Small subunit rDNA and Bayesian inference reveal *Pectenophilus ornatus* (Copepoda *incertae sedis*) as highly transformed Mytilicolidae, and support assignment of Chondracanthidae and Xarifiidae to Lichomolgoidea (Cyclopoida)

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Phylogenetic analysis of newly obtained data from the complete small subunit rDNA (18S) nuclear gene of a wide range of copepods placed the enigmatic *Pectenophilus ornatus* firmly in the Cyclopoida. Both maximum parsimony tree reconstruction, and Bayesian analysis operating under the GTR + I + Γ model of nucleotide substitution, gave identical solutions and placed *P. ornatus* at the base of the poecilostome families, in apposition to the mytilicolid taxa. The recently suggested assignment to the Siphonostomatoida on the basis of a tubular mouth cone in the pygmy male was rejected not only by the molecular data but also by new morphological observations. Scanning electron microscopy revealed that the appendage previously interpreted as the mandible was in reality the maxilla, the presumptive 'labium' only an intermaxillary outgrowth of the ventral cephalic sclerite bearing the widely separated paragnaths, and that there was no basal fusion between the labrum and the 'posterior lip' as in genuine siphonostomatoids. Absence of mandibles and their functional replacement by the anteriorly displaced maxillae is a unique and robust apomorphy for the Mytilicolidae and placed unequivocally *P. ornatus* in that family. The morphology of male *Pectenophilus* probably evolved as a result of global progenesis, involving early sexual maturation at the metanauplius stage and the complete cessation of somite and limb development. The molecular data were also employed to examine the relationships of two other highly modified parasitic families, the Xarifiidae (inhabiting hard corals) and the Chondracanthidae (parasitic on marine demersal fishes). Our analyses rejected the previously proposed relationship between Xarifiidae and Vahiniidae and strongly supported an Anchimolgidae + (Rhynchomolgidae + Xarifiidae) clade as sister group to the Sabelliphilidae within a monophyletic Lichomolgoidea. The obtained topology suggests that the common ancestor of this clade had already established a symbiotic relationship with scleractinian corals and that host switching occurred only secondarily in the Rhynchomolgidae, involving predominantly other cnidarian and occasionally noncnidarian hosts. Reassessment of the morphology of *Parangium* provided new evidence for a relationship with the xarifiids, rendering its current position in the Serpulidicolidae extremely unlikely. Both parsimony and Bayesian analyses revealed an unexpected but strongly supported relationship between the Chondracanthidae and Pseudanthessiidae. This result contrasts with earlier views advocating affinity to the Synapticolidae or Lichomolgidae, but was congruent with the previously unnoticed morphological similarity in antennary armature patterns in the first copepodid stage. The morphological grounds used to establish the Lernaeosoleidae were shown to be secondarily derived characters shared with one or several chondracanthid genera. Particularly the similarity between the Lernaeosoleidae and *Markevitchielinus* demonstrated that the former evolved from a mesoparasitic ancestor within the Chondracanthidae and consequently should sink as a synonym of the latter. © 2006 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2006, **87**, 403–425.

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INTRODUCTION

The morphological plasticity and disparity expressed in body form and shape within the Copepoda are arguably unrivalled among the Crustacea. Many adult parasitic copepods such as the bizarre Herpyllobiidae and Xenocoelomatidae (both parasitizing polychaetes) exemplify a major divergence from the podoplean body plan (i.e. prosome–urosome boundary located between fourth and fifth pedigerous somites), lacking any external trace that could positively identify their crustacean identity. Without recourse to molecular sequence data, such radically divergent taxa continue to defy our attempts to force them into convenient or traditionally accepted taxonomic entities. *Pectenophilus ornatus* Nagasawa, Bresciani and Lützen is one of the most strikingly transformed parasitic copepods known, lacking any indication of tagmosis or segmentation in either sex, and exhibiting a peculiar life cycle. Adult females are globular in shape and attach to the pectinid bivalve host by a tubular stalk formed from hypertrophied host ctenidial tissue. The mouth opens into a blood lacuna in the middle of the stalk and connects via a compartmentalized pharynx to a spacious blind midgut. The ovoid pygmy males, one to six per female, are enclosed within a vesicle, which is connected to the capacious incubatory pouch of the female. They possess rudimentary antennules, antennae and one pair of mouthparts, and deposit their spermatophores in the males' vesicle from where sperm are transferred to the seminal receptacle. Eggs hatch as nauplii inside the incubatory pouch (see Fig. 2A, B, below) and are released to the exterior through a minute unpaired birth pore. It is unknown whether the nauplius or an as yet undiscovered copepodid (or onychopodid as in *Gonophysema* Bresciani & Lützen; Bresciani & Lützen, 1961) represents the infective stage.

Pectonophilus ornatus is a serious pathogen of the Japanese scallop, *Patinopecten yessoensis* (Jay), and is also known to infect two other members of the bivalve family Pectinidae, the prickly scallop *Chlamys farreri nipponensis* Kuroda, and Farrer's scallop *C. f. farreri* (Jones & Preston) (Tahara & Hirose, 1989, 1990; Nagasawa *et al*., 1993). *Patinopecten yessoensis* is one of the most commercially important shellfish in Japan, its annual production currently exceeding 200 000 metric tons (Nagasawa, 1999). According to Nagasawa *et al*. (1991) *P. ornatus* may infest up to 100% of the aquacultured populations, reduce the scallop's marketability (Elston, Wilkinson & Burge, 1985), and have a negative effect on its fatness factor (Takahashi *et al*., 1973; Nagasawa & Nagata, 1992). *P. ornatus* appears to be endemic to Japan and its distribution is so far restricted to some coastal waters along the Pacific coast of Honshu, and both north and south shores of the Tsugaru Strait (Nagasawa *et al*., 1993; Nagasawa, 1999).

The radically divergent external morphology, in conjunction with the total lack of limb differentiation in the adult female, led earlier workers to assume that *P. ornatus* was either a member of the Rhizocephala (Takahashi *et al*., 1973; Takahashi, Tanaka & Ito, 1974) or at least affiliated to this group of parasitic cirripedes (Elston *et al*., 1985). The absence of frontal horns or filaments in the nauplius, the arrangement of sperm cells within the spermatophore, and the presence of individualized pygmy males with typical antennules, antennae and mouthparts demonstrated unequivocally the copepodan identity of the parasite (Nagasawa, Bresciani & Lützen, 1988). These latter authors refuted a possible affinity with other aberrant bivalve parasites, such as *Teredoika* Stock and *Axinophilus* Bresciani & Ockelmann, and regarded the shared presence of a brood pouch with the cyclopoid families Notodelphyidae, Ascidicolidae and Gastrodelphyidae as a product of convergence. However, they were unable to make a firm recommendation for assignment to any existing family or order. Bresciani's (1991) detailed scanning and transmission electron microscopy (SEM and TEM) study of the male failed to provide robust characters of phylogenetic significance, except for the presence of an incipient oral cone (not a true siphon) which could point to a possible position within the Siphonostomatoida. However, Bresciani (1991) admitted that a detailed topographical study of the cephalic extrinsic musculature is essential before such a relationship can be corroborated. Boxshall & Halsey (2004) also provisionally assigned *Pectenophilus* to the Siphonostomatoida, based solely on the presence of a 'tubular mouth' in the dwarf male. The presence of an internal incubatory pouch prevented them from assigning it to any existing family.

From the perspective of host utilization, the currently accepted ordinal position of *Pectenophilus* can be viewed with some scepticism since only a negligible fraction of the 1750+ species of Siphonostomatoida are known to utilize mollusc hosts. Some representatives of the Dirivultidae exhibit a certain predilection for hydrothermal vent bivalves (Humes & Segonzac,

1998) but it is conceivable that they feed only inter-mittently or on particulate matter, maintaining a rather loose association with their hosts (Tsurumi, de Graaf & Tunnicliffe, 2003). The large family Astero-cheridae (> 175 spp.) utilizes a wide range of hosts but only *Obesiella lyonsiellae* Ridewood and some species of the genus *Scottocheres* Giesbrecht are known to be associated with molluscs (Ridewood, 1903; Norman & Scott, 1906). Records of *Artotrogus* Boeck (Artotrogidae) using nudibranch gastropods (Monod & Dollfus, 1932) are anecdotal in nature and require confirmation. The Indo-Pacific *Anchicaligus nautili* Willey is parasitic on the deep-water nautiloid *Nautilus pompilus* Linn. and represents an exceptional case of host switching within the fish-parasitizing family Caligidae (Ho, 1980). Finally, various pelagic gastropod molluscs serve as first host for the developmental stages of the pennellid *Cardiodectes medusaeus* (Wilson), which utilizes mesopelagic and bathypelagic fishes as final hosts (Ho, 1966; Perkins, 1983; Boxshall, 1998).

Due to the difficulty in finding sufficient morphological evidence to reach a consensus view on the position of *Pectenophilus* among the Copepoda, we decided to test its position independently of morphology using a molecular approach. There have been very few efforts to use molecular data to recover interordinal relationships within the Copepoda (Braga *et al.*, 1999; Kim & Kim, 2000), and all of them suffer from inadequate taxon sampling or sequence ambiguity. In the tradition of modern molecular phylogenetics we used nuclear ribosomal genes because they exhibit semiconserved domains interspersed with divergent regions, and allow phylogenetic reconstruction over a wide range of taxonomic levels. We have generated complete small subunit (SSU, also referred to as 18S) nuclear rDNA sequences of 41 species, representing the four major copepod orders (Calanoida, Cyclopoida, Harpacticoida and Siphonostomatoida) (Huys & Boxshall, 1991), and analysed them using both maximum parsimony and Bayesian tree reconstruction. In this study we used our SSU rDNA gene sequence data (1) to confirm whether *Pectenophilus* represents another case of unusual host switching (like *Anchicaligus*) or an independent lineage within a more encompassing mollusc-associated clade, and (2) to examine the relationships of two other highly modified parasitic families, the Chondracanthidae (parasites of marine demersal fishes) and the Xarifiidae (inhabiting scleractinian corals).

