Tantulocarida versus Thecostraca: inside or outside? First attempts to resolve phylogenetic position of Tantulocarida using gene sequences

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Abstract

Complete 18S rDNA sequences of two species of the Tantulocarida Articicarcicarpus pertsovii (Basipodellidae) and Microdajus tchesunovi (Microdajidae) were obtained and used for estimating the relationship of the class with other Crustacea. This constitutes the first use of tantulocaridan gene sequences, and we conclude that the Tantulocarida are very close relatives of the class Thecostraca, which comprise cirripedes, ascothoracidans and the enigmatic Facetotecta. With much lower confidence, the Tantulocarida are also indicated as nested within the Thecostraca, being sister group to the Cirripedia. We therefore discuss morphological similarities and differences between tantulocaridans and the thecostracans in search of potential synapomorphies, including a possible relation to the parasitic barnacles (Rhizocephala). We conclude that the cement gland of the tantulus larva and the cirripede cyprid might be homologous structures, but that similarities in host infection and root systems between the Tantulocarida and the Rhizoccephala are, on present evidence, likely to be homoplasies evolved by convergent evolution into advanced parasitism. The precise position of the Tantulocarida in relation to or within the Thecostraca must be pursued by a more extensive database of genetic markers.

Key words: Tantulocarida – Cirripedia – Thecostraca – phylogeny – molecular genetics – 18S rDNA – cement adhesion – rootlet system

Introduction

Tantulocaridans are parasitic crustaceans with a remarkably complex life cycle, but their precise phylogenetic position within Crustacea remains very problematic. Their late recognition as a separate class (Boxshall and Lincoln 1983) was largely due to their minute size and meiofaunal habitat, but they now prove to be both species diverse and widespread. Tantulocaridans parasi- tize a variety of other crustaceans including Tanaidacea, Copepoda, Ostracoda and Amphipoda, but their complex life history is far from fully understood. The life cycle includes both free-swimming forms and ectoparasitic stages fixed to their hosts while reproduction involves both sexual forms and parthenogenesis (Boxshall and Lincoln 1987; Huys et al. 1993; Kolbasov and Savchenko 2010). The complex life cycle, the minute size of all stages and the severe difficulty in sampling live material from their meiofaunal habitats all combine to impede comprehensive studies of these parasitic Crustacea.

Due to their parasitic mode of life, tantulocaridans present few morphological characters that could be used to indicate their relationship among Crustacea. This is very apparent in the absence of any cephalic appendages, except for unsegmented antennules, unsegmented hysteresis antennules used for settlement and carrying maximally two aesthetasc setae, large frontal hysteresis antennules used for settlement and carrying maximally two aesthetase setae, large frontal filaments and paired compound eyes with three crystalline cones (Grygier 1987; Hoeg 1985; Boxshall and Lincoln 1987). The Ascothoracans also include three groups of obligatory parasites. The Cirripedia, Rhizoccephala are highly specialized parasites on other Crustacea and sports a complex life cycle where the method of host infection might present some similarity to the tantulocaridans (Hoeg 1985; Boxshall and Lincoln 1987). The Ascothoracans are parasites in anthozoans and echinoderms. Finally, the enigmatic Facetotecta were recently proved to be advanced parasites in yet to be identified hosts (Glenner et al. 2008). These several parasitic groups within the Thecostraca obviously call for several characters in the cypridid larvae that initiate the sessile adult life found in all members of the class. These include the presence of so-called lattice organs based on a carapace covering most of the body, specialized prehensile antennules used for settlement and carrying maximally two aesthetase setae, large frontal filaments and paired compound eyes with three crystalline cones (Grygier 1987; Hoeg and Kolbasov 2002; Pérez-Losada et al. 2004, 2009; Hoeg et al. 2009).

The best-known thecostracan group is the suspension feeding stalked and sessile barnacles (Cirripedia Thoracica), but thecostracans also include three groups of obligatory parasites. The Cirripedia, Rhizoccephala are highly specialized parasites on other Crustacea and sports a complex life cycle where the method of host infection might present some similarity to the tantulocaridans (Hoeg 1985; Boxshall and Lincoln 1987). The Ascothoracans are parasites in anthozoans and echinoderms. Finally, the enigmatic Facetotecta were recently proved to be advanced parasites in yet to be identified hosts (Glenner et al. 2008). These several parasitic groups within the Thecostraca obviously call for studies on a possible relationship between any of these and the similarly parasitic Tantulocarida.

