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Extraordinary host switching in siphonostomatoid copepods and the demise of the Monstrilloida: Integrating molecular data, ontogeny and antennulary morphology

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Abstract

Copepods exhibit an astounding variety of lifestyles, host associations and morphology, to the extent that their crustacean affinities may be obscured. Relationships among the ten copepod orders based on morphological characters remain equivocal. Here we test the ordinal status of the enigmatic Monstrilloida using SSU rDNA gene sequences, comparative morphological data (antennulary sensory interface) and ontogenetic data (caudal ramus setation patterns). Bayesian analysis unexpectedly revealed the Monstrilloida are nested within a fish-parasitic clade of the Siphonostomatoida and share a common ancestor with the stem species of the caligiform families (sea-lice). This unforeseen relationship is congruent with both antennulary and caudal ramus morphology. The divergence of the monstrilloids from an ectoparasitic, vertebrate-associated ancestor involved radical changes in host utilization, body plan and life cycle strategy, a combination rarely observed and probably unique in metazoan parasites. Adult monstrilloids secondarily returned to a free-living, predator-exposed mode of life and we postulate the pressure on maintaining a functional approaching-predator detection system has progenetically delayed the suppression (as in post-copepodid caligiform instars) of the 5-point antennulary sensory array. The homoplastic evolution of the frontal filament in Siphonostomatoida is discussed.

Keywords: Monstrilloida; Caligiform families; SSU rDNA; Morphology; Host switching; Phylogenetic inference

1. Introduction

No group of plants or animals on Earth exhibits the range of morphological diversity as seen among the extant Crustacea (Martin and Davis, 2001). This structural disparity is best demonstrated by the Copepoda, which by virtue of their immense vertical distribution—from the abyss to the high Himalayas, spanning three quarters of the possible vertical range on Earth—are also arguably the most abundant metazoans (Hardy, 1970; Huys and Boxshall, 1991). Earlier workers depicting the evolutionary history of the Copepoda considered primarily body tagmosis and major modifications of the cephalic feeding structures (Thorell, 1859; Giesbrecht, 1892; Sars, 1903–1921; Kabata, 1979), often being more concerned with the ecological radiation of the group than with a rigorous application of the concept of homology (Dussart, 1984; Marcotte, 1982, 1986; Stock, 1991). The advent of cladistic methodology provided a conceptual turning point in the study of copepod interrelationships and culminated with the publication of a large-scale phylogenetic analysis of the ten copepod orders (Huys and Boxshall, 1991). Some challenges to this phylogenetic scheme have arisen because of revised interpretations of the original data set (Ho, 1994; Ho et al., 2003), the discovery of new material (Ho et al., 1998; Huys et al., 2002), and the upgrading of certain subsets of taxa (Ho et al., 2003; Dahms, 2004).

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However, with the continuous arrival of new morphological data, the study of higher copepod phylogeny is so carried by its own momentum that it seems presumptuous to consider where and how new insights will arrive. There is an obvious, auspicious direction to the study of gene sequences, an area in copepod research that has not yet received the attention it deserves (Huys et al., 2006). Another promising route, despite its long cultivation, concerns the elucidation of ontogenetic processes and patterns, as a basis for further insights on character transformation in phylogeny (Boxshall and Huys, 1998). Likewise, the explanatory power of individual setation elements continues to offer new evidence (Huys, 2001; Huys and Boxshall, 1991; Huys et al., 2002). Given the limitations in traditional morphological approaches, the time appears ripe to untangle the intricate relationships of free-living and symbiotic copepods with a diverse arsenal of data and systematic methods. A better map of copepod evolution provides a critical framework for addressing vexed issues on zoogeography, host switching, habitat colonization and the connection between phylogeny and character transformation.

One of the major contentious issues in copepod evolution concerns the position of the Siphonostomatoida, a very diverse order that has established symbiotic relationships with marine mammals (Cetacea), fish (Myxini, Holocephali, Elasmobranchii, Actinopterygii), and no less than eight invertebrate phyla (Porifera, Cnidaria, Annelida, Mollusca, Arthropoda, Bryozoa, Echinodermata, Urochordata). Most authors now accept the monophyly of the group [e.g. Boxshall (1986), although see Marcotte (1982) for a dissenting opinion] based on the shared presence of an oral cone or siphon. Faced with the homoplastic evolution of this character in other copepod orders (reviewed in Huys et al., 2006), an independent assessment of siphonostomatoid monophyly is required as well as of the alleged sistergroup relationship with the enigmatic Monstrilloida. Members of the latter order have a bizarre, protelean life history, comprising endoparasitic naupliar stages and free-swimming, non-feeding, pelagic adults. Unlike most other copepods, that either produce egg sacs or spawn freely in the water column, monstrilloid females attach their eggs onto long ovigerous spines by means of a mucous substance secreted by the terminal part of the oviduct. Huys and Boxshall (1991) showed that egg masses are produced iteratively, the ovigerous spines growing accordingly when a new batch is being spawned. Eggs hatch into lecithotrophic nauplii that locate a mollusc or polychaete host and burrow into its tissues. After undergoing a remarkable metamorphosis in the host's blood system, the endoparasitic sac-like naupliar stage bears virtually no resemblance to other crustacean larvae (Malaquin, 1901; Caullery and Mesnil, 1914; Pelseneer, 1914). Once development is complete the monstrilloid leaves its host as a last copepodid and undertakes a single moult into the adult, which lacks all cephalic appendages except for the antennules. The known hosts include pyramidellid (Pelseneer, 1914; Gallien, 1934) and vermetid (R. Huys, unpublished) prosobranch gastropods, bivalves (Boxshall and Halsey, 2004), sponges (R. Huys, unpublished) and polychaete worms (e.g. Malaquin, 1901; Caullery and Mesnil, 1914; Huys and Boxshall, 1991; Nishi in Grygier, 1995).