MATERIAL AND METHODS

DISSECTION

The intimate association between *P. yessoensis* and *P. ornatus* poses particular problems with regard to avoiding host-tissue contamination. *Pectenophilus ornatus* is a haemophagous gill parasite which taps the pectinid's blood circulation and pumps host blood into its digestive system by muscular action. Nagasawa *et al.* (1988) showed that the capacious midgut of the copepod is often filled with coagulated proteins and host blood corpuscles. In addition, *P. ornatus* is, strictly speaking, an endoparasite, being completely clothed by the scallop's ciliated ctenidial epithelium (except for the birth pore). Similarly, the conical stalk consists largely of host proliferative connective tissue and contains a central lacuna through which the host's blood is sucked into the oesophagus of the adult female. In order to minimize the risk of host contamination only large ovigerous specimens (> 6 mm) of *P. ornatus* were used for tissue preparation. Stalks and enveloping ctenidial tissues were removed prior to extraction of the egg mass from the dorsal incubatory brood pouch. Small volumes of the egg mass were used for genomic extraction. Pygmy males dissected out of the vesicle were prepared for SEM (see below).

TAXON SAMPLING AND EXTRACTION OF GENOMIC DNA

Representatives of the two major parasitic orders, Cyclopoida (which now embraces the Poecilostomatoida; see Boxshall & Halsey (2004)) and Siphonostomatoida were collected from various vertebrate and invertebrate hosts. Two species of the order Harpacticoida were also included as well as a range of Calanoida as this order is widely accepted as being the most primitive one within the Neocopepoda (Huys & Boxshall, 1991; Ho, 1994). Finally, we added available GenBank sequences for three noncopepodan outgroup taxa, a primitive malacostracan (*Squilla empusa*) and two basal Thecostraca (*Berndtia purpurea* and *Ulophysema oeresundense*).

The Appendix provides a taxonomic listing of the exemplar species analysed, their collection localities and sequence accession numbers. A small number of published sequences from previous studies based on complete SSU rDNA (Abele *et al.*, 1992; Spears & Abele, 1998) were also included (L81939, L34046, AF208263). Representative voucher specimens, when available, have been deposited in the Natural History Museum (Appendix). 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 genomic DNA concentration, the elution volume was then reduced to 50 μL in a vacuum centrifuge.

POLYMERASE CHAIN REACTION AMPLIFICATION AND SEQUENCING

SSU rDNA sequence fragments (~600 bp) were amplified using primers 18Sf (5′-TAC CTG GTT GAT CCT GCC AG-3′) and 18Sr (5′-TAA TGA TCC TTC CGC AGG TTC AC-3′), and internal primers 554f (5′-AAG TCT GGT GCC AGC AGC CGC-3′) and 1150f(p2) (5′-ATT GAC GGA AGG GCA CCA CCA G-3′), as well as primers 1282r (5′-TCA CTC CAC CAA CTA AGA ACG GC-3′) and 614r (5′-TCC AAC TAC GAG CTT TTT AAC C-3′). Polymerase chain reaction (PCR) amplifications (50 μL) were performed using a HotStarTaq DNA Polymerase kit developed by QIAGEN, using 25 μL HotStarTaq Master Mix (containing HotStarTaq DNA polymerase, PCR buffer (with Tris-Cl, KCl, $(NH4)_2SO_4$, 3 mM $MgCl_2$; at pH 8.7) and 400 μM each dNTP), 2–5 μL genomic extract and 10 μM each PCR primer using the following thermocycling profile: 15 min denaturation at 95 °C, followed by 35 cycles of: 1 min at 94 °C, 1 min at 55 °C (fragment 1), 59 °C (fragment 2) or 57 °C (fragment 3), and 2 min at 72 °C, with a final 10-min extension hold at 72 °C. 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 Analyser. 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 numbers AY626994–7032 and AY629258–9 (Appendix).

SEQUENCE ALIGNMENT

Sequences were aligned by eye using BioEdit Sequence Alignment Editor (Hall, 1999; ver. 5.0.9) and MacClade (Maddison & Maddison, 2002; ver. 4). Regions of ambiguous alignment were delimited by identifying the first parsimony-uninformative nucleotide on each side of an unalignable region and these were excluded from subsequent phylogenetic analyses. Regions containing gaps in a majority of taxa were also excluded from analyses even if they were alignable among the minority of taxa possessing the insertions. The complete alignment has been deposited

with EBI and is available by anonymous FTP from ftp://ftp.ebi.ac.uk in directory pub/databases/embl/align and via the EMBL-Align database via SRS at http://srs.ebi.ac.uk, under the following accession: ALIGN_000697. The character exclusion set is added as notes and the alignment may be adapted as a NEXUS file.

PHYLOGENETIC ANALYSES

Phylogenetic analyses were conducted using the methods of maximum parsimony and Bayesian inference. Maximum parsimony analysis was conducted with PAUP* (Swofford, 2001; ver. 4.0b10) and Bayesian inference analysis with MrBayes (Huelsenbeck & 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 set unordered with equal weights 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 & 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) to provide the best fit to the data. This was true when evaluating the 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 maximum parsimony analysis. Bayesian inference analysis consequently used the following parameters: nst = 6, rates = invgamma, ncat = 4, shape = estimate, inferrates = yes and basefreq = 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 chains (nchains = 4) with every 100th tree saved (samplefreq = 100). Default values were used for the Markov Chain Monte Carlo parameters. A 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 & Ronquist,

2001). The burn in was set to 300, corresponding to trees saved during the first 30 000 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). We accepted a clade in the Bayesian tree at around 95% posterior probability (Murphy *et al.*, 2001; Wilcox *et al.*, 2002), while accepting values around 60–70% in the nonparametric bootstrap tree (Hillis & Bull, 1993). Nodal support was also assessed by decay analysis (Bremer, 1994) using AutoDecay ver. 5 (Eriksson, 2001).

The Shimodaira–Hasegawa test as implemented in PAUP* was used to test whether an alternative topology with the appropriate constraint enforced was significantly less well supported by the data than that found in our Bayesian inference analysis and could be rejected confidently. The resampling estimated log-likelihood (RELL) approximation method (Kishino & Hasegawa, 1989) was used with 10 000 bootstrap replicates as well as the slower FULL optimization with 1000 bootstrap replicates.

SCANNING ELECTRON MICROSCOPY

Females and pygmy males of *P. ornatus* and adult females of *Mytilicola intestinalis* Steuer were examined with a Philips XL 30 scanning electron microscope. Specimens were prepared by dehydration through graded acetone, critical point dried, mounted on stubs and sputter-coated with palladium. Scales on SEM photographs (i.e. Figs 2–5, below) are in μm.

RESULTS

PHYLOGENETIC POSITION OF *PECTENOPHILUS*

Full SSU rDNA sequences were determined for 41 taxa, including *P. ornatus*, providing 47 sequences in total for analysis. The alignment comprised 1882 nucleotide positions of which 1673 were unambiguously alignable, 651 were variable and 490 were parsimony-informative. Parsimony analysis found 66 equally parsimonious solutions (length = 2417 steps; consistency index = 0.4278; retention index = 0.5794), with the Calanoida as the earliest divergent taxon standing in apposition to the Podoplea. The majority-rule consensus tree of these trees (not shown) showed a monophyletic Podoplea but failed to resolve the deep divergences within the group, with the Harpacticoida, *Nanaspis tonsa* + Cancerillidae, Cyclopoida and the remaining Siphonostomatoida forming a basal polytomy. Both maximum parsimony and Bayesian methods of phylogeny reconstruction placed unequivo-cally *Pectenophilus* in the Cyclopoida, at the base of the poecilostome complex of families (Fig. 1). Two mytilicolids, *M. intestinalis* and *Trochicola entericus*, were recovered as the sister group of *P. ornatus*. This placement was conserved in both strict and majority-rule consensus trees.

Monophyly was supported by strict consensus of the Cyclopoida, 'Poecilostomatoida' and the superfamily Lichomolgoidea. Bootstrap and Bayesian support for the interrelationships between the lichomolgoidean families was generally high with the exception of some deeper nodes (e.g. position of Synapticolidae) and the relationships within the *Pachos* + *Vahinius* + *Stellicola* clade. *Xarifia* and the two chondracanthid taxa (*Chondracanthus lophii* and *Lernentoma asellina*) were recovered consistently as members of the Lichomolgoidea. Both analyses strongly supported a relationship between the Pseudanthessiidae and the Chondracanthidae and provided overwhelming nodal support for a predominantly scleractinian-associated clade comprising the Anchimolgidae, Rhynchomolgidae and Xarifiidae. *Sabelliphilus elongatus* was recovered as the sister to this group whereas the only vahiniid species included in the analysis showed no direct relationship with this clade.

