Wilkinson, 1999) given the set of topologies resulting from the combined Bayesian analysis: a total of 19,200 tree topologies not including those estimated during burn-in, using a Python program developed by T. Hill (Natural History Museum, London). Leaf stability of a taxon is based on the average of the support (here maximum posterior probabilities) for each subtree containing that taxon and any other pair of taxa.

2.4. Coding and analysis of morphology

2.4.1. Coding

The morphological characters on which we have focused are based on those of Beveridge et al. (1999). However, a number of the characters associated with tentacle armature have been refined into more discrete and thus unambiguously codeable units. Four additional characters were added to accommodate the expanded taxon sampling used here. A listing of the characters and their states is provided in Table 2. A matrix of morphological characters (Supplementary Data S1) was constructed based, with a few exceptions, on observed features of the specimens used for sequencing (i.e. hol– or paragenophores). In cases in which specimens were damaged, incomplete or unavailable, codings were based on recent descriptions of the species.

2.4.2. Character analysis

The morphological character state changes of a subset of the 45 characters in Table 2 were mapped onto the topology of the Bayesian inference tree resulting from combined analysis of 18S and 28S rDNA data using MacClade with accelerated transformation. Character state changes shown in bold in Table 2 were those of key importance to previous trypanorhynch classification schemes and/or based on our results, showed systematic utility (i.e. help to define natural groups).

2.5. Analysis of host associations and phylogenetic dating

2.5.1. Host association matrix construction

A matrix of host associations coded at the levels of host family, host order and a division between batooids and sharks was constructed based, with a few exceptions, on observed features of the specimens used for sequencing (i.e. hol– or paragenophores). In cases in which specimens were damaged, incomplete or unavailable, codings were based on recent descriptions of the species.
constructed using MacClade to examine the distribution of host associations with respect to the phylogeny of trypanorhynch. Host associations for each species consisted of the actual hosts, in the cases of specimens collected from definitive hosts, in combination with previously reported definitive hosts as compiled in Palm (2004). Those trypanorhynch species infecting multiple host taxa were coded as multistate for any and all relevant taxonomic levels. For two taxa collected as larvae, Pseudogilquinia pillersi and Pseudotobothrium dipsacum, definitive hosts have yet to be identified (Palm, 2004) and thus character states for these taxa were coded as question marks. The matrix is shown in Supplementary Data S1.

2.5.2. Ancestral character state reconstructions (ACSRs)

The absence of a comprehensive molecular data set that includes the same species of elasmobranchs that host the parasite species analyzed herein prevented any type of congruence analysis of independently-derived host and parasite phylogenies using methods such as TreeMap (Page, 1994) or Jungles (Charleston, 1998). Instead, the distribution of known, contemporary host associations across the parasite phylogeny was used to identify the most likely ancestral host ‘states’ for nodes subtending major clades. ACSR s were estimated for the three hierarchical levels at which host associations were coded in the matrix in Supplementary Data S1 (i.e. family, order and shark versus batoid) using BayesTraits (Pagel et al., 2004; ver. 1.0, available from www.evolutions.rdg.ac.uk) with the multistate model of evolution. By this method, the degree of uncertainty in the hypothesized ancestral host states could be estimated from the data.

2.5.3. Phylogenetic dating

Divergence dates under strict and relaxed clock models were estimated by Bayesian analysis using BEAST (Drummond and Rambaut, 2007; ver. 1.4, available from www.bio.ed.ac.uk). In the absence of any direct evidence of tapeworm ancestry (such as fossilized specimens), calibration was achieved using proxy dates based on the fossil record of the host groups. Although we are aware that the estimated dates of first appearance of elasmobranch groups differ among authors (e.g. see Maisey et al., 2004), the relatively broad taxon coverage provided in a single work by Cappetta et al. (1993) made comparisons across families feasible. Thus all the age at the root of the ingroup was estimated as the age of the earliest known (i.e. minimum age) representative of the extant host groups, as reported by Cappetta et al. (1993). This was 183 million years ago (Myr) based on the oldest known fossil of the Hexanchidae. Thus the ingroup clade was defined as a taxon set and the prior distribution for the age of its root was set to an exponential curve with a zero offset of 183 Myr and an exponential mean of 10 Myr. Such a distribution is considered to be most appropriate for minimum age dates as it avoids sampling more recent dates whilst accommodating for uncertainty in the fossil record (see Ho, 2007). The speciation tree priors was set to a Yule birth process and other priors set as defaults. The substitution model was the GTR + I + G with four rate categories and empirical base frequencies, as employed above using MrBayes, and the length of the chain (i.e. No. generations) was set initially to default values of 10,000,000 with samples saved every 1000 generations.