Huys and Boxshall (1991) considered Monstrilloida and Siphonostomatoida sistertaxa based on the shared presence of a cephalothorax incorporating the first pedigerous somite, and, a similar leg 5 (loss of intercoxal sclerites, formation of baseoendopod in the female). These characters, however, have evolved convergently in other copepod orders and, consequently, can hardly be considered a secure base for common ancestry. The problem is further compounded by the nature of the autapomorphies used to define the Siphonostomatoida. At present, monophyly of the group rests exclusively on derived character states displayed by the cephalic appendages. Unfortunately all these characters had to be scored as missing data in its sistertaxon, the Monstrilloida (Huys and Boxshall, 1991) which lacks antennae, mandibles, maxillules, maxillae and maxillipeds. The monstrilloid body plan offers few morphological clues of phylogenetic significance since (1) the infective nauplius stage is too specialized for meaningful inferences to be made (Grygier and Ohtsuka, 1995), and (2) the swimming leg setation pattern in the planktonic adults is more derived than the ancestral state found in most other copepod orders (Huys and Boxshall, 1991). Consequently, the anterior antennules and the posterior caudal rami remain the only potentially informative appendages that can partly substitute for the missing head appendages in efforts towards resolving the origin and relationships of this order. In this study we test the ordinal integrity of the Monstrilloida using comparative morphological data derived from the antennulary sensory interface, ontogenetic data based on caudal ramus setation patterns, and molecular data from almost complete SSU rDNA genes. In the tradition of modern molecular phylogenetics we used nuclear ribosomal genes because they exhibit semiconserved domains interspersed with divergent regions, and hence allow phylogenetic reconstruction over a wide range of taxonomic levels. We also use our molecular data to test siphonostomatoid monophyly, potential host switching events and the single origin of vertebrate parasites in this group.

2. Material and methods

2.1. Taxon sampling and extraction of gDNA

Representatives of the three major symbiotic orders, Cyclopoida (which now embraces the Poecilostomatoida; see Boxshall and Halsey (2004)), Monstrilloida and Siphonostomatoida were collected from various vertebrate and invertebrate hosts, or from plankton samples. A range of Calanoida was also included since this order is widely accepted as the most primitive within the Neocopepoda

(Huys and Boxshall, 1991; Ho, 1994). Finally, we added available GenBank sequences for two non-copepodan outgroup taxa, a primitive malacostracan (*Squilla empusa* Say) and a basal thecostracan (*Berndtia purpurea* Utinomi). Our analysis focuses on the relationship between Siphonostomatoids and monstrilloids and does not intend to present an ordinal phylogeny of the Copepoda as this would be beyond the scope of the paper. Secondly, it should be noted that this paper does not aim at an analysis of the phylogenetic relationships between siphonostomatoid families. The Ecbathyriontidae and Dirivultidae were selected as basal invertebrate-associated families because they are likely to be more primitive than the Asterocheridae, for example in the presence of a basal enditic seta on the maxilla. Preferably, the Asterocheridae (and other families such as the Brychiopontiidae, Scottomyzontidae, Coralliomyzontidae and Atrotrogidae, all of which may be nested within the former) should be subjected to a separate phylogenetic analysis—both morphologically and molecularly—before it can be included in a broader scale analysis. It was consequently excluded here.

Table 1 provides a taxonomic listing of the exemplar species analysed, their collection localities and accession numbers of the sequences. Published sequences from previous studies based on complete SSU rDNA (Spears and Abele, 1998; Huys et al., 2006) were also included. Newly collected specimens were fixed live in the field using 95–100% EtOH and stored in 95% EtOH at −20 °C. Whole specimens or subsamples (when total specimen volume > ~4 mm^3) were transferred to 1.5-ml microcentrifuge tubes and left at 70 °C for a few minutes to eliminate residual EtOH. Tissue homogenization was achieved by physical maceration using a teflon pestle and/or by freeze fracturing (exposure to liquid N_2). Genomic DNA was extracted using a 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 200 µl. In order to increase the gDNA concentration, the elution volume was reduced to 50 µl in a vacuum centrifuge.

2.2. PCR amplification and sequencing

PCR amplifications (50 µl) were performed using a HotStarTaq® DNA Polymerase kit (QIAGEN®), using 25 µl HotStarTaq Master Mix (containing HotStarTaq DNA Polymerase, PCR buffer (with Tris–Cl, KCl, $(NH_4)_2SO_4$, 3 mM $MgCl_2$; at pH 8.7) and 400 µM of each dNTP), 2–5 µl of genomic extract and 10 µM of each PCR primer using the following thermocycling profile: 15 min denaturation hold at 95 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C (fragment 1), 59 °C (fragment 2) or 57 °C (fragment 3), 2 min at 72 °C; and 10 min extension hold at 72 °C. Three overlapping SSU rDNA sequence fragments (~600 bp) were amplified using primers 18Sf (5′-TAC CTG GTT GAT CCT GCC AG-3′) and 1282r (5′-TCA CTC CAC CAA CTA AGA ACG GC-3′) for fragment 1, 554f (5′-AAG TCT GGT GCC AGC AGC CGC-3′) and 614r (5′-TCC AAC TAC GAG CTT TTT AAC C-3′) for fragment 2, and 1150f(p2) (5′-ATT GAC GGA AGG GCA CCA CCA G-3′) and 18Sr (5′-TAA TGA TCC TTC CGC AGG TTC AC-3′) for fragment 3. PCR amplicons were either gel-excised or purified directly using QIAGEN® QIAquick® spin columns, cycle-sequenced from both strands using Applied Biosystems BigDye™ chemistry, alcohol-precipitated and run on an Applied Biosystems Prism 377™ Automated Sequencer or an Applied Biosystems 3730™ DNA Analyzer. SSU rDNA products were sequenced in both directions using the six PCR primers. Contiguous sequences were assembled and edited using Lasergene ver. 4.0.3 (DNASTAR, Inc. Madison) and submitted to GenBank under Accession No. DQ538495–509 (Table 1).