EXTERNAL MORPHOLOGY OF ADULT MALES

Adult males lie freely in the vesicle; their bodies are ovoid (Fig. 2C), about 500 μm long, and without trace of thoracic appendages but with sparse integumental sensillae (Fig. 3D). The cephalic region is very small (arrowed in Fig. 2C), located on the prominence (Figs 2D, 3D) at the anteroventral pole of the male.

The rostrum is comparatively large (Fig. 2D), trapezoid in shape and wider than long; the anterior margin is incised and deflected ventrally. The dorsal surface has two pairs of sensillae, a median pore anteriorly and two additional pores adjacent to the anteriormost pair of sensillae (Fig. 3A).

The antennule is short, stubby and unsegmented (Fig. 3B); the distal portion has three setae (Figs 2D, 3D), two blunt nonarticulating spines (arrowed in Fig. 3B) and around 25 spinule-like elements (possibly representing vestigial setae).

The antenna is strongly developed (Figs 2D, 3C) and indistinctly three-segmented, comprising a coxobasis and two-segmented endopod. The coxobasis is partly fused to the proximal endopod segment (Fig. 3D); the original segmentation is marked by an incomplete transverse surface suture on the anterior surface. The proximal endopod segment has a short seta along the inner margin and few spinules on the anterior surface. The distal endopod segment is produced into a terminal claw (fully incorporated into the segment) and bears a short seta on both inner and outer margins.
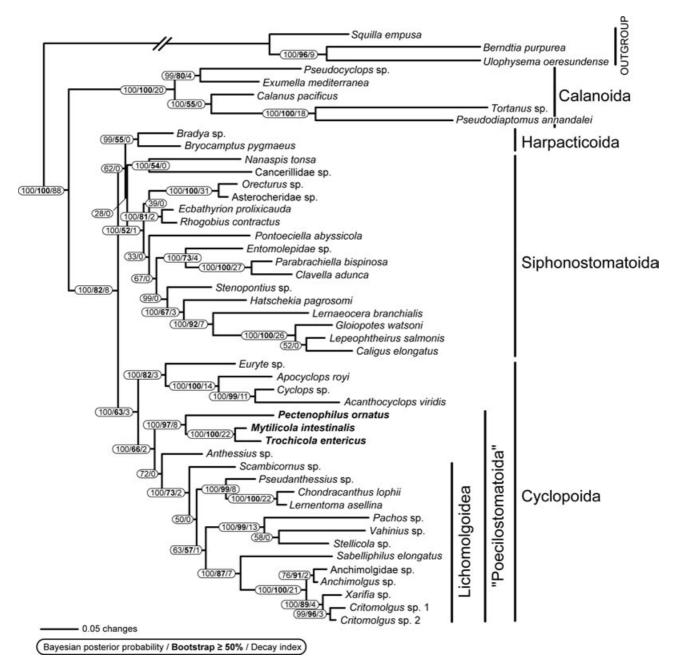
Figure 1. Species-level phylogram (47 taxa) based on Bayesian inference using the GTR + I + Γ model of nucleotide substitution. Nodal support based on posterior probabilities (Bayesian inference), bootstrapping (maximum parsimony) and decay indices. Mytilicolid taxa indicated in bold typeface.

The labrum (Fig. 4A, B) is fleshy, semicircular, and unarmed; the posterior surface has a series of minute secretory pores.

Both mandible and maxillule are absent.

The maxilla (Fig. 4A, B) is well developed, positioned in between the labrum and paragnaths; it is two-segmented, comprising the syncoxa and allobasis. The syncoxa is large and without endites; the maxillary gland exits on the posterior surface via a large slit

or opening (arrowed in Fig. 4A, D). The allobasis is a small, slender segment, produced into a unilaterally serrate process distally and has a short seta along the outer margin.

Paragnaths are represented by small, widely separated, rounded lobes set on a large semicircular lip-like outgrowth (Fig. 4A, B); the posterior 'lip' is fused on either side to the maxillary syncoxae (Figs 4A, 5A; arrowed in Fig. 4C).
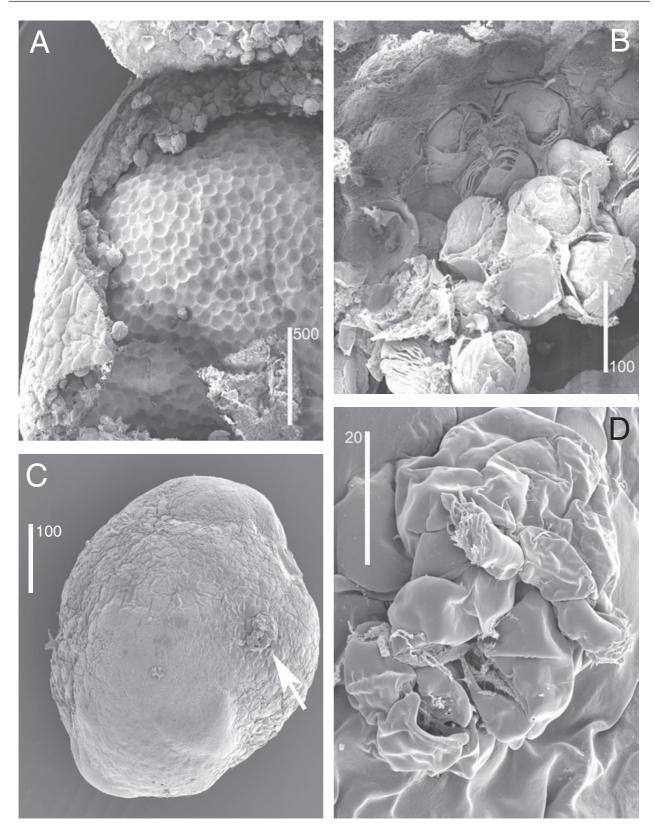
Figure 2. *Pectenophilus ornatus*. A, adult female with body wall partly removed to reveal honeycomb-structured wall of incubatory pouch; B, nauplii inside brood pouch at different stages of eclosion; C, pygmy male, ventral [cephalic prominence arrowed]; D, head region of male. Scale bars shown are in µm.
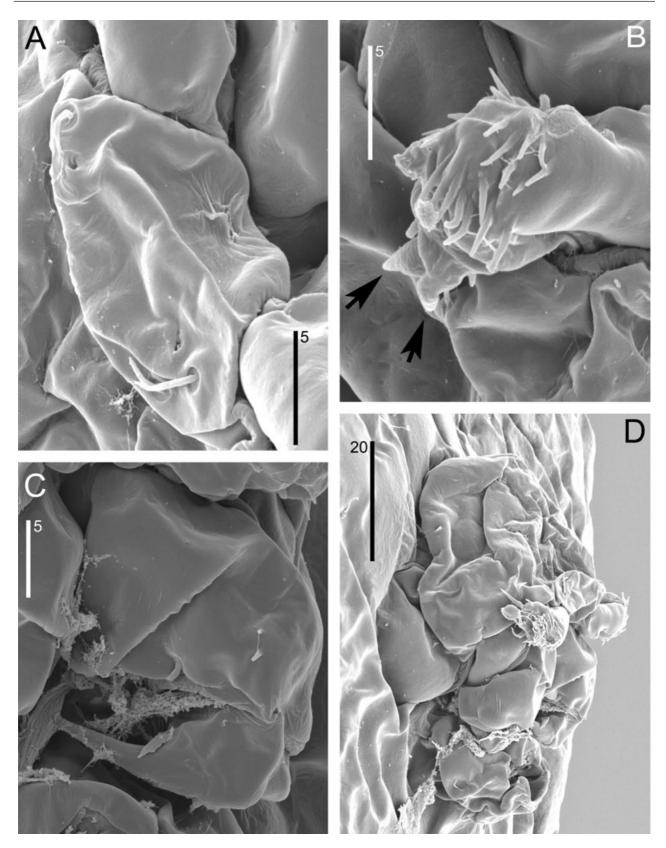
Figure 3. *Pectenophilus ornatus*, pygmy male. A, rostrum showing paired integumental pores and two pairs of sensillae, dorsal; B, antennule [spinous elements on apex arrowed]; C, antenna, anterior; D, head region, lateral. Scale bars shown are in µm.
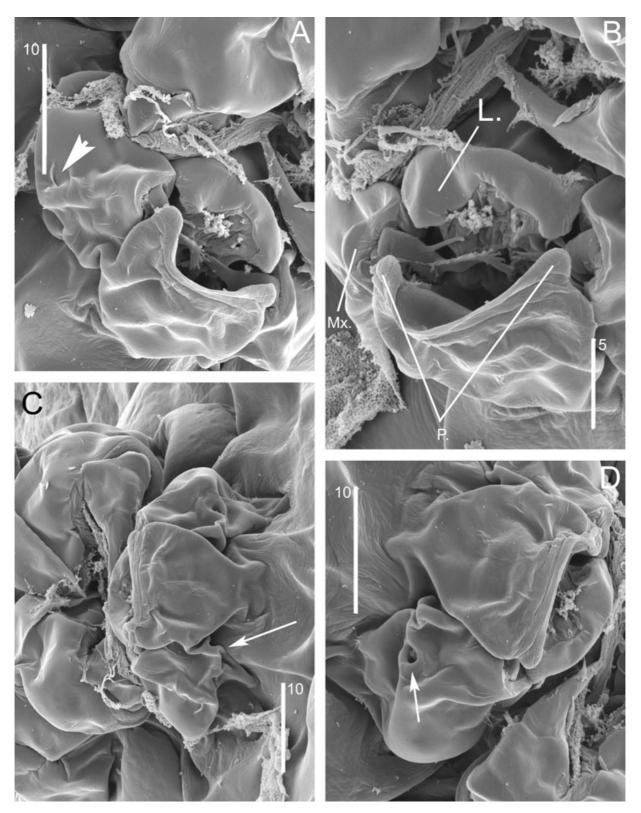
Figure 4. *Pectenophilus ornatus*, pygmy male. A, oral region, showing labrum, paragnaths and maxillae, ventral [opening of maxillary gland arrowed]; B, close-up of oral region, showing labrum (L.), paragnaths (P.) and maxilla (Mx.); C, oral region, posterior [arrow indicates fusion between maxillary syncoxa and basal portion of paragnaths; D, right maxilla and paragnaths, posterolateral [opening of maxillary gland arrowed]. Scale bars shown are in μm.
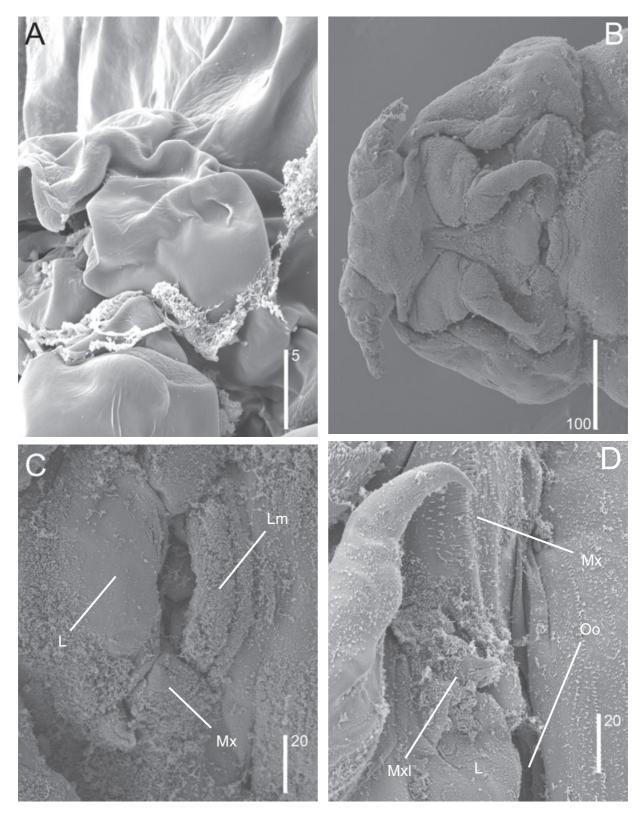
Figure 5. *Pectenophilus ornatus*, pygmy male. A, posterolateral view of right maxila showing fusion between syncoxa and paragnath. *Mytilicola intestinalis*, adult female. B, head region, ventral; C, oral area showing labrum (L), reduced paragnaths (Lm) and maxillary syncoxa (Mx); D, left half of oral area showing oral opening (Oo), maxillule (Mxl) and syncoxa of maxilla (Mx). Scale bars shown are in μm.