The SD of the relaxed uncorrelated lognormal clock model (i.e. ‘ulclstddev’ parameter) provides an indication of how clock-like divergence rates are, with values of zero indicating no variation in rates, and values over one (i.e. SD > mean rate) indicating high degrees of rate heterogeneity (Drummond et al., 2006). For our combined data set that value was 0.618, suggesting that our data are neither highly clock-like nor highly heterogeneous, and thus we chose to conduct and evaluate separate analyses using the strict as well as relaxed clock models. Resulting prior and posterior distributions and effective sample sizes (ESSs) were examined using Tracer, and consensus topologies and 95% confidence intervals for the nodal ages calculated using TreeAnnotator (both programs part of the BEAST software package). ESSs of some parameters were found to be insufficient after 10,000,000 generations for the relaxed clock analysis and thus this analysis was rerun with the chain increased to 15,000,000. The first 1000 and 1500 saved trees, for the strict and relaxed clock analyses respectively, were removed as burn-in prior to consensus analysis using TreeAnnotator. The resulting linearized consensus trees were drawn using FigTree ver. 1.0 (Rambaut, A., Edinburg, UK, tree.bio.ed.ac.uk/software/); and further annotated using Adobe Illustrator.

3. Results

3.1. Analysis of 18S and 28S rRNA

Both individual (Fig. 4) and combined (Fig. 5) analyses resulted in well-resolved and strongly supported topologies characterized by a pattern consisting of two major lineages, one (upper clade in Fig. 5) consisting of the Eutetrarhynchidae and one family of the former Tentacularioidea (i.e. the Tentaculariidae), and the other (lower clade in Fig. 5) consisting of the remaining trypanorhynch taxa. These two major lineages showed marked differences in divergence rates that can be seen in their relative branch lengths: the upper clade (Fig. 5) is characterized by relatively long internal and terminal branches, subtended by comparatively short basal internal branches, resulting in well-supported clades, albeit with less support for the interrelationships of the clades themselves. In contrast, the lower clade (Fig. 5) is characterized by considerably shorter but nevertheless more equitable internal and terminal branches, providing less support for the monophyly of individual clades but greater support for their interrelationships. These differences are reflected in their stability profiles, such that the more equitable branch lengths that characterized members of the lower lineage also led to greater stability in the positions of individual taxa and thus higher leaf stability values (Fig. 5). Both lineages, however, showed high clade stability (as indicated by the thick lines in Fig. 5) suggesting that membership of the individual clades in each lineage is stable. Thus extinct lineages notwithstanding, molecular data strongly suggest that the trypanorhynchs split into two separate major lineages early in their evolution, after which their diversification followed disparate evolutionary tempos.

3.2. Analysis of morphology

Seventeen of the 45 morphological characters in Table 2 have or now appear to have potential systematic utility. These are shown in Fig. 6 mapped onto the Bayesian inference tree resulting from combined analysis of 18S and 28S rDNA data. Mappings of only three of these (Characters 3, 8 and 45, the latter being the apomorphic loss of the rhyncheal apparatus) were found to be non-homoplasic.

3.3. Analysis of host associations and estimated divergence dates

ACSRs suggest that the two major lineages of trypanorhynchs diverged together with the two major lineages of neoselchians, the Batoidea (rays) and Selachimorpha (sharks) (Fig. 7), and thus support recent molecular estimates of elasmobranch phylogeny that hypothesize a basal divergence of shark and ray lineages (see Fig. 3). Host switching from ray to shark hosts and from shark to ray hosts is seen in both the ‘ray-inhabiting clade’ (RIC) (upper clade) and ‘shark-inhabiting clade’ (SIC) (lower clade), respectively, but estimates show such events to be restricted largely to the re-
cent past. At least one exception is seen in the clade encompassing the major- 
ity of tentacularioid species (i.e. Nybelinia and its relatives) which is esti-
mated to have evolved from a host switch from rays to sharks that occurred
~40 Myr, with multiple, subsequent independent host switches back to rays
(Fig. 7). ACSRs of the host orders estimated the ancestral host of the RIC to be a member of the
Rajiformes, whereas the likelihood of it being a member of another 
batoid group (i.e. Pristiformes or Torpediniformes) was neg-
ligible (i.e. <1%; see pie-charts in Fig. 7). The SIC, the most likely ancestral host was estimated to be a member of the Carcharhini-
forms, although the case is less clear cut than that above, as the
squaliform and lamniform sharks showed notable likelihood val-
ues. At the level of host family, the likelihoods become highly dis-
persed and there is no evidence that either the finer scale
diversification of the host orders or families are reflected in the
parasite phylogeny (but see Section 4).