2.3. Sequence alignment

Sequences were aligned by eye using BioEdit Sequence Alignment Editor (Hall, 1999, ver. 5.0.9) and MacClade (Maddison and Maddison, 2002, ver. 4.06). Regions of ambiguous alignment were delimited by identifying the first parsimony-uninformative nucleotide on each side of an unalignable region and were excluded from subsequent phylogenetic analyses. The alignment comprised 1941 nucleotide positions of which 1631 were unambiguously alignable, 632 variable and 499 parsimony informative.

2.4. Phylogenetic analyses

Phylogenetic analyses were conducted using the methods of maximum parsimony and Bayesian inference. Maximum parsimony (MP) analysis was conducted with PAUP* (Swofford, 2001, ver. 4.0b10) and Bayesian inference (BI) analysis with MrBayes (Huelsenbeck and Ronquist, 2001, ver. 2.01). Maximum parsimony analysis was performed using a heuristic search strategy with random addition sequences followed by tree-bisection-reconnection branch-swapping (TBR) on 10,000 search replicates (MULTREES was in effect and only one tree in each replicate was saved), with all characters unordered, equally weighted, and with gaps treated as missing data. This strategy was adopted to enable searching in a wide area of tree space, maximizing the chances of finding multiple islands of equally parsimonious trees. All trees from different islands were used as starting trees for further TBR searches with MAXTREES effectively unlimited.

Evaluation of the various models of nucleotide substitution using ModelTest (Posada and Crandall, 1998, ver. 3.06) showed the most parameter rich model (i.e. general-time-reversible including estimates of invariant sites and gamma distributed among site rate variation) provided the best fit to the data. This was true when evaluating the

Table 1
Taxonomic listing of exemplar taxa, their classification, host taxon (if applicable; —: original host unknown), voucher specimen registration number, SSU rDNA sequence accession numbers (GenBank/EMBL), used in this study

Exemplar taxon	Classification	Host taxon	NHM reg. no	GenBank Accession No.
Squilla empusa (Say, 1818)	Malacostraca, Stomatopoda		—	L81946
Berndtia purpurea (Utinomi, 1950)	Thecostraca, Ascothoracida		—	L26511
Calanus pacificus (Brodsky, 1948)	Calanoida, Calanidae		—	L81939
Pseudocyclops sp.	Calanoida, Pseudocyclopidae		2005.42	AY626994*
Exumella mediterranea (Jaume and Boxshall, 1995)	Calanoida, Ridgewayiidae		2005.44	AY629259*
Tortanus sp.	Calanoida, Tortanidae		2007.7–8	AY626995*
Euryte sp.	Cyclopoida, Cyclopidae	*Halimeda* washings	—	AY626996*
Apocyclops royi (Lindberg, 1940)	Cyclopoida, Cyclopidae		2005.46	AY626997*
Macrocyclops albidus (Jurine, 1820)	Cyclopoida, Cyclopidae		—	DQ538505†
Acanthocyclops viridis (Jurine, 1820)	Cyclopoida, Cyclopidae		—	AY626999*
Anchimolgidae gen. nov. §	Cyclopoida, Anchimolgidae	*Catalaphyllia jardinei*	2005.47	AY627000*
Anchimolgus sp. §	Cyclopoida, Anchimolgidae	*Polyphyllia talpina*	2005.48	AY627001*
Anthessius sp. §	Cyclopoida, Anthessiidae	*Tridacna squamosa*	2005.49	AY627002*
Chondracanthus lophii (Johnston, 1836) §	Cyclopoida, Chondracanthidae	— (probably *Lophius piscatorius*)	—	L34046
Lernentoma asellina (Linn., 1758) §	Cyclopoida, Chondracanthidae	*Chelidonichthys gurnardus*	2005.50	AY627003*
Lichomolgidium sp. §	Cyclopoida, Lichomolgidae	*Polycarpa clavata*	2007.9-18	DQ538504†
Stellicola sp. §	Cyclopoida, Lichomologidae	*Culcita novaeguineae*	2005.51	AY627004*
Mytilicola intestinalis (Steuer, 1902) §	Cyclopoida, Mytilicolidae	*Mytilus edulis*	2005.52	AY627005*
Pectenophilus ornatus (Nagasawa et al., 1988) §	Cyclopoida, Mytilicolidae	*Chlamys farreri nipponensis*	2005.53	AY627032*
Trochicola entericus (Dollfus, 1914) §	Cyclopoida, Mytilicolidae	*Calliostoma zizyphinum*	—	AY627006*
Pseudanthessius sp. §	Cyclopoida, Pseudanthessiidae	*Filograna* sp.	2005.54	AY627007*
Critomolgus sp. 1 §	Cyclopoida, Rhynchomolgidae	*Chromodoris* sp.	2005.55	AY627008*
Critomolgus sp. 2 §	Cyclopoida, Rhynchomolgidae	*Actinodendron* cf. *globeratum*	2005.56	AY627009*
Sabelliphilus elongatus (M. Sars, 1862) §	Cyclopoida, Sabelliphilidae	*Sabella pavonina*	2005.57	AY627010*
Scambicornus sp. §	Cyclopoida, Synapticolidae	*Thelenota ananas*	2005.58	AY627011*
Vahinius sp. §	Cyclopoida, Vahiniidae	*Antipathes* sp.	2005.59	AY627012*
Xarifia sp. §	Cyclopoida, Xarifiidae	*Acropora millepora*	2005.60	AY627013*
Cymbasoma sp.	Monstrilloida, Monstrillidae	[plankton]	—	DQ538498†
Monstrilla clavata (G.O. Sars, 1921)	Monstrilloida, Monstrillidae	[plankton]	—	DQ538495†
Monstrilla sp.	Monstrilloida, Monstrillidae	[plankton]	2007.19	DQ538496†
Monstrillopsis sp.	Monstrilloida, Monstrillidae	[plankton]	—	DQ538497†
Gloiopotes watsoni (Kirtisinghe, 1934)	Siphonostomatoida, Caligidae	*Makaira nigricans*	2005.64	AY627019*
Lepeophtheirus salmonis (Krøyer, 1837)	Siphonostomatoida, Caligidae	— (probably *Salmo salar*)	—	AF208263
Lepeophtheirus hippoglossi (Krøyer, 1837)	Siphonostomatoida, Caligidae	*Hippoglossus hippoglossus*	2007.20–24	DQ538503†
Caligus elongatus (von Nordmann, 1832)	Siphonostomatoida, Caligidae	*Gadus morhua*	2005.65	AY627020*
Cancerillidae sp.	Siphonostomatoida, Cancerillidae	unidentified Ophiuroidea	—	AY627021*
Aphotopontius mammilatus (Humes, 1987)	Siphonostomatoida, Dirivultidae	*Bathymodiolus thermophilus*	2007.25–34	DQ538508†
Ceuthoecetes sp.	Siphonostomatoida, Dirivultidae	*Bathymodiolus thermophilus*	2007.35–40	DQ538506†
Rhogobius contractus (Humes, 1987)	Siphonostomatoida, Dirivultidae	*Bathymodiolus thermophilus*	2005.67	AY627023*
Dissonus manteri (Kabata, 1966)	Siphonostomatoida, Dissonidae	*Plectropomus leopardus*	2007.41	DQ538500†
Ecbathyrion prolixicauda (Humes, 1987)	Siphonostomatoida, Ecbathyriontidae	washings of vent invertebrates	—	AY627024*
Hatschekia pagrosomi (Yamaguti, 1939)	Siphonostomatoida, Hatschekiidae	*Sparus auratus*	2005.69	AY627026*
Hatschekia sp.	Siphonostomatoida, Hatschekiidae	*Sufflamen fraenatus*	2007.42	DQ538507†