DISCUSSION

MOLECULAR ANALYSIS

Huys & Boxshall's (1991) morphology-based phylogenetic analysis favoured a sister-group relationship between the Poecilostomatoida and the Monstrilloida + Siphonostomatoida (MS). The morphological characters used to unite this clade were the compound segment XXIV-XXV in the male antennule, the fusion of the maxillulary coxa, basis and rami, the loss of the maxillulary basoexite, maxillary endopod and maxillipedal praecoxal endite, and the fusion of coxa and basis in the male fifth legs. Subsequent analyses (Ho, 1994; Ho *et al*., 2003) also revealed strong support for a Poecilostomatoida + MS clade but such a sister-group relationship was not recovered in our SSU rDNA-based analysis. Instead, the poecilostomatoid taxa were resolved as a sister group to the Cyclopoida with moderately low bootstrap support (63%) but a high Bayesian posterior probability value (100%). This scenario does not necessarily conflict with the emerging morphology-based concept of a paraphyletic Cyclopoida with the poecilostomatoid taxa being nested within this order (Huys *et al*., 2002; Boxshall & Halsey, 2004), because in our study cyclopoids were represented only by the family Cyclopidae. Rather than analysing the data further, it seems obvious that inclusion of the more basal cyclopoid families Cyclopinidae, Notodelphyidae and Mantridae and denser sampling of the remaining 'Poecilostomatoida' are needed in an attempt to recover the paraphyletic status of the 'gnathostome' Cyclopoida, as their morphology would lead us to believe.

Our molecular analyses indicate that *Pectenophilus* is not a member of the Siphonostomatoida but nests within the poecilostome complex of the Cyclopoida. Both maximum parsimony and Bayesian analyses provided overwhelming statistical support for the sister-group relationship between *P. ornatus* and two mytilicolids, *M. intestinalis* and *T. entericus*. This result is extremely robust because it is congruent with the newly acquired morphological evidence derived from the dwarf male, demonstrating that *Pectenophilus* is a highly derived member of the Mytilicolidae (see below).

Humes & Boxshall's (1996) analysis of the Lichomolgoidea indicated that within the 'Poecilostomatoida' there is generally a strong (but not universal) congruence between common ancestry and host utilization, but Huys (2001) showed that caution is required when accommodating morphologically divergent taxa in monophyletic clades solely on the basis of their shared host affiliation. Among the families utilizing molluscs there exists a broad spectrum of variation in body morphology, ranging from cyclopiform, as in the primitive Myicolidae (bivalves) and Anthessiidae (bivalves, gastropods), to moderately modified, as in the Mytilicolidae (bivalves, gastropods) and Philoblennidae (gastropods), to unsegmented and highly transformed, as in the Micrallectidae (gymnosome pteropods), Splanchnotrophidae (nudibranchs) and Chitonophilidae (aplacophorans, prosobranchs, cocculiniform limpets). Most recently, Boxshall & Halsey (2004) remarked that the presence of at most two leg pairs during development may represent an important synapomorphy linking the highly modified genera *Axinophilus* and *Teredoika* together with a larger group of taxa, including the derived families Micrallectidae, Philoblennidae and Splanchnotrophidae. This character effectively excludes the Chitonophilidae from the 'splanchnotrophid group of families', because the infective copepodid in members of this family bears four pairs of swimming legs. Such circumstantial evidence appears to substantiate a recent comparative study (Huys *et al*., 2002) based on antennulary morphology and antennary segmentation, demonstrating that the Chitonophilidae cannot be placed in the poecilostome complex but should be regarded as a more basal cyclopoid lineage that established symbiotic relationships with mollusc hosts independently. Boxshall & Halsey (2004) also claimed that most families of the splanchnotrophid superfamilial grouping display an anthessiid-like mandible, at least in the first copepodid, implying that the majority of mollusc-associated poecilostome copepods are derived from a common myicolid/anthessiid ancestral stock. Although a denser sampling of the highly transformed taxa is required for greater confidence, we used the Shimodaira–Hasegawa test to see whether the alternative hypothesis of a monophyletic mollusc-associated clade (Mytilicolidae + *Anthessius* sp.) was supported or rejected by the data. We found that our null hypothesis, constraining *M. intestinalis*, *T. entericus*, *P. ornatus* and *Anthessius* sp. to group together, was not significantly worse than was the Bayesian tree ($\Delta \ln L = 4.13$, $P = 0.262$ for RELL and 0.238 for FULL optimization approach). The hypothesis that the Mytilicolidae are derived from an anthessiid (or myicolid) ancestral stock can therefore not be rejected at the moment. Furthermore, this calls for renewed efforts in morphological studies, as too few characters, especially at the ontogenetic level, have been examined thoroughly across the mollusc-associated families.

OTHER CLADE-SPECIFIC FINDINGS IN THE CYCLOPOIDA

Monophyly of the Lichomolgoidea

Humes & Stock (1972, 1973) established the superfamily Lichomolgoidea to accommodate the newly defined Lichomolgidae, Sabelliphilidae and Pseudanthessiidae, and two new families, the Rhyncho-

molgidae and Urocopiidae. Humes & Boxshall (1996) thoroughly revised the existing family concepts, excluded the Urocopiidae from the lichomolgoidean core, and established six new families: Anchimolgidae, Kelleriidae, Macrochironidae, Octopicolidae, Synapticolidae and Thamnomolgidae. Although their analysis of the phylogenetic relationships between the ten families was based on a robust morphological dataset, the results should be regarded as provisional because of the lack of an appropriate outgroup (Lubbockiidae) and the omission of several lichomolgoidean families, such as the Intramolgidae, Urocopiidae and Sapphiriniidae (and Polyankyliidae; Ho & Kim, 1997), and some floating taxa with lichomolgoid affinities (e.g. *Pachos* Stebbing, *Octophiophora* Stock, *Stockia* Sebastian & Pillai). In addition, the superfamily has never been diagnosed properly by autapomorphic character states and was consequently not adopted by Martin & Davis (2001) in their new classification of the Crustacea. Our molecular analyses showed that the six lichomolgoidean families included here (exemplars of Octopicolidae, Macrochironidae, Kelleriidae and Thamnomolgidae were not included) form a monophyletic group with reasonably high bootstrap (73%) and maximum Bayesian (100%) support, but only when some other taxa, not previously placed in the Lichomolgoidea, were included. Humes & Boxshall (1996) remarked that the Synapticolidae are probably related distantly to the other families of the lichomolgoid complex. Although the position of the only included synapticolid (*Scambicornus* sp.) as a basal offshoot was not supported here, it likewise did not group confidently with any other lichomolgoidean clade, indicating that only wider sampling can resolve its phylogenetic affinity.