Both the relaxed (Fig. 7) and strict (results not shown) clock ana-
lyses estimate the split between the two major trypanorhynch
clades around the Triassic–Jurassic boundary (210 Myr), or slightly
older than the calibration date of 183 Myr. Diversification of the
RIC is estimated to have occurred near the start of the Cretaceous
(140 Myr), whereas diversification of the SIC is estimated to have 
happened later, in the latter half of the Cretaceous (~100 Myr),
based on a relaxed clock model. The strict clock model estimated
even less equitable divergence dates, pushing the RIC back into the
Upper Jurassic and the SIC forward into the Upper Cretaceous
(data not shown). However, the greatest discrepancy between
the two models was found in the size of the error estimated, with
the relaxed clock producing far larger error bars than the strict
model. For example, whereas the strict clock estimated an error for
the time of the diversification of the SIC that spans roughly
the latter half of the Cretaceous, the same error bar based on the
relaxed model spans from the Upper Jurassic to the end of the Cre-
taceous, thus overlapping with the error associated with the origin
of the RIC (Fig. 7). The relaxed model thus incorporates far greater
uncertainty in the divergence time estimates.
Fig. 5. Tree resulting from combined analysis of 18S and 28S rDNA by Bayesian inference (BI) showing nodal support as posterior probabilities and bootstrap percentages. Thick branches indicate clades found in 100% of the 19,200 topologies estimated by BI; values shown on (or to the left of) the thick branches show the average stability value for the members of the clade (i.e., the internal stability of the clade). Histogram on the right shows the relative Leaf Stability Value of each taxon. Host orders are indicated with symbols adjacent to terminal taxon names; question marks indicate species for which the elasmobranch (definitive) host is unknown. EUT, Eutetrarhynchoidea; GYM, Gymnorhynchoidea; LAC, Lacistorhynchoidea; OTO, Otobothrioidea; TBA, Trypanobatoida; TEN, Tentacularioidea; TSE, Trypanoselachoida.
Fig. 6. Character state distributions of selected morphological characters (with states in parentheses) mapped using accelerated transformation onto the Bayesian inference topology resulting from combined analysis of 18S and 28S rDNA data, as shown in Fig. 5. † indicates species for which morphological data were not coded. For character descriptions see Table 2. Asterisks indicate characters that are non-homoplasious. Character ambiguities are mapped on the tree at their basal-most potential position.
4. Discussion

4.1. Implications of the molecular data for trypanorhynch classification

The trypanorhynchcs have been divided historically into two suborders based primarily on whether they possess a plerocercoid or plerocercus larva. Referred to as the Acstidea and Cystidea, respectively, by Guirat (1927), he later replaced these names with Atheca and Thecaphora to avoid redundancy with existing echino-derm names (Guirat, 1931), this system, but with the former terminolgy, was followed in several important subsequent works (e.g. Fuhrmann, 1931; Schmidt, 1986). However, recognition of these two suborders, or of suborders in trypanorhynchcs in general, has not been widely endorsed. In fact, in four of the most comprehensive treatments of the order (Dollfus, 1942; Campbell and Beveridge, 1994; Beveridge et al., 1999; Palm, 2004), suborders were not recognized because the system of Guirat was thought to be non-natural. Following Pintner (1913, 1931), Dollfus (1942) focused his efforts to provide a classification of the trypanorhynchcs on features associated with the size, shape and arrangement of the tentacle hooks, coining the formal terminology for hook features that remains in use today (e.g. Campbell and Beveridge, 1994). The result of Dollfus' efforts was the recognition of four major groups, which he referred to as the Homéacanthes, Heteracanthes, typical, Heteracanthes atypical and Pécilacanthes. These groups were slightly redefined by Campbell and Beveridge (1994) to re-
flect hook pattern symmetry, and were recognized formally as the superfamilies Homeacanthoidea, Heteracanthoidea, Otobothrioidea and Poecilacanthoidea. Palm (2004), believing that tentacle armature should not be used to define higher trypanorhynch taxa owing to the potential homoplasies associated with similar modes of attachment, took a somewhat different approach. His classification emphasized other features of the scolex such as bothrial pits, prebulbar organs, complete or partly reduced rows of hooks and larval type to define five superfamilies: Tentacularioidea, Gymnorhynchoidea, Lacistorhynchoidea, Otobothrioidea and Eutetrarhynchoidea, the memberships of which had little correspondence to the superfamilies of Campbell and Beveridge (1994). Palm et al. (2009) persisted in recognizing these taxa despite the fact that the topology of the trees resulting from their molecular work show three of the five families to be non-natural as circumscribed by Palm (2004).