(*continued on next page*)

Table 1 (*continued*)

Exemplar taxon	Classification	Host taxon	NHM reg. no	GenBank Accession No.
Kroyeria sp.	Siphonostomatoida, Kroyeriidae	*Negaprion acutidens*	2007.43	DQ538499†
Parabrachiella bispinosa (von Nordmann, 1832)	Siphonostomatoida, Lernaeopodidae	*Chelidonichthys gurnardus*	2005.70	AY627027*
Clavella adunca (Strøm, 1862)	Siphonostomatoida, Lernaeopodidae	*Melanogrammus aeglefinus*	2005.71	AY627028*
Nanaspis tonsa (Humes and Cressey, 1959)	Siphonostomatoida, Nanaspididae	*Thelenota ananas*	2005.72	AY627029*
Choniosphaera maenadis (Bloch and Gallien, 1933)	Siphonostomatoida, Nicothoidae	*Carcinus maenas*	2007.44–47	DQ538509†
Dinemoura latifolia (Steenstrup and Lütken, 1861)	Siphonostomatoida, Pandaridae	*Isurus oxyrinchus*	2007.48–51	DQ538501†
Pandarus smithii (Rathbun, 1866)	Siphonostomatoida, Pandaridae	*Isurus oxyrinchus*	2007.52	DQ538502†
Lernaeocera branchialis (Linn., 1767)	Siphonostomatoida, Pennellidae	*Merlangius merlangus*	2005.73	AY627030*

Poecilostomatoid taxa indicated by §. *GenBank sequences submitted by Huys et al. (2006). New sequences indicated by †.

models over a neighbour-joining topology (as implemented in ModelTest) or when using a strict consensus topology of the equally parsimonious trees resulting from prior MP analysis. Bayesian inference analysis consequently used the following parameters: nst = 6, rates = invgamma, ncat = 4, shape = estimate, inferrates = yes and base freq = empirical, corresponding to the model estimated (general-time-reversible including estimates of invariant sites and gamma distributed among-site rate variation). Posterior probabilities were approximated over 5,000,000 generations (ngen = 5,000,000) via four simultaneous Markov Chain Monte Carlo (MCMC) chains (nchains = 4) with every 100th tree saved (samplefreq = 100). Default values were used for the MCMC parameters. A majority rule consensus tree with mean branch lengths was constructed using the 'sumt' command with the 'contype = allcompat' option and ignoring the initial topologies saved during 'burn in' (the initial *n*-generations before log-likelihood values and substitution parameters plateau) (see Huelsenbeck and Ronquist, 2001). Based on a *X*-*Y* plot of posterior probabilities vs generations the burn in was set to 400, corresponding to trees saved during the first 40,000 generations.

Maximum parsimony nodal support was estimated by bootstrap analysis (full heuristic; 3000 replicates of 100 random additions), and as posterior probabilities in the Bayesian inference analyses (Huelsenbeck et al., 2001). Nodal support was also assessed by decay analysis (Bremer, 1994) using AutoDecay ver. 5 (Eriksson, 2001).

3. Results

Full SSU rDNA sequences were determined for 15 taxa, including four monstrilloids, providing 50 sequences in total for analysis. Parsimony analysis found eight equally parsimonious solutions (length = 2316 steps; CI = 0.4380; RI = 0.6168) with the Calanoida as the earliest divergent taxon standing in apposition to a monophyletic Podoplea (Cyclopoida + Siphonostomatoida). Competing topologies provided different hypotheses for the phylogenetic position of *Scambicornis* sp. and *Anthessius* sp. in the Cyclopoida, and for the interrelationships of the three dirivultid taxa (*Rhogobius contractus*, *Ceuthoecetes* sp., *Aphotopontius mammilatus*) in the Siphonostomatoida. The strict consensus tree of these trees supported monophyly of Cyclopoida (including the poecilostomatoid taxa, indicated by § in Table 1), but identified the Siphonostomatoida as a paraphyletic assemblage (Fig. 1b). Both maximum parsimony and Bayesian methods of phylogeny reconstruction placed the monstrilloid taxa in the Siphonostomatoida, as the sistergroup of the caligiform families (here represented by the Pandaridae, Dissonidae and Caligidae) (Fig. 1). The only difference between the BI tree (Fig. 1a) and the MP strict consensus tree (Fig. 1b) is the relative position of the nicothoid *Choniosphaera maenadis* which is either nested within a hydrothermal vent clade (Ecbathyriontidae + Dirivultidae)