Anchimolgidae–Xarifiidae–Rhynchomolgidae (AXR): a scleractinian-associated clade

The most strongly supported clade in Humes & Boxshall's (1996) analysis was defined by the sexual dimorphism of leg 1, in which there is, primitively, an apical seta on the third endopodal segment in females (formula I,5) and an apical spine in males (formula I,I,4). Kim (2003) showed that this transformation occurred at the last moult from male copepodid V to adult male, giving further support to the assumption that the sexual dimorphism of leg 1 is involved in spermatophore transfer or in precopulatory mate behaviour (Humes & Boxshall, 1996). Based on this and five additional characters, Humes & Boxshall proposed to group together the Kelleriidae, Macrochironidae, Rhynchomolgidae and Anchimolgidae. In our analyses the latter two families were also recovered as a very strongly supported clade with maximum bootstrap resampling value; however, the highly derived Xarifiidae occupied an intermediate position, being

the sister group of the two rhynchomolgids (*Critomolgus* spp.). Virtually all members (with one exception) of the Anchimolgidae and Xarifiidae are associates of scleractinian corals, and only two of the 43 genera currently classified in the Rhynchomolgidae utilize noncnidarian hosts (17 live in association with Scleractinia). Our molecular-based topology (Fig. 1) suggests strongly that the common ancestor of this AXR-clade had already established a symbiotic relationship with scleractinians and that host switching occurred only secondarily within the Rhynchomolgidae, involving predominantly other cnidarian (Actiniaria, Alcyonacea, Antipatharia, Corallimorpharia, Gorgonacea, Hydrozoa, Pennatulacea, Telestacea) and occasionally noncnidarian host groups. In contrast to Humes & Boxshall's (1996) morphological solution, which places the Sabelliphilidae at the base of the Lichomolgidae–Thamnomolgidae–Pseudanthessiidae clade, this family was consistently recovered as the sister group of the AXR-clade in our molecular analyses.

Boxshall & Halsey (2004) speculated that the Xarifiidae may be closely related to the Vahiniidae (or possibly the antheacherid family group which includes the Antheacheridae, Corallovexiidae, Lamippidae and Mesoglicolidae). Such a relationship of the Vahiniidae was not supported by our molecular analysis, which placed *Vahinius* in a strongly supported clade with *Pachos* and *Stellicola*. Wider sampling of lichomolgoidean taxa is required before the more precise relationships of the Vahiniidae can be resolved. On the basis of morphology alone (antenna, male maxilliped), the family Thamnomolgidae, which likewise utilize antipatharians (only the monotypic *Forhania* Humes uses gorgonians) as hosts, appears to be the most likely candidate for this sister-group position.

Humes (1985a) described a new genus, *Parangium*, known from a single female associated with a scleractinian coral. Humes pointed out that there was a similarity in the mouthparts with those of the Lichomolgoidea but in the absence of the male was unable to assign it to any particular family. Based on mandibular morphology, Humes & Boxshall (1996) considered a position in the rhynchomolgid/anchimolgid group to be unlikely but did not exclude the possibility of a relationship with the Thamnomolgidae. Boxshall & Halsey (2004) finally placed *Parangium* in the Serpulidicolidae (ectoparasites of serpulid polychaetes) on the basis of synapomorphies displayed in the antennule, mandible, maxillule, swimming legs and the laterally directed caudal rami of the female. However, some of these similarities are not as robust as they seem (i.e. antenna with different armature, maxillule without terminal spatulate spine, leg 1 positioned closely to maxillipeds and not laterally directed) and additional differences in tagmosis

(*Parangium* has a cephalothorax instead of a cephalosome as in serpulidicolids), gonopore position (clearly dorsal in *Parangium*; dorsolateral in Serpulidicolidae), maxilliped (basis with two setae and endopod one-segmented instead of asetose basis and two-segmented endopod) and host utilization cast further doubt on its current position. Given our new rDNA evidence in support of a scleractinian associated clade, the position of *P. abstrusum* can now be reassessed on morphologial grounds. One of the most distinctive apomorphies of the Xarifiidae is the form and armature of the three-segmented antennary endopod. The proximal segment is longest and bears one seta, the middle segment is shortest and has two setae, and the distal segment typically possesses an outer seta and an inner claw at its tip. This family diagnostic is also displayed by *Parangium*, except that the middle and distal segments are partially fused, a condition that has also evolved secondarily within the Xarifiidae. Both taxa also share the form of the mandible (reduced to single blade), the bisetose maxillule (according to Humes's (1985b) family diagnosis, xarifiids can have two to three setae but all reports of a trisetose state are unconvincing; see, e.g. *Xarifia fissilis* Humes), the maxilla with digitiform allobasis, the maxilliped with rounded endopod, the poorly defined external segmentation of the body, the dorsal position of the female genital apertures and the reduction of the swimming legs. This morphological evidence suggests that either *Parangium* is the sister group of the Xarifiidae or it occupies a basal position within this family. However, it remains disputable whether family rank should be attributed to the Xarifiidae since the morphology of some of the more derived genera in the Rhynchomolgidae (such as *Kombia* Humes and *Calonastes* Humes & Goenaga) indicates that xarifiids may well be an advanced clade within the latter. Denser sampling of genes and molecules, as well as more morphological work across the Rhynchomolgidae, is deemed necessary before its paraphyletic status can be corroborated or refuted.

Relationships between Pseudanthessiidae and Chondracanthidae

The Chondracanthidae present a large number of autapomorphies and, except for the unjustified exclusion of *Pharodes* Wilson by Ho (1971), their monophyly has never been questioned on morphological grounds (Østergaard, Boxshall & Quicke, 2003). All 161 species are highly modified parasites of marine demersal fishes, occurring predominantly in the oral and branchial cavities of their hosts (Boxshall & Halsey, 2004). This host affiliation has led some workers to suggest that chondracanthid relationships may lie with other fish-parasitic families. Hogans & Benz (1990) pointed to a close affinity with the lernaeosoleids and Boxshall

& Halsey (2004) recently suggested that the Chondracanthidae belong to a larger group of families including the Lernaeosoleidae, Philichthyidae and Shiinoidae, all of which utilize marine fishes as hosts. According to Boxshall & Halsey the main diagnostic characters for this family group are found in the form of the antenna in the first copepodid (with one geniculate claw on the second and two geniculate claws on the third endopodal segment), the structure of the mandibular gnathobase, forming one (or two) bilaterally spinulate or dentate blade(s), and the reduction of legs 4 and 5. However, the first character cannot be confirmed at present for the Lernaeosoleidae and Shiinoidae because information on developmental stages is as yet unavailable for these two families. Also, although the mandible shows a gross similarity between the Chondracanthidae and Philichthyidae, it is radically divergent in the Shiinoidae and completely absent in the Lernaeosoleidae. The shiinoid mandibular gnathobase, having a serrate lash and a bipectinate seta (Kabata, 1968; West, 1986), is much more reminiscent of the condition found in some primitive mollusc-associated families such as the Anthessiidae and Myicolidae (e.g. Ho & Kim, 1992). Although the affinity of the Chondracanthidae, Lernaeosoleidae and Philichthyidae appears to be well-established, confirmation of the position of the Shiinoidae must await the arrival of ontogenetic and sequence data.

Various workers have suggested a relationship between the Chondracanthidae and one or several lichomolgoid families. Using comparative antennary morphology, Ho (1984) suggested that chondracanthids had evolved from a group of *Scambicornus*-like sabelliphilids associated with holothurians (the eight genera Ho referred to were collectively transferred to a new family, Synapticolidae, by Humes & Boxshall (1996)). Izawa (1987), employing naupliar characters, recognized an 'Antelichomolgus Group' which included the Lichomolgidae, Sabelliphilidae, Philoblennidae, Myicolidae, Anthessiidae, Philichthyidae and Chondracanthidae. This group was largely recovered by Ho's (1991) analysis based on 117 female morphological characters; however, the Chondracanthidae were placed in a most unorthodox subgroup, embracing the Eunicicolidae (sister group), Xarifiidae, Anomoclausiidae and Philoblennidae, and standing in apposition to the Pseudanthessiidae. Kim & Kim (2000), who explored poecilostomatoid interrelationships with SSU rDNA sequence data, claimed a strongly supported relationship between the Chondracanthidae and Lichomolgidae. Our analysis showed that chondracanthids are not related to the Synapticolidae as suggested by Ho (1984), or to the Lichomolgidae as proposed by Kim & Kim (2000), but revealed them as the sister group to the Pseudanthessiidae with very high bootstrap support (99%).