The results of the molecular analyses conducted here (Fig. 5) are inconsistent with both the suborders Atheca and Thecaphora, and deviate substantially from the superfamily classification of Campbell and Beveridge (1994). Our results are most consistent with the scheme proposed by Palm (2004) and subsequently endorsed by Palm et al. (2009), but even here several major differences exist. These are due in part to differences in tree topology, but also result from whether monophyly of higher taxa is required. For example, Palm et al. (2009) accepted the Otobothrioidea as a superfamily, despite its derived placement among the Lacistorhynchoidea. In our analyses, the Lacistorhynchoidea grouped as sister to the Otobothrioidea and thus we recognize both taxa as monophyletic superfamilies. Palm et al. (2009) persisted in the acce- tance of a paraphyletic Eutetrarhynchoidea with their recognition of the Tentacularioidea as an independent superfamily, despite its derived position among the Eutetrarhynchoidea on their trees. In an effort to circumcribe monophyletic superfamilies, our scheme would include the taxa previously assigned to the Eutetrarhynchoidea and the Tentacularioidea in the same superfamily. However, given that Tentaculariidae is the older of the two names and thus has priority, the group, if recognized as a single superfamily, should be referred to as the Tentaculariidae. Based on our results, we concur with Palm et al. (2009) that the Gymnorhynchoidea is monophyletic only if it is considered to include the Sphyriocephalidae, a family formerly placed in the Tenta- cularioidea (e.g. see Campbell and Beveridge, 1994; Palm, 2004). Our results provide little support for the family-level classification of Palm (2004) and amplified in Palm et al. (2009). Neither the Lacistorhynchoidea nor either of its subfamilies (Lacistorhynchoidea and Grillotiinae) are monophyletic. Similarly, neither of two families of Eutetrarhynchoidea included here (Eutetrarhynchoidea and Rhinoptericolidae) is monophyletic. In fact, these results suggest that both of the other families of Gymno- rhynchoidea (Gymnorhynchoidea or Gilquinidae) as defined by Palm (2004) and Beveridge and Justine (2006) is monophyletic. Whereas our results suggest that the Tentaculariidae is monophyletic as currently circumscribed, recognition of that family raises serious issues with the familial level classification of species in some of its immediate sister genera such as Rhinoptericola and Oncomegas, which do not collectively form a monophyletic assem- blage. In addition, and contrary to Palm et al. (2009), our results suggest that both the Otobothriidae and Pseudobothriidae are monophyletic.

Our work also calls into question the monophyly of many of the genera included in this study suggesting that the generic-level classification of trypanorhynchs in most superfamilies is in need of revision. Phylogenetic analyses revealed the following genera to be para- or in a few cases polyphyletic, both here and by Palm et al. (2009): Nybelinella, Kotorella, Dolflisiella, Tetranhynchobothrium, Parachristianella, Prochristianella, Poemotobothrium, Caliitetrarhynchus and Floriceps. This is despite the fact that the latter two genera were said to be monophyletic by Palm et al. (2009). In the case of Caliitetrarhynchus gracilis, whereas the two specimens collected from sharks were found to group together on our tree, the third specimen, collected as a larva from a bonyfish, grouped well away from the adult specimens of its conspecific. In an attempt to re- solve this apparent anomaly, the relevant voucher specimens were compared with all specimens of C. gracilis available in museum collections (British Museum of Natural History, UK; Muséum National d’Histoire Naturelle, France; United States National Parasite Collection, USA; South Australia Museum, Australia; Natural History Mu- seum Vienna, Austria; Musée d’Histoire Naturelle Genève, Switzerland) but no discrete morphological differences were detected among specimens. However, the formal investigation of the monophyly of these and other trypanorhynch genera was beyond the scope of this study and will require more focused sampling efforts.

4.2. Nomenclatural recognition of two primary lineages

The most conspicuous result of the molecular analyses con- ducted here is the suggestion that trypanorhynchs in fact comprise two independent lineages. One of these consists of the Eutetrarhynchoidea and one family of tentacularioid, both of which should now be considered to belong to a single superfamily. The other consists of the Lacistorhynchoidea, Otobothrioidea, Gymno- rhynchoidea as well as the sphyriocephalid genera Sphyriocephalus and Hepatosyilon, which were considered by Palm (2004) to belong to the Tentaculariidae but should now be considered with the Gymnorhynchoidea. Although supported by unique suites of char- acters discussed below, host associations of these two lineages are perhaps their most consistent distinguishing features (see Fig. 7). We thus propose that they be formally recognized as suborders, and in recognition of the ray associations of the first lineage pro- pose the name Trypanobatoida, and in recognition of the shark associations of the second lineage, propose the name Trypanoselachia. These names reflect not only the most common host asso- ciations within each lineage, but are hypothesized via ACSRs to be their ancestral host groups (see below).