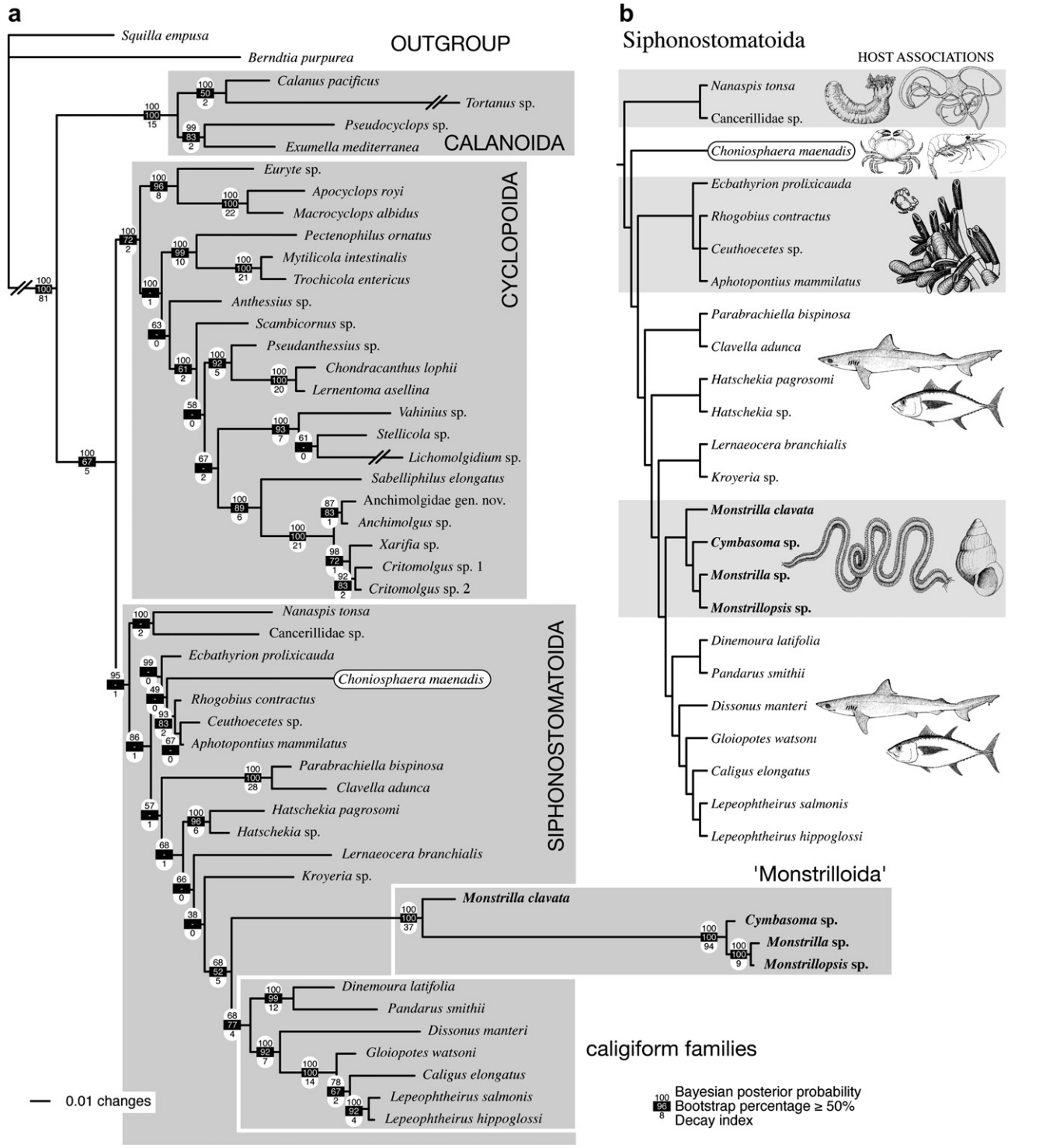

Fig. 1. (a) Phylogenetic position of Monstrilloida and caligiform families based on Bayesian analysis of SSU rDNA sequences using the GTR+I+Γ model of nucleotide substitution. Nodal support indicated by posterior probabilities (Bayesian inference), bootstrap values (maximum parsimony) and decay indices. (b) Maximum parsimony strict consensus tree showing interrelationships of siphonostomatoid families and their host association. Note the different position of *Choniosphaera maenadis* (enclosed) in (a) and (b).

(BI) or occupies a transitionary position between the echinoderm associates (Nanaspididae + Cancerillidae) and the residual siphonostomatoid taxa (MP). Although maximum parsimony bootstrapping failed to resolve the deep divergences within the Siphonostomatoida, Bayesian inference provided high or maximum support for some basal nodes including Siphonostomatoida + 'Monstrilloida' (95%).

4. Discussion

4.1. Monophyly of Siphonostomatoida and origin of fish parasitism

The clade Siphonostomatoida circumscribes those copepods that have an oral cone or siphon (Kabata, 1979; Huys and Boxshall, 1991). Its monophyly was severely questioned by Marcotte (1982) who considered a diphyletic origin more plausible, the families associated with fish and other vertebrate hosts being derived from a different cyclopoid-like ancestral stock. This hypothesis was not parsimony-based and is strongly rejected by our SSU rDNA based analyses, which favours a single origin for invertebrate and vertebrate associated siphonostomatoids (Fig. 1), but renders the Siphonostomatoida in Kabata's (1979) sense paraphyletic exclusive of the Monstrilloida. Given this new view on relationships, the conical structure on the ventral surface of the cephalothorax in adult monstrilloids (Huys and Boxshall, 1991: Figs 2.5.9–10) can now be re-interpreted as an incipient siphon or oral cone. Although both bootstrap and Bayesian support values were generally low, and denser sampling of the more basal families is required for greater confidence, both MP and BI analyses consistently recovered the fish parasitic families as a monophyletic group (but including the monstrilloids), a lineage traditionally defined by the shared loss of the mandibular palp. The basal position of the two lernaeopodid taxa (*Parabrachiella bispinosa*, *Clavella adunca)* in this clade is of particular significance since the Lernaeopodidae and the closely related Sphyriidae (not sampled here) are the only families parasitic on vertebrates that have retained the antennary exopod and do not have the apomorphic uniseriate type of egg sac in which discoid eggs are tightly packed to form a cylindrical egg string.