Pseudanthessiids are associated primarily with echinoderm hosts, and have a cyclopiform body shape in both sexes and a much more primitive morphology than do chondracanthids. Although the adult morphology lends little support to this unexpected relationship, the basic mandible morphology of the Pseudanthessiidae is not fundamentally different from the bilaterally toothed chondracanthid type. Similarly, deriving the chondracanthid maxilla and maxilliped from their respective counterparts in the Pseudanthessiidae would not involve any major transformations. Recently obtained ontogenetic data have, however, provided us with a valuable and compelling source of phylogenetically significant information. Costanzo, Crescenti & Calafiore (1996) described the copepodid stages of *Pseudanthessius gracilis* Claus, showing that the antennary morphology in the first copepodid conforms with the pattern displayed by the Philichthyidae (Izawa, 1973, 1975) and the Chondracanthidae (Izawa, 1986) (Fig. 6). In all three families the antenna has primitively one geniculate spine (a) on the second and two geniculate spines (b-c) on the third endopodal segment; given their significance in attachment to the host these elements are generally more strongly developed and claw-like in the fish-parasitic families. In four of the five pseudanthessiid genera, this condition is obscured in the adult because the spine on the second segment is replaced by a seta; however, some species of *Pseudanthessius* Claus retain the geniculate spine (e.g. *P. gracilis*,
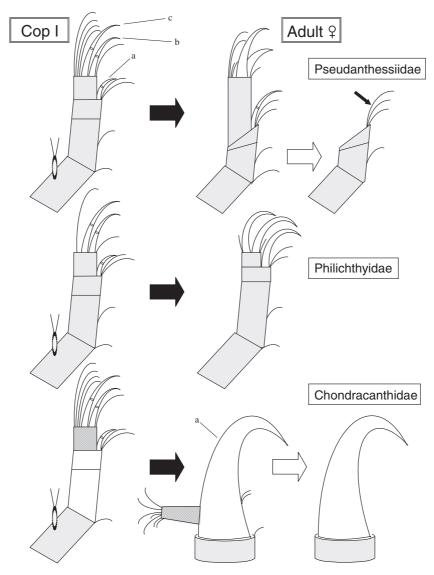

Figure 6. Generalized transformation in antennary development of Pseudanthessiidae, Philichthyidae and Chondracanthidae (Cop I, first copepodid; a, b-c, geniculate claws on second and third endopodal segments, respectively).

P. aestheticus Stock, Humes & Gooding, *P. nemertophilus* Gallien) throughout ontogeny. In the majority of pseudanthessiids, the distal geniculate elements develop into strong claws in the adult. The transformation is much more dramatic in the Chondracanthidae, with the antenna being used as the primary attachment device (Fig. 6). Izawa (1986) demonstrated that in *Acanthochondria yui* Shiino, antennary shape and armature change abruptly during the moult to copepodid II, resulting in a two-segmented antenna comprising a ring-like basal segment (coxobasis) and a heavily sclerotized terminal hook. The latter is a compound derivative of the geniculate claw (a) and the fused proximal and middle endopodal segments; in some genera a setose remnant of the distal endopod segment remains on the outer surface of the hook, also known as the 'accessory antennule' (Ho, 1984). The antenna in the Philichthyidae follows the primitive pattern of the Pseudanthessiidae, except that the number of endopodal setae is reduced and the middle and distal segments have become largely condensed into a single functional unit bearing the three large claws.

The resemblance in juvenile antennary morphology alone does not necessarily persuade acceptance of a Pseudanthessiidae–Philichthyidae–Chondracanthidae clade (PPC) as this potential synapomorphy may have been conserved in other families that display it in the adult (Kelleriidae, Octopicolidae, Polyankyliidae) but for which ontogenetic and molecular data are as yet lacking. Ho's (1984) suggestion that chondracanthids may have evolved from a synapticolid-like ancestor led us to test whether the Synapticolidae are related to the PPC clade. As our null hypothesis, we constrained *Scambicornus* sp. (Synapticolidae) and the PPC clade to group together. Using the Shimodaira–Hasegawa test we found that this null tree was not significantly worse (Δ ln$L$ = 4.06344, P = 0.1549 for RELL and 0.150 for FULL optimization approach) and consequently we cannot reject the hypothesis that synapticolids are at the base of the PPC clade as their shared presence of a claw on the second endopodal segment would indicate.

Validity of Lernaeosoleidae

Although Hogans & Benz (1990) established the Lernaeosoleidae as a new family, it is Yamaguti (1963) who has to be credited with the authorship, having used it as a new subfamilial name in his Lernaeidae (= Pennellidae) (Boxshall & Halsey, 2004). Both monotypic lernaeosoleid genera, *Lernaeosolea* Wilson and *Bobkabata* Hogans & Benz, are mesoparasites of fishes and their relationships are thought to lie with the Chondracanthidae on account of the sparsely armed, indistinctly segmented antennules, the prehensile antennae consisting of a terminal hook articulating with a basal, rotatable socket, and the genitoabdominal trunk morphology (Hogans & Benz, 1990; Benz & Braswell, 1998). Evidence in support of the recognition of the Lernaeosoleidae as a distinct family originally included the lack of a distinct mouth and associated oral appendages, and the absence of legs 1–4 (Hogans & Benz, 1990). Since the original proposal, however, Benz & Braswell (1998) have shown that at least in *B. kabatabobbus* Hogans & Benz a small mouth opening is probably present, and Benz, Nagasawa & Wetmore (2002) demonstrated the presence of a vestigial leg 1 and possible rudiments of two additional legs represented by transverse sclerites with muscle attachments. Given the shared apomorphic conditions in the female antennule and antenna, we find the justification for maintaining the Lernaeosoleidae as distinct from the Chondracanthidae flimsy and unconvincing. All of the diagnostic character states used by Hogans & Benz (1990) are highly derived and the majority of them is also found in one or several chondracanthid genera. For example, complete reduction of legs 1–4 occurs in six chondracanthid genera (*Apodochondria* Ho & Dojiri, *Brachiochondria* Shiino, *Immanthe* Leigh-Sharpe, *Markevitchielinus* Titar, *Rohdea* Kabata, *Strabax* von Nordmann) and Østergaard *et al.*'s (2003) analyses suggest that this condition has evolved independently at least three times (based on female and male partitions combined) within the Chondracanthidae. Among these 'leg-less' taxa the monotypic genus *Markevitchielinus* is of particular interest since it may represent a potential link in the evolution towards the lernaeosoleid body plan. Kabata's (1979) redescription of *M. anchoratus* Titar (translated in Østergaard (2003)) showed that the mouth is minute and difficult to discern, the paragnaths, maxillules and maxillae are absent, and the maxillipeds are strongly reduced. *M. anchoratus* is (like adult female lernaeosoleids) a mesoparasite that embeds virtually the entire cephalothorax in the tissue of its host, the sea raven *Hemitripterus villosus* (Pallas). In addition, there is a striking resemblance with *Lernaeosolea lycodis* in the morphology of the cephalothorax, which is divided into a transversally extended anterior part bearing the cephalic appendages and a contractile cylindrical posterior part, and also in the shape of the trunk, which possesses paired cylindrical posterodorsal processes. We interpret all of these similarities as evidence that the lernaeosoleids are merely a highly derived lineage that evolved within the Chondracanthidae from an ancestral mesoparasitic stock (of which *Markevitchielinus* is also a present-day descendant). As the Chondracanthidae, as currently constituted, represent a paraphyletic assemblage, we formally relegate the Lernaeosoleidae to a junior subjective synonym of the former.

The presence of a stylet-like mandible typically contained within an oral cone or siphon, formed by the labrum and the medially fused paragnaths (the labium), is a diagnostic autapomorphy for the Siphonostomatoida (Boxshall, 1986; Huys & Boxshall, 1991). In a comparative study, Boxshall (1990) showed that adaptive radiation in feeding mechanisms, such as fluid feeding (e.g. Entomolepididae, Pontoeciellidae) or surface grazing (Caligidae), involves mainly modification of the oral cone and maxillules. Even in highly transformed Nicothoidae such as *Rhizorhina* Hansen and *Nicorhiza* Lincoln & Boxshall, the specialized absorptive rootlet system embedded in the crustacean host can be homologized convincingly with the labrum and labium of the mouth cone (Lincoln & Boxshall, 1983). Comparative study of fish-parasitic and invertebrate-associated siphonostomatoids revealed the similarity in the arrangement and trajectory of the labral and levator muscles to be so undeniably close (Boxshall, 1986, 1990) that a possible polyphyletic origin of the order Siphonostomatoida (Marcotte, 1982) can now be ruled out.

However, the applicability of the oral cone as a high-level taxonomic discriminant is subject to two caveats. First, its diagnostic value as a siphonostomatoid attribute is not absolute because several families in the Harpacticoida, such as the Superornatiremidae, Rotundiclipeidae, Leptopontiidae and Novocriniidae, exhibit a well-developed oral cone, comprising a labrum, labium and stylet-like mandibles which extend down the cone (Huys, 1988, 1996; Huys & Conroy-Dalton, 1996; Huys & Iliffe, 1998). The close similarity in oral cone morphology between these harpacticoid taxa and primitive siphonostome families such as the Asterocheridae and Dirivultidae is clearly the result of convergence and indicative of a similar feeding mode. Second, an unpaired posterior lip is not necessarily a genuine labium derived by medial fusion of the paired paragnaths. In several harpacticoids and some poecilostome families in the Cyclopoida, the paragnaths have undergone extreme reduction and lost their lobate appearance, leaving behind only an undifferentiated fold around the posterior margin of the mouth. Such an unpaired structure, which is derived by bilateral reduction and not medial fusion, is clearly not homologous with the labium as defined by Boxshall (1986, 1990). In other families, such as the Lamippidae, the mouth is usually located on a buccal swelling and the presence of a prominent buccal cone in the subfamily Lamippinae made Stock (1988) assign this family to the Siphonostomatoida. In reality, the posterior margin of the buccal cone is formed by an intermaxillipedal swelling and does not represent a labial derivative. Another variation on the oral cone theme is demonstrated by the as yet unplaced genus *Endocheres* Bocquet & Stock, in which the buccal cone appears to be derived from paired structures meeting in the ventral midline rather than from the anterior labrum and posterior labium (Boxshall & Halsey, 2004).