4.3. Implications of molecular data for interpretation of character evolution

Characters that have been employed historically in the circum- scription of subgroups (i.e. suborders, superfamilies, families) have included the number of bothria (e.g. Blainville, 1828; Southwell, 1929; Pintner, 1931; Dollfus, 1942; Campbell and Beveridge, 1994; Palm, 2004), larval type (e.g. Dissing, 1850; Braun, 1894; Guiart, 1927; Schmidt, 1986; Palm, 2004), presence or absence of ciliated bothrial pits (= sensory flossettes) (e.g. Linton, 1889; Southwell, 1929; Campbell and Beveridge, 1994; Palm, 2004), presence or absence of pre-formed uterine pores (e.g. Pintner, 1913; Campbell and Beveridge, 1994), presence or absence of prebulbar organs (e.g. Palm, 2004), and an extensive suite of char- acters associated with tentacular armature patterns (e.g. Pintner, 1913, 1931; Dollfus, 1942; Campbell and Beveridge, 1994). It is on these kinds of characters that we therefore focus our morpho- logical discussions. Such characters of interest are shown in Fig. 6 mapped onto the topology of the combined molecular tree topology (Fig. 5).

Among the principal insights provided by these analyses was the fact that features employed previously to recognize suborders and/or superfamilies are unreliable indicators of genealogy. For
example, larval type (Character 2) fails to circumscribe the two major clades and although the derived state of this character (i.e. lacking a blastocyst) more or less defined the Tentacularioidea, this condition also occurred in two genera that should be considered members of the superfamily Gymnorhynchoidea (i.e. Hepatoxylon and Sphyriocephalus, both in the Sphyriocephalidae). This explains the lack of support for the family-level classification of Palm (2004) in which the Sphyriocephalidae was placed in Gymnorhynchoidea and not the Tentacularioidea. Similarly, the major hook features including armature type (Character 29), hook pattern (Character 30), hook symmetry (Character 32) and chaînette type (Character 34) were found to be homoplastic and fail to define major groups. For example, homoplasy in hook pattern reveals the Heteracanthes of Dollfus (1942), Homeacanthoidea of Campbell and Beveridge (1994), Tentacularioidea of Palm (2004), as well as the Heteracantha of Dollfus (1942) and Heteracanthoidea of Campbell and Beveridge (1994) to be polyphyletic. Some of this homoplasy, however, is likely to result from difficulties in interpreting hook patterns and this has been mooted previously. Bussieras (1970) and Beveridge and Campbell (1996) noted that whereas the metabasal armature of the Sphyriocephalidae and Hepatoxylidae was homeoacanthous, their basal armature was heteroacanthous. Homoplasy in intercalary hooks (Character 33) reveals the Heteracantha of Dollfus (1942) and the Otobothrioidea as circumscribed by Campbell and Beveridge (1994) also to be polyphylectic. Homoplasy in the presence of a chaînette (Character 34) suggests that the Péclacanthes of Dollfus (1942) and the Péclacanothoidea of Campbell and Beveridge (1994) are polyphylectic. The notion that there might be homoplasy in chaînettes was first advanced by Beveridge and Campbell (1989) who recognized that there is considerable diversity in hook types and arrangements currently considered to comprise a “chaînette”. We suspect that a more careful examination of the criteria employed to recognize this feature would do much to resolve at least some of the apparent homoplasy.

There are a number of additional apparently homoplastic characters which, although not incorporated into formal classification schemes, have been considered of importance to trypanorhynch classification. For example, solid hooks (Character 28) appear multiple times within the Trypanobatoida and only once within the Trypanoselachoida. The evolution of four bothria (Character 4), an important character for distinguishing families once within the Trypanoselachoida, but within this clade are conspicuously absent at the bases of the tentacular bulbs. In contrast, the Trypanobatoida lack prebulbar organs and gland cells within the tentacular bulbs and all Trypanobatoida exhibit retractor muscles that originate at the bases of the tentacular bulbs. Furthermore, it could be argued that while the Trypanobatoida exhibit simple terminal genital ducts, the Trypanoselachoida exhibit more complex terminal genital ducts that include hermaphroditic ducts (Lacistorhynchoidea and Otobothrioidea), accessory seminal vesicles (Gilquiniidae and Gymnorhynchoidea) and extensions of the internal seminal vesicle beyond the muscular wall of the cirrus sac into which the retracted cirrus extends (Grillotia, Gymnorhynchus), none of which have been observed in the former suborder. In the Trypanobatoida, modifications of the terminal genital ducts are restricted to the presence of paired internal seminal vesicles (e.g. Tetrarhynchobothrium) and vaginal diverticula (Zygorynchus) neither of which have been considered in the present analysis. Although the morphological underpinnings of the proposed suborders require additional investigation, strong molecular support for these primary lineages suggests that reciprocal illumination may reveal additional characters.