4.2. Position of the Monstrilloida: congruence between molecules and morphology

The placement of the Monstrilloida as sister of the caligiform families provides an unexpected hypothesis for the origin and phylogenetic position of this enigmatic order within the Copepoda. Pinpointing the origins of morphologically highly modified groups such as the Monstrilloida is always onerous, if not a contentious issue, except when a measure of concordance among different sources of data can be acquired. Despite the body plan between caligiforms and monstrilloids being radically divergent, two previously unexploited sources of morphological data generate patterns that are convincingly congruent with our molecularly based results.

4.2.1. Antennulary morphology

Monstrilloid antennules are indistinctly 4-segmented in the female and have at most six segments in the male. Too few setation elements remain to allow identification of segmental homologies in the proximal part of the limb but the geniculation in the male provides an unequivocal reference point, corresponding with the boundary between segments XX and XXI of the hypothetical ancestral copepod condition (Huys and Boxshall, 1991). The setation of the compound segment (XXI–XXVIII) distal to this articulation is highly conserved in copepods, both in ontogeny and phylogeny: typically a maximum of three setae is added to the distal array during the entire copepodid phase. This morphological conservatism was interpreted as evidence of the functional continuity of the distal setal array as a mechanosensory system providing early warning of approaching predators (Boxshall and Huys, 1998). Monstrilloid males exhibit four types of antennules (Huys and Boxshall, 1991), primarily differing in the modification of the distal segment. In the most plesiomorphic type (e.g. *Monstrilla longicornis* Sars; Huys and Boxshall, 1991: Fig. 2.5.5A), the setal array of the compound apical segment consists of 12 elements, shown schematically in Fig. 2. A total of seven unmodified elements (1–7) are arranged around the anterior and apical margins, the two distal ones (1–2) being typically spiniform. The posterior margin has an array of five 3-dimensionally branched setae (A–E); the second proximalmost seta (E) is displaced to the dorsal surface of the segment.

Within the Copepoda similarly branched setae have thus far only been reported in the family Caligidae (order Siphonostomatoida). The caligid life cycle (based on *Caligus* Müller and *Lepeophtheirus* von Nordmann but conceivably applicable to all Caligidae and caligiform families) comprises two nauplius stages, an infective copepodid, four to six chalimus stages, one or two preadults and the adult (e.g. Lin et al., 1996). The antennule is 2-segmented throughout ontogeny but only in the copepodid does the

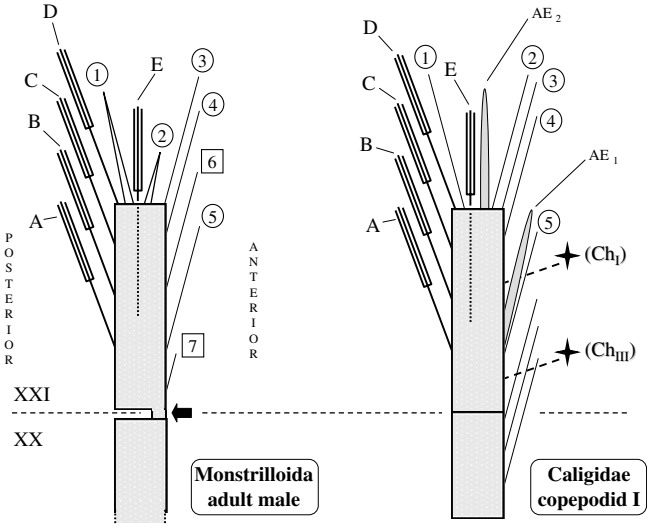

Fig. 2. Schematic diagram comparing distal setal array on antennule of adult male monstrilloid and caligid copepodid I (ventral views). Arrow indicates position of neocopepodan geniculation, corresponding to boundary between ancestral segments XX and XXI. [A–E: branched setae; 1–7: unmodified setae; AE_{1-2}: aesthetascs; $Ch_{I,III}$: chalimus stages I, III].

apical segment possess branched elements on the distal segment. The homology of this segment with the apical segment of male monstrilloids is unequivocally authenticated by reference to Boxshall and Huys's (1998) model of antennulary development, which demonstrated that the articulation separating ancestral segments XX and XXI is the earliest expressed boundary (as early as the naupliar phase). The antennulary setal array of the infective copepodid (based on *Caligus multispinosus* Shen; Lin et al., 1997: Fig. 2B) is represented schematically in Fig. 2. Virtually complete concordance is found with the adult monstrilloid pattern, including the number and relative position of branched setae (E displaced ventrally). The two differences encountered are the presence of two aesthetascs (AE_1 derived from ancestral segment XXI, AE_2 from XXVIII) and of only five unmodified setae (1–5) instead of seven along the anterior margin. The remaining two setae, probably derived from ancestral segments XXII and XXIV are added later in ontogeny, typically in the chalimus stages I, II or III (e.g. Kim, 1993; Lin et al., 1996, 1997). The delayed expression of these setae is in accord with the model proposed by Boxshall and Huys (1998) but the exact timing of their appearance differs slightly due to the addition of intercalary stages in the caligid life cycle.