Bresciani (1991) placed *Pectenophilus* in the Siphonostomatoida exclusively on the basis of the oral cone in the male, but failed to confirm whether the two lips were confluent at their bases as in genuine siphonostomatoids. He also expressed reservations as to the identity of the appendages positioned between the anterior and posterior lips, as siphonostomatoid mandibles enter the cone obliquely whereas in *P. ornatus* the presumptive gnathobases are medially directed and do not extend down the incipient oral cone. Our re-examination has demonstrated that the appendage, which was previously homologized as the mandible on positional grounds, is in reality the maxilla. Not only does its articulated, two-segmented condition rule out possible mandibular identity, but the discovery of the exit of the maxillary gland at the posterior surface of the basal segment also unequivocally identifies it as the maxilla. The structure erroneously identified as the mandibular gnathobase in reality represents the maxillary allobasis and the proximal segment described as the 'squared base' by Bresciani (1991) is interpreted here as the syncoxa. The position of the maxillae in between the labrum and the posterior 'lip' is unique within the Copepoda (but see below) and results from considerable anterior displacement. Similar displacement of cephalic appendages has been reported for other copepod families but in none of them has the maxilla functionally replaced and superseded the mandible. In the Micrallectidae, for example, the position of the maxillule is prelabral instead of postmandibular (Huys, 2001), and in the Dichelinidae the mandibular palp is located posterior to the maxillule (Boxshall & Ohtsuka, 2001). Our SEM observations have also revealed that the posterior 'lip' of the tubular mouth, tentatively interpreted as the labium (Bresciani, 1991), is an intermaxillary outgrowth of the ventral cephalic sclerite, bearing the widely separated paragnaths at its free margin (Fig. 4B) and being fused largely to the maxillary syncoxae (Figs 4, 5A). The absence of genuine mandibles (and maxillules) in conjunction with the lack of fusion between the labrum and the posterior lip (Figs 4B, 5A) demonstrate that the tubular mouth region in *Pectenophilus* is not homologous to the siphonostomatoid oral cone, and, corroborating our molecular-based findings, that it is not a member of the Siphonostomatoida.

MORPHOLOGICAL COMPARISON BETWEEN
PECTENOPHILUS AND MYTILICOLIDAE

The family Mytilicolidae was established by Bocquet & Stock (1957a, b) to include the genera *Mytilicola*

Steuer, *Trochicola* Dollfus & *Piratasta* Leigh-Sharpe. Monod & Dollfus (1932) described the genus *Cerastocheres* and placed it in the Lernaeidae but Boxshall & Halsey (2004) recognized it is a member of the Mytilicolidae. Known records demonstrate that mytilicolids are typically parasites of bivalve molluscs although some species of *Mytilicola* (Ho & Kim, 1992) and *Trochicola* (Dollfus, 1914; Bocquet & Stock, 1957a) have been reported from trochid gastropods (topshells). Unlike *Pectenophilus*, adult mytilicolids are intestinal parasites and there is no gross disparity in size and dimorphism between the sexes, nor is there any significant difference in feeding strategy.

Like all molecular systematic studies, the strength of interpretation relies on comparative morphological data to make biological sense. Our DNA analysis recovered a strongly supported relationship between the as yet unplaced genus *Pectenophilus* and the Mytilicolidae, raising the question as to whether there is any morphological congruence between the two taxa. The absence of postmaxillary limbs in *P. ornatus* necessarily restricts such comparison to characters associated with the cephalic appendages and their ontogeny. Various workers have attempted to elucidate the life cycle of mytilicolids, but there appears to exist a great deal of inconsistency regarding the number of stages and both the appendages and the segmentation that characterizes them (Steuer, 1903; Pesta, 1907; Caspers, 1939; Hockley, 1951; Costanzo, 1960; Bocquet, Stock & Kleeton, 1963; Gee & Davey, 1986). However, based on studies of *M. intestinalis* and *T. entericus* (Bocquet *et al.*, 1963; Gee & Davey, 1986), it is now widely accepted that mytilicolids typically pass through one naupliar, one metanaupliar, five copepodid, and a sexually immature adult stage before the life cycle is complete and egg production commences. Both naupliar stages have well-developed uniramous antennules and biramous antennae and mandibles. Given its absence in *Pectenophilus* the fate of the mandible in postnaupliar mytilicolid stages is of particular interest. Early workers (Steuer, 1903; Pesta, 1907; Caspers, 1939; Costanzo, 1960) identified the third cephalic limb in *M. intestinalis* as the mandible and, based on the position of the maxillary gland opening, homologized the fourth limb with the maxilla, thus implying that the maxillule was absent. Dollfus (1914) initially arrived at the same serial arrangement for *T. entericus* but later (Monod & Dollfus, 1932) concluded that the mandible is absent in all copepodid stages, the reduced third cephalic limb being the homologue of the maxillule. The absence of the mandible was confirmed by Bocquet & Stock (1957a, b), who claimed that this condition was diagnostic for all mytilicolid genera. However, Hockley (1951) described and illustrated the mandible (and maxillule) as being present in the

'second parasitic instar' of *M. intestinalis*. Although it is not clear exactly which stage Hockley was referring to, his interpretation must have been based on an observational error as Gee & Davey's (1986) thorough examination of all stages convincingly demonstrated that the mandible is never expressed during postnaupliar development. Our SEM observations not only confirmed the absence of the mandible but also revealed that the rudimentary maxillule is preoral in position (Fig. 5D). Most importantly, we were able to demonstrate that not only are the maxillae, as in *Pectenophilus*, displaced anteriorly, but they have also functionally supplanted the mandibles, with their allobases directed into the oral cavity (Fig. 5B–D). This unique topology, involving the functional replacement of the mandibular gnathobase by the maxillary allobasis, is a robust synapomorphy of the Mytilicolidae and manifestly demonstrates that *Pectenophilus* must be included in this family. Additional morphological similarities supporting such an affiliation include: (1) short antennules bearing stubby or reduced armature elements around the apex, (2) powerful antennae with a robust coxobasis and an endopod terminating in a strong claw or hook-like process, and (3) maxilla showing a disproportionate size difference between the syncoxa and allobasis, with allobasis produced into a serrate process or spine.

The life cycle of the male *Pectenophilus* is highly abbreviated compared with that of other mytilicolids, which typically pass through seven instars before reaching sexual maturity. Although the preadult is not known with certainty, it is likely to be a metanauplius and not a copepodid for two reasons. First, sexually mature males of *P. ornatus* have a volume approximately 33 times that of nauplii, and the duct leading into the male vesicle is very narrow compared with the diameter of adult males (Nagasawa *et al.*, 1988). It is therefore conceivable that males enter the duct as a small-sized instar, and do not undergo their final moult and subsequent substantial increase in size until their arrival in the vesicle. A similar phenomenon has been reported in another mollusc parasite, *Nucellicola holmanae* Lamb, Boxshall, Mill & Grahame (Chitonophilidae), in which the adult male undergoes not only extreme transformation at the final moult but also gross size increase as a result of hypermorphosis (Huys *et al.*, 2002). Coincidentally, the adult male of this species is also contained within a membranous vesicle (however, at the posterior end of the female), and the extent of hypermorphosis involved is evidenced by comparison with the exuvium size of the late copepodid (III) enclosed with it.

The second line of evidence making the existence of a transitionary copepodid stage in the life cycle unlikely is offered by the morphology of the adult male. Its gross morphology, including the distinctive

lack of segmentation, tagmosis, maxillipeds and swimming legs, and the presence of fully functional maxillae, is reminiscent of that of an early metanauplius. It is extremely rare in copepods to see such global progenesis, resulting in early sexual maturation at the metanauplius stage and the complete cessation of somite and limb development, which normally progresses during the copepodid phase. The only other case of similarly extreme paedomorphic development was reported recently for the Micrallectidae, which utilize gymnosome pteropod molluscs as hosts but do not show any significant hypermorphosis in the adult (Huys, 2001).

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APPENDIX

Appendix. Taxonomic listing of exemplar taxa, with classification following Martin & Davis (2001). **Exemplar taxon* Ex. *Host taxon* (host group), collection locality (country), collector (date), [voucher specimen accession number] SSU rDNA sequence accession number. [a]Previously published GenBank sequences. NHM, Natural History Museum.