4.4. Morphological characters underpinning recognition of the Trypanobatoida and Trypanoselachoida

Beyond the robust molecular support for the proposed suborders Trypanobatoida and Trypanoselachoida, these lineages can be circumscribed to some extent by unique suites of morphological characters. For example, all Trypanobatoida except the tentacularoids, possess prebulbar organs and gland cells within the tentacular bulbs and all Trypanobatoida exhibit retractor muscles that originate somewhere near the middle of the tentacular bulbs. Furthermore, it could be argued that while the Trypanobatoida exhibit simple terminal genital ducts, the Trypanoselachoida exhibit more complex terminal genital ducts that include hermaphroditic ducts (Lacistorhynchoidea and Otobothrioidea), accessory seminal vesicles (Gilquiniidae and Gymnorhynchoidea) and extensions of the internal seminal vesicle beyond the muscular wall of the cirrus sac into which the retracted cirrus extends (Grillotia, Gymnorhynchus), none of which have been observed in the former suborder. In the Trypanobatoida, modifications of the terminal genital ducts are restricted to the presence of paired internal seminal vesicles (e.g. Tetrarhynchobothrium) and vaginal diverticula (Zygorynchus) neither of which have been considered in the present analysis. Although the morphological underpinnings of the proposed suborders require additional investigation, strong molecular support for these primary lineages suggests that reciprocal illumination may reveal additional characters.

4.5. Implications for previous interpretations of trypanorhynch evolution

Existing explicit morphology-based phylogenetic hypotheses for the trypanorhynchs (e.g. Campbell and Beveridge, 1994; Beveridge et al., 1999; Palm, 2004) are highly incongruent. It is likely that differences in the methods employed in the generation of these trees account for much of this incongruence. For example, the tree presented by Campbell and Beveridge (1994) was meant merely to summarize their thoughts on trypanorhynch phylogenetic relationships; on this tree they mapped 13 characters they considered to be of use in supporting these relationships. In contrast, the trees of Beveridge et al. (1999) and Palm (2004) were the result of matrix-based phylogenetic analyses. However, the
characters on which these trees were based differed. Whereas the former was based on 44 characters, 17 of which concerned tentacle armature, the latter expanded the characters to a total of 60, 24 of which concerned tentacle armature. In addition, these authors handled the challenges imposed by the unique nature of many of the features of trypanorhynchs differently. Whereas Beveridge et al. (1999) assumed that the simplest condition of each of their characters was plesiomorphic, Palm (2004) assumed (with a few exceptions) the most common state of each character to be plesiomorphic.

Beyond the level of major subgroups, all of these morphological hypotheses also differ substantially from the trees resulting from the molecular work conducted here. Certainly our formal use of outgroup comparisons is likely to contribute to some of these differences. However, the taxonomic category of interest among these studies also differs. Whereas our analyses were specimens (and thus species) based, Campbell and Beveridge (1994) presented their results in the context of families, and both Beveridge et al. (1999) and Palm (2004) focused on genus-level taxa, thereby using combined codings for their terminal taxa. The latter approaches are clearly invalid in cases where families and genera have been shown to be non-natural.

It is similarly difficult to compare the results of the character analysis employed here, based on formal coding and parsimony mapping of 45 characters for each of our 79 ingroup taxa, with the discussion of the 13 morphological characters assumed to represent important morphological synapomorphies in Palm et al. (2009). This is not only because the method employed to obtain the character mapping presented by the latter authors is not articulated, but also because the mappings presented do not appear to be consistent. For example, the character “solid hooks” is indicated as a synapomorphy for their clades Eutetrarhynchoida and Tantacularioidea, but no indication is given that one of the relatively major clades of this group (including a diversity of Dolfusiella and Tetrarhynchobothrium and Paraoncomegas spp.) is comprised of species that exhibit hollow hooks. Similarly, the symbolic coding of the armature pattern “homeoacanthous” is inconsistent with the actual mapping of homeoacanthous armature presented on the tree itself. Nevertheless, some similarities were found. For example, number of bothria, which was shown to be remarkably homoplasic by Palm et al. (2009), was similarly homoplasic in our analysis, and features such as prebacular organs, found to represent a synapomorphy for the batoïd-infecting clade was similarly synapomorphic in our study.