4.2.2. Ontogeny of caudal rami

Adult copepod caudal rami primitively possess seven setae (Huys and Boxshall, 1991) but the ontogenetic trajectory of each of these elements and the potential phylogenetic signal they may hold have never been analysed. Comparative analysis of the development of caudal ramus setation patterns across five orders of podoplean copepods revealed numerous common features not shared by their gymnoplean counterparts, the calanoids (S. Conroy-Dalton and R. Huys, unpublished data). These features can be summarized in a generalized podoplean model for caudal ramus development shown schematically in Fig. 3. In this model, setae are gradually added in a regular pattern during the naupliar phase, resulting in a total of five setae (II, III, IV, VI, VII) in nauplius VI. The moult from nauplius VI to copepodid I is marked by the addition of two setae, the anterolateral seta (I) and the inner terminal seta (V), completing the full array of caudal setae. Seta V appears as a short element, which is fused at the base to the long terminal accessory seta VI, forming a bifid setal complex. At the moult to copepodid II the setal complex unfuses, seta VI reduces dramatically in size and seta V becomes the principal seta. This pattern persists in all subsequent copepodid stages, including the adult. Within the Podoplea only the Siphonostomatoida deviate from this baseline model. In the primitive family Asterocheridae (observations based on *Asterocheres echinicola* (Norman); see also Ivanenko and Ferrari (2003) for data on *Dermatomyzon nigripes* (Brady and Robertson)) and the closely related Scottomyzontidae (Ivanenko et al., 2001) and Dirivultidae (Ivanenko, 1998) seta IV is modified into a flattened hyaline spine in the early developmental stages

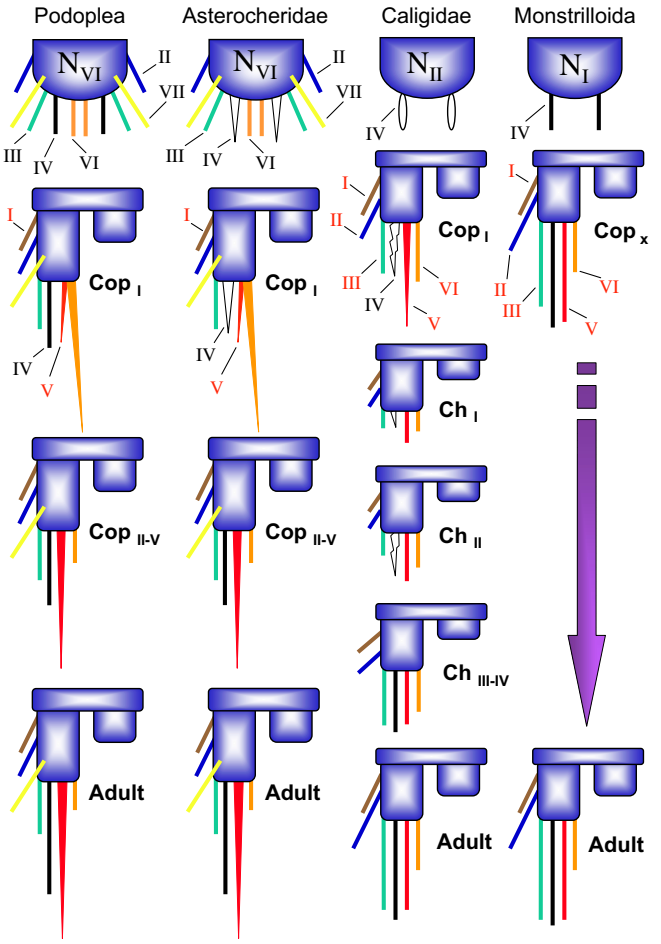

Fig. 3. Schematic diagram showing ontogenetic trajectories of individual setation elements on caudal ramus of generalized podoplean, two siphonostomatoid families (Asterocheridae, Caligidae) and Monstrilloida. Newly added setae indicated by red Roman numerals. Colour coding refers to homologous elements in successive stages [$N_{I,II,VI}$: nauplius stages; Cop_{I-V}: copepodid stages; Ch_{I-IV}: chalimus stages].

and only attains its setiform nature at copepodid II. Unpublished observations on other primitive families such as the Dirivultidae confirm that this modification is an autapomorphy for the Siphonostomatoida. However, in the Caligidae (observations based on *Lepeophtheirus salmonis* (Krøyer); see also Lin et al., 1997 for data on *C. multispinosus*) the pattern is radically divergent due to the abbreviated naupliar phase, comprising only two instars, and the intercalation of chalimus stages in the copepodid phase. The caligid nauplius II is characterized by the presence of only one seta, which is typically flaccid and swollen. Examination of intermoult stages (S. Conroy-Dalton and R. Huys personal observations) revealed that this so-called "balancer" represents the positional homologue of seta IV in other podopleans. At the moult to copepodid I five setae are added (I–III, V, VI), of which seta V is longest and not forming a setal complex with VI. Seta IV retains its flaccid character in the copepodid, degenerates during the early chalimus stages (I–II) and finally becomes setiform from chalimus III onwards. The major divergence from the

podoplean baseline is that the dorsal seta VII is never expressed during the entire ten-stage life cycle, resulting in a total of only six setae in the adults. The highly specialized monstrilloid life cycle offers only two informative stages for comparative analysis: the infective nauplius (Grygier and Ohtsuka, 1995) and the free-swimming adult. As in the caligiform families the nauplius stage possesses only seta IV, the earliest appearing seta in podoplean caudal ramus development. Adult monstrilloids show considerable variation in the number of caudal setae, ranging from three to six. The primitive six-setae condition is displayed by e.g. *Monstrilla grandis* Giesbrecht and *M. minuta* Isaac. Re-examination of these species revealed that the dorsal seta VII is absent, being concordant with the caligid pattern.

4.3. Host switching

Results from our SSU rDNA analysis are fully compatible with antennulary morphological data and caudal ramus setal patterns, and serve to illustrate the extraordinary morphological and adaptive versatility within the fish parasitic siphonostomatoids. Using the principle of parsimony, we believe the common ancestor of the monstrilloid-caligiform clade not only had acquired a vertebrate host but also was initially ectoparasitic. The subsequent divergence of the monstrilloids involved dramatic alterations in host utilization (vertebrate vs invertebrate), body plan (transformed nauplii, non-feeding adults) and life cycle strategy (ectoparasitic juveniles vs endoparasitic; parasitic adults vs free-living), a combination rarely observed and probably unique in metazoan parasites.