OUTGROUP
  Class Malacostraca
    Subclass Hoplocarida
      Order Stomatopoda
        *Squilla empusa Say, 1818 L81946[a]
  Class 'Maxillopoda'
    Subclass Thecostraca
      Infraclass Ascothoracida
        *Berndtia purpurea Utinomi, 1950 L26511[a]
        *Ulophysema oeresundense Brattstrøm, 1936 L26521[a]
COPEPODA
  Order Calanoida
    Family Calanidae
      *Calanus pacificus Brodsky, 1948 L81939[a]
    Family Pseudocyclopidae
      *Pseudocyclops sp., sledge net, off Nagannu Island (Japan), S. Ohtsuka (28 May 2000), [NHM reg. no. 2005.42] AY626994
    Family Pseudodiaptomidae
      *Pseudodiaptomus annandalei Sewell, 1919, fish grow-out ponds, Tungkang (Taiwan), S.-H. Cheng (13 Jul 2002), [NHM reg. no. 2005.43] AY629258
    Family Ridgewayiidae
      *Exumella mediterranea Jaume & Boxshall, 1995, anchialine cave, Cova de na Barxa, Mallorca (Spain), D. Jaume (21 April 2001), [NHM reg. no. 2005.44] AY629259
    Family Tortanidae
      *Tortanus sp., plankton tow, Lifou (New Caledonia), R. Huys (01 Nov 2000), [NHM reg. no. 2005.45] AY626995
  Order Cyclopoida
    'Gnathostome families'
      Family Cyclopidae
        *Euryte sp., *Halimeda* washings, Lifou (New Caledonia), R. Huys (04 Nov 2000), AY626996
        *Apocyclops royi (Lindberg, 1940), fish grow-out ponds, Tungkang (Taiwan), S.-H. Cheng (13 Jul 2002), [NHM reg. no. 2005.46] AY626997
        *Cyclops sp., wildlife garden pond NHM, London (England), R. Huys (04 Apr 2000), AY626998
        *Acanthocyclops viridis (Jurine, 1820), wildlife garden pond NHM, London (England), R. Huys (04 Apr 2000), AY626999
    'Poecilostome families' = 'Poecilostomatoida'
      Family Anchimolgidae
        *Anchimolgidae gen. nov. Ex. Cataphyllia jardinei (Scleractinia), Baie Ste Marie (New Caledonia), R. Huys & S. Conroy-Dalton (16 Oct 2001), [NHM reg. no. 2005.47] AY627000
        *Anchimolgus sp. Ex. *Polyphyllia talpina* (Scleractinia), Baie Ste Marie (New Caledonia), R. Huys & S. Conroy-Dalton (16 Oct 2001), [NHM reg. no. 2005.48] AY627001

Family Anthessiidae

Anthessius sp. Ex. *Tridacna squamosa* (Bivalvia), Lifou (New Caledonia), R. Huys (08 Nov 2000), [NHM reg. no. 2005.49] AY627002

Family Chondracanthidae

Chondracanthus lophii Johnston, 1836, L34046[a]

Lernentoma asellina (Linn., 1758) Ex. *Chelidonichthys gurnardus* (Actinopterygii: Scorpaeniformes), Bell Rock, 56°27.69′N 02°15.05′W (Scotland), P. Østergaard (27 May 2000), [NHM reg. no. 2005.50] AY627003

Family Lichomologidae

Stellicola sp. Ex. *Culcita novaeguineae* (Asteroida), Ilot Brun (New Caledonia), R. Huys & S. Conroy-Dalton (19 Oct 2001), [NHM reg. no. 2005.51] AY627004

Family Mytilicolidae

Mytilicola intestinalis Steuer, 1902, Ex. *Mytilus edulis* (Bivalvia), Beggars Island in Lynher River (U.K.), J.M. Gee (25 Oct 2002), [NHM reg. no. 2005.52] AY627005

Trochicola entericus Dollfus, 1914, Ex. *Calliostoma zizyphinum* (Gastropoda), Ambleteuse (France), R. Huys (27 Sep 2003), AY627006

Family Pseudanthessiidae

Pseudanthessius sp. Ex. *Filograna* sp. (Polychaeta), Ilot Maître (New Caledonia), R. Huys & S. Conroy-Dalton (17 Oct 2001), [NHM reg. no. 2005.54] AY627007

Family Rhynchomolgidae

Critomolgus sp. 1 Ex. *Chromodoris* sp. (Gastropoda), Lifou (New Caledonia), R. Huys (09 Nov 2000), [NHM reg. no. 2005.55] AY627008

Critomolgus sp. 2 Ex. *Actinodendron* cf. *globeratum* (Actiniaria), between Ilot Ste Marie and Ilot Poirée (New Caledonia), R. Huys & S. Conroy-Dalton (09 Oct 2001), [NHM reg. no. 2005.56] AY627009

Family Sabelliphilidae

Sabelliphilus elongatus M. Sars, 1862, Ex. *Sabella pavonina* (Polychaeta), Looe beach, Cornwall (England), P. Tompsett (20 Aug 2001), [NHM reg. no. 2005.57] AY627010

Family Synapticolidae

Scambicornus sp. Ex. *Thelenota ananas* (Holothuria), Passe de Boulari (New Caledonia), R. Huys & S. Conroy-Dalton (18 Oct 2001), [NHM reg. no. 2005.58] AY627011

Family Vahiniidae

Vahinius sp. Ex. *Antipathes* sp. (Antipatharia), Ilot Brun (New Caledonia), R. Huys & S. Conroy-Dalton (19 Oct 2001), [NHM reg. no. 2005.59] AY627012

Family Xarifiidae

Xarifia sp. Ex. *Acropora millepora* (Scleractinia), Lifou (New Caledonia), R. Huys (11 Nov 2000), [NHM reg. no. 2005.60] AY627013

Family *incertae sedis*

Pachos sp., plankton tow, off Akuseki Island (Japan), S. Ohtsuka (30 May 2000), AY627014

Order Harpacticoida

Family Canthocamptidae

Bryocamptus pygmaeus (G.O. Sars, 1863), Scio Pond in Wimbledon (England), R. Huys (28 Apr 2002), [NHM reg. no. 2005.61] AY627015

Family Ectinosomatidae

Bradya sp., triangle dredge, 166-170 m, Iqpik, Disko (Greenland), R.M. Kristensen (12 Jul 2002), AY627016

Order Siphonostomatoida

Family Asterocheridae

Orecturus sp. Ex. unidentified Gorgonacea, Lifou (New Caledonia), R. Huys (03 Nov 2000), [NHM reg. no. 2005.62] AY627017

Asterocheridae sp. Ex. *Chironephthya* sp. (Alcyonacea), Passe de Boulari (New Caledonia), R. Huys & S. Conroy-Dalton (18 Oct 2001), [NHM reg. no. 2005.63] AY627018

Family Caligidae

Gloiopotes watsoni Kirtisinghe, 1934, Ex. *Makaira nigricans* (Actinopterygii: Perciformes), off Port Stephens (Australia), A. Ingram (Feb 2000), [NHM reg. no. 2005.64] AY627019

Lepeophtheirus salmonis (Krøyer, 1837) AF208263[a]

Caligus elongatus (von Nordmann, 1832), Ex. *Gadus morhua* (Actinopterygii: Gadiformes), Bell Rock (Scotland), R. Bray (12 May 1997), [NHM reg. no. 2005.65] AY627020

Family Cancerillidae

Cancerilla sp. Ex. unidentified ophiuroid, Lifou (New Caledonia), R. Huys (09 Nov 2000), AY627021

Family Dinopontiidae
  Stenopontius sp. Ex. *Callyspongia* cf. *hispidoconulosa* (Porifera), Baie Ste Marie (New Caledonia), R. Huys & S. Conroy-Dalton (16 Oct 2001), [NHM reg. no. 2005.66] AY627022
Family Dirivultidae
  Rhogobius contractus Humes, 1987, washings of *Bathymodiolus thermophilus* (East Pacific Rise, 9.5° E, 2507 m), J. Zekely (30 Nov 2002), [NHM reg. no. 2005.67] AY627023
Family Ecbathyriontidae
  Ecbathyrion prolixicauda Humes, 1987, washings of *Calyptogena, Riftia, Bathymodiolus thermophilus* (East Pacific Rise, 9.5° E, ~2500 m), J. Zekely (14 Dec 2002), AY627024
Family Entomolepidae
  Entomolepidae sp. Ex. *Catalaphyllia jardinei* (Scleractinia), New Caledonia, R. Huys & S. Conroy-Dalton (16 Oct 2001), [NHM reg. no. 2005.68] AY627025
Family Hatschekiidae
  Hatschekia pagrosomi Yamaguti, 1939, Ex. *Sparus auratus* (Actinopterygii: Perciformes), Coffs Harbour, New South Wales (Australia), K. Rohde (Sep 1996), [NHM reg. no. 2005.69] AY627026
Family Lernaeopodidae
  Parabrachiella bispinosa (von Nordmann, 1832), Ex. *Chelidonichthys gurnardus* (Actinopterygii: Scorpaeniformes), Beatrice, 58°07'N 2°56'W (Scotland), P. Østergaard (19 May 2001), [NHM reg. no. 2005.70] AY627027
  Clavella adunca (Strøm, 1862), Ex. *Melanogrammus aeglefinus* (Actinopterygii: Gadiformes), Fair Isle, 59°13'N 01°25'W (Scotland), P. Østergaard (20 May 2001), [NHM reg. no. 2005.71] AY627028
Family Nanaspididae
  Nanaspis tonsa Humes & Cressey, 1959, Ex. *Thelenota ananas* (Holothuria), Passe de Boulari (New Caledonia), R. Huys & S. Conroy-Dalton (18 Oct 2001), [NHM reg. no. 2005.72] AY627029
Family Pennellidae
  Lernaeocera branchialis (Linn., 1767), Ex. *Merlangius merlangus* (Actinopterygii: Gadiformes), Moray Firth, 58°06'N 03°03'W (Scotland), P. Østergaard (19 May 2001), [NHM reg. no. 2005.73] AY627030
Family Pontoeciellidae
  Pontoeciella abyssicola (T. Scott, 1893), ORI plankton net, off Okinoerabu Island (Japan), S. Ohtsuka (26 May 2000), AY627031
Copepoda *incertae sedis*
  Pectenophilus ornatus Nagasawa, Bresciani & Lützen, 1988, Ex. *Chlamys farreri nipponensis* (Bivalvia), Kesen-numa Bay (Japan), A. Oshino (05 Dec 2002), [NHM reg. no. 2005.53] AY627032