4.6. Host–parasite coevolution and the Hypnosqualea hypothesis

That the phylogeny of the trypanorhynchs is characterized by a basal split leading to two major lineages, the ancestral hosts of which were alternatively a carcharhiniform shark or a rajiform batoid, we find to be compelling evidence in support of rejection of the Hypnosqualea hypotheses, or the notion that rays represent a lineage of derived sharks. This finding is congruent with the most recent molecular phylogenetic studies of elasmobranchs as illustrated in Fig. 3 (i.e. Douady et al., 2003; Winchell et al., 2004; Naylor et al., 2005; Human et al., 2006), and provides a source of evidence derived independently from the hosts themselves. Beyond this basal diversification, however, the phylogeny of the parasites does not appear to be strongly influenced by the pattern of their host’s evolution (i.e. to the extent the latter is known). In the diversification of the Trypanoselachoida, for example, it could be argued that a cladogenic event in the Middle Cretaceous led to the evolution of a squaliform-hosted lineage, the Gymnorrhynchoidea. However, ACRs of the host groups predict the ancestral host of the Gymnorrhynchoidea to be a member of the Lamniformes, despite the fact that squaliform sharks are the more common contemporary hosts in this clade (see Fig. 7). This is in contrast to our understanding of elasmobranch phylogeny in which the lamniform sharks are considered to represent the sister taxon of the carcharhiniform sharks (see Fig. 3), whereas the squaliform sharks are thought to share a more recent common ancestor with the angel sharks (Squatinaformes), saw sharks (Pristiphoriformes) and related groups (see Fig. 3). Thus even such major diversification events in the host phylogeny do not appear to be mirrored by the parasite phylogeny. However, these data remain to be evaluated in the context of a comprehensive, molecular-based phylogeny of the elasmobranchs, such that topological congruency analyses can be conducted without the confounding problem of missing data.

The level of fidelity shown to the final, elasmobranch hosts has been shown to be highly variable among trypanorhynchs (Palm and Caira, 2008), and thus one would predict different evolutionary influences (i.e. phylogenetic versus ecological) depending on whether the specific trypanorhynch group examined showed high or low levels of host specificity. The Trypanoselachoida and Trypanobatoida each contain examples of both extremes of host specificity, and include species (although rare) that parasitize both sharks and rays (e.g. species of Dolfusiella, Parachristianella and Lactorhrynchus). In most cases, host switching inferred from the parasite phylogeny can be readily explained by ecological, rather than phylogenetic, influences. For example, the clade within the Trypanoselachoida including species of Grilliotia and Pterobothrium contains species parasitising rays that are closely related to species parasitising pristiophoriform and orectolobiform sharks, both host groups including many bottom-feeding taxa whose ecological niche overlaps extensively with that of the batoids (Wetherbee and Cortés, 2004). The opportunity for host-switching in such groups is thus great, and evidence of ecologically driven host-switching combined with the existence of generalist species that can infect a broad spectrum of elasmobranchs suggest that any physiological differences between sharks and rays have not provided a significant barrier to colonization. Such differences are therefore unlikely to explain a dichotomous phylogenetic pattern separating the Trypanoselachoida from the Trypanobatoida, and thus this early diversification is best explained through coevolution, whereas subsequent diversification events are more likely to be explained by ecological factors.

As with the morphological analyses, direct comparisons with the treatment of Palm et al. (2009) are difficult to make due to the differences in their considered and methodologies employed. For example, the families of definitive hosts considered by Palm et al. (2009) represent only a subset of the families of elasmobranchs known to host species in the trypanorhynch genera under consideration in our analyses (see Palm, 2004); omitted were the Etmopteriidae, Hemiscylliidae, Heterodontidae (and its order Heterodontiformes), Hexanchidae, Parascylliidae, Scyliorhinidae, Somniosidae and Urotrygonidae. Moreover, in a number of instances different suites of host families are presented for parasite genera replicated across the tree topology. Of perhaps greater significance, the fact that no attempt was made to determine ancestral host states or ages of divergence, leaving conclusions to be made on the basis of the assumption that common equals primitive, which as demonstrated above, can be misleading (e.g. ancestral hosts of the Gymnorrhynchoidea).