Protelean life cycles combining a parasitic juvenile phase and a free-living adult phase have been documented for other groups such as the Fecampiidae (Platyhelminthes), Nematomorpha, Mermithidae (Nematoda), Tantulocarida (Crustacea) and even Copepoda (Thaumatopsyllidae) but there is as yet no evidence suggesting that any of these lineages evolved from ancestors with obligatory parasitic adults. The evolutionary shift in mode of life from obligatory parasitic to free-living in adult monstrilloids is not only unique but also offers an explanation for their striking similarity in antennulary morphology with juvenile caligids. The underlying conservatism of the distal array of antennulary setae through ontogeny indicates a requirement for functional continuity in these sensory elements. Boxshall and Huys (1998) suggested that this array is involved in detecting approaching predators, forming the main component of the mechanosensory early-warning system of all copepods and eliciting an appropriate escape response. Sensitivity of setae may be correlated with the surface area presented to the environment. The five 3-dimensionally branched setae found in the infective copepodid stage of caligiform copepods provide a larger surface, a greater coupling with the external aquatic medium, and consequently a higher sensitivity. It is also conceivable that the 5-point array of branched setae with different orientations provides directionality in detecting signals. Obviously such predator-detection system has to be functional only until a host is found which explains why it is shed at the next moult to chalimus I. In monstrilloids the branched setal array also makes its first appearance in the copepodid (which develops inside the endoparasitic nauplius), however, it is conserved when the monstrilloid leaves the host and undertakes a single moult into the free-swimming adult. We postulate the pressure on maintaining a functional approaching-predator detection system in adult monstrilloids has delayed its suppression (as in post-copepodid caligiform instars) beyond the final moult, the 5-point array of branched setae being a juvenile attribute persisting in the adult by progenesis.

Switching from vertebrate to invertebrate hosts is extremely rare in symbiotic copepods. Only two other cases are known in the Siphonostomatoida, the caligid *Anchicaligus nautili* (Willey) which is parasitic on the deep-water nautiloid *Nautilus pompilus* Linn. in the Indo-Pacific (Ho, 1980), and the pennellid *Cardiodectes* spp. whose developmental stages utilize pelagic gastropod molluscs as intermediate hosts (Ho, 1966; Perkins, 1983). Similarly, two isolated host switching events are known from the Cyclopoida. Within the Ergasilidae, the monotypic genus *Teredophilus* Rancurel and a single species of *Paraergasilus* Markevich, *Paraergasilus rylovi* Markevich, are parasitic on the gills of brackish-water or freshwater (such as *Anodonta piscinalis* Nils) bivalve molluscs (e.g. Rancurel, 1954; Chernysheva, 1988). Within the Taeniacanthidae, a well-defined group comprising three genera and 14 species utilizes sea urchins as hosts (Dojiri and Humes, 1982); all remaining taeniacanthids (>80 spp.) are parasites of marine fishes. However, in none of these exceptions does host switching involve dramatic changes in body plan or life cycle strategy. The alternative view that the monstrilloid stem species had a two-host life cycle (by integration of an invertebrate intermediate host) is implausible since there seems no reason to postulate the loss of the vertebrate host. Given the congruence between our molecular, morphological and ontogenetic data, a secondary return to a free-living mode of life in the adult phase of the life cycle is more parsimonious.

4.4. Frontal filaments and position of Nicothoidae

In many siphonostomatoid families associated with fish hosts, the infective copepodid stage develops internally a frontal filament, which is used by the subsequent chalimus stages (and occasionally by the preadults) as a temporary tether attaching themselves to the host's gills or external surface. A frontal filament is present in the caligiform families Caligidae (e.g. Lin et al., 1997), Cecropidae (Grabda, 1974) and Dissonidae [Anderson and Rossiter's (1969) claim that it is absent in *Dissonus nudiventris* Kabata has been questioned by Kabata (1981)]. It has also been confirmed for the Lernaeopodidae (Kabata and Cousens, 1973) and the Pennellidae (Ho, 1966), is presumed to be

present in the Sphyriidae (Jones and Matthews, 1968), and, according to Schram and Aspholm's (1997) recent report of a frontal cephalic organ in *Hatschekia hippoglossi* (Cuvier), is almost certainly present in juvenile Hatschekiidae. However, at least some basal fish parasitic families such as the Dichelesthiidae (Kabata and Khodorevskii, 1977; Benz et al., 2002) and the Lernanthropidae (Cabral et al., 1984) lack it, suggesting that this innovative mode of attachment (and the chalimus phase) evolved within the fish parasitizing clade, probably soon after the explosive diversification and radiation of actinopterygian hosts.

Huys and Boxshall (1991) remarked that the Nicothoidae is the only invertebrate-associated family to have a cephalic frontal filament attaching the infective copepodid to its host (Hansen, 1897). Nicothoids are typically small, highly modified ectoparasites of other crustaceans and are virtually the only siphonostomatoids associated with this host group (some Dirivultidae can be found in the gill chambers and around the mouths of hydrothermal vent crabs and shrimps; Humes, 1996). Inspired by the shared presence of the frontal filament, Huys and Boxshall (1991) hinted at a possible sistergroup relationship between the Nicothoidae and the families parasitic on vertebrates, however, given the secondary acquisition of this character within the latter, the alternative scenario of within-clade host switching would appear more plausible. Our molecular analyses refute either hypothesis, indicating instead that nicothoids are only distantly related to the fish parasites. Although nodal support in the basal part of the siphonostomatoid tree is generally low, *C. maenadis* was consistently recovered within the invertebrate associated families, either as the second offshoot after the echinoderm associates (MP; Fig. 1b) or as sister of the Dirivultidae (BI; Fig. 1a), suggesting a homoplastic evolution of the frontal filament. Hansen (1897) showed that the frontal filament is not an exclusively larval attribute but can persist in adult males, which attach themselves either to the host's marsupial plates or to conspecific adult females prior to mating. This dual function is interpreted here as circumstantial evidence for the independent evolution of the frontal filament in nicothoids.

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