4.7. Divergence time estimates

Without a fossil record, calibrating the phylogenetic tree of a parasite group can only be done by assuming that they are at least as old as their hosts, which not only leaves considerable room for error but cannot be tested directly. In fact, simply estimating a date of divergence between sharks and rays is highly tenuous despite
their relatively extensive fossil record. The resulting uncertainty associated with the divergence estimates makes support for most scenarios permissible and thus we simply cannot draw robust conclusions. From a relative standpoint, the divergence dates of the two major lineages of trypanorhynchs is at least consistent with the fossil record of the hosts in that, according to Cappetta et al. (1993), the earliest evidence of the Rajiformes comes from the Toarcian (Lower Jurassic; ~183 Myr) with the appearance of the Rhinobatidae, whereas the Carcharhiniformes first occur slightly later in the Bathonian (Upper Jurassic; ~167 Myr) with the appearance of the Scylliorhinidae, and not until the Turonian (Middle Cretaceous; ~93.5 Myr) do we first see the appearance of carcharhiniform sharks that host contemporary trypanorhynchs (i.e. Triakidae) (Cappetta et al., 1993). Diversification of the Trypanoselachoida thus correlates well with the first appearance of the Triakidae in the Middle Cretaceous (see Fig. 7), whereas diversification of the Trypanobatoidea is estimated to have occurred ~40 Myr earlier around the Jurassic–Cretaceous boundary. The latter date falls significantly later than that the first appearance of the contemporary ray groups (even given a 60 Myr error bar), but nevertheless predates the diversification of the Trypanoselachoida, which is consistent with the host record in a relative sense.

Whereas the ancestral host group of the Trypanobatoidea is estimated to be a member of the Rajiformes with effectively no support for alternative batoid orders (i.e. Pristiformes or Torpediniformes), that of the Trypanoselachoida is more equivocal, with the Carcharhiniformes having a likelihood of only ~52%, the Lamniformes receiving ~34%, the Squiliformes ~10%, and the remaining shark orders ~1% each. With regard to the fossil record, this node corresponds equally well with first appearances of both the carcharhiniform (i.e. Triakidae) and lamniform (i.e. Lamnidae) shark hosts of contemporary trypanorhynchs, albeit error associated with this estimate is great enough to encompass the first appearance of the carcharhiniform sharks (i.e. Squilidae; Upper Jurassic) as well. Thus while the Squiliformes is calculated to have a very low likelihood of being the ancestral host group of the Trypanoselachoida, and while its date of first appearance shows less correspondence with the estimated divergence of the Trypanoselachoida than with either the Carcharhiniformes or Lamniformes, the level of uncertainty associated with the analysis does not allow us to dismiss it.

An obvious question concerning divergence rates is whether or not the disparity seen in the parasite phylogeny, in which the Trypanobatoidea shows a markedly greater average divergence than that of the Trypanoselachoida (Figs. 4–7), is reflected in differing rates between sharks and rays. The question is difficult to answer given our limited understanding of the host evolution. The most comparable data set is that of Winchell et al. (2004) who also used a combination of lsrDNA and ssrDNA and thus the comparison is not confounded by choice of genes. In their work the batoids do appear to show a greater divergence than the majority of the shark lineages (their Fig. 2). A major exception, however, is the carcharhiniform sharks that show even greater divergence than the rays, whereas this disparity is not reflected in the parasite phylogeny. Moreover, studies based on mitochondrial genes (i.e. Douady et al., 2003; Human et al., 2006) do not show this disparity either between sharks and rays or between the carcharhiniform and other sharks. Thus the lack of correspondence in the disparity of rates in the parasite and host phylogenies remains an outstanding question.

4.8. Host-parasite coevolution

Because copeciation events become masked through time via host switching, extinction and lineage sorting, comparisons of independently-derived host and parasite phylogenies fail more often than not to show significant levels of congruence. This presumably accounts for the fact that the field of host-parasite coevolution has generated greater advances in methodology than in the generation of empirical data: very few actual data sets have been found that show clear coevolutionary patterns (see Page, 2003). Even the ubiquitously used example, pocket gophers and their chewing lice (Hafner and Page, 1995), has been shown to exhibit an unusually high level of coevolution when chewing lice and their mammalian hosts are compared more broadly (Taylor and Purvis, 2003). Although few people would argue that the process of coevolution has been a non-existent or inconsequential part of parasite evolution (especially among parasites having little independent means of dispersal, such as chewing lice), finding empirical evidence has proven exceedingly difficult in most groups. For these reasons, the simple lack of congruence itself, as noted by Page (2003), does not necessarily signal lack of cospieciation. However, it obviously provides no evidence for it.

Advancing the work presented here would benefit from additional sampling of the trypanorhynchs fauna, particularly where that would introduce new genera or host associations not represented herein, but given our broad sampling of their diversity, will depend chiefly on obtaining a more comprehensive understanding of elasmobranch evolution. Ideally what is required is a comprehensive and well calibrated tree incorporating multiple molecular divergence rate estimates as well as multiple calibration points based on fossil evidence. Most such data are available today, except with regard to the molecular characterization of elasmobranchs, in which different genes are available for different subsets of taxa. A concerted effort is thus needed to produce a more comprehensive analysis of their evolution, which in turn will have utility for understanding the evolution of the flatworm, arthropod, annelid, nematode, 'protozoan' and other parasitic animals for which the great diversity of elasmobranchs play host (Caira, 1990).
Appendix A. Supplementary data


References