In a more general perspective, Crustacea is one of the most diverse groups of arthropods, and both their intrinsic phylogeny and the relationship to other arthropods are under intense debate based on both morphological and molecular evidence (Wheeler et al. 2004; Regier et al. 2008, 2010; von Reumont et al. 2009; Koenemann et al. 2010). Since not only the number of genetic markers but also taxon sampling is critical to molecular phylogeny (Regier et al. 2008; Oakley et al., 2013), inclusion of the Tantulocarida in these studies could well have a significant effect on both Crustacea and perhaps even for Arthropoda as a whole.
Here, we present the first use of 18S rDNA nucleotide sequences to study phylogenetic relationships of the Tantulocarida and review existing morphological evidence and presenting new SEM- and TEM-based data. We also discuss their possible relation to the thecostracan groups under the caveat that any conclusions based on a single gene analysis at this deep level of crustacean evolution must necessarily be given with caution.

Material and Methods

Molecular analysis

Material including two species of the Tantulocarida (Arcticotantulus pertzovi Kornev, Tchesnov, Rybnikov, 2004 and Microdajus tchesnovi Kolbasov and Savchenko 2010) was collected off the White Sea Biological Station of Moscow State University, Velikaya Salma Strait, Kandalaksha Bay, (66°31′41″N, 33°1108″E) in August 2010. Sediment samples were obtained with a hyperbenthic Ockerman dredge from depths of 20–50 m and rinsed through a 50-μm sieve. For DNA extraction of each species, 10 to 12 parthenogenetic females at late stage of development were pooled. The parasites were detached very carefully from their hosts to be kept in lysis buffer for 2 h. Standard techniques of 3D extraction using Promega Wizard DNA extraction kit were applied (with some modifications). The complete 18S rDNA gene was amplified in three overlapping regions, using the following primer pairs: 18SIF (TACCTCGGTGATCTCCGCA/TGAAAC)/18S9R (GATCCTTCC (TACCTGGTTGATCCTGCCAGTAG)/18S5R (CTTGGCAAATGCTTT

For the analysis of our data from the Tantulocarida, we used the data set developed by Koenemann et al. (2010). In that study, multiple sequence alignment methods were based on an algorithm implemented in MUSCLE (v.3.7) (Edgar 2004) followed by manual secondary-structure optimization, as described in detail in the study described by Koenemann et al. (2010).

Our 18S rDNA sequences were placed manually into the prealigned data set from Run 5 in Koenemann et al. (2010). In our tree, Arcticotantulus pertzovi and Microdajus tchesnovi sit as sister groups with 100% bootstrap support (Fig. 4). This is hardly surprising considering the many morphological synapomorphies that unite the Tantulocarida (Boxshall and Lincoln 1983, 1987). The long branch leading to the Tantulocarida could be explained by a accelerated evolutionary tempo that might be typical for parasitic taxa (Dowton and Austin 1995; Page et al. 1998). Although recent investigations show that this statement may be far from always true (Gilman et al. 2012; Kaltenpoth et al. 2012).

High-level phylogeny of arthropods is a challenging task and it is still not completely resolved. It was shown that alignment optimization of ribosomal markers based on secondary-structure information can have a radical impact on phylogenetic reconstruction, and within several multiple alignments only few data sets recover monophyletic Arthropoda (excluding Onychophora), Pancrustacea, Malacostraca, Insecta, Myriapoda and Chelicerata (Koenemann et al. 2010). Because we have not attempted to reconstruct deep phylogeny of Arthropoda, but rather aimed to properly allocate the Tantulocarida branch within the Crustacean tree, we closely followed algorithms described for its best data set in this paper (Run 5). We present and discuss only Bayesian analysis, because our results are consistent with those of Koenemann et al. (2010) that ML analysis of the same data set leads to poorly resolved tree.

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The Tantulocarida are also, but with only 86% bootstrap support, placed inside the Thecostraca as a sister group to the Cirripedia. These results call for a morphological comparison between the Tantulocarida and the Thecostraca in search of possible synapomorphies (see also Heeg et al. 2009). Although the support was low, the alleged relationship to the Cirripedia calls for a special comparison with this taxon.

Comparison of our tree with the original one (Koenemann et al. 2010) reveals some significant differences. Addition of the Tantulocarida in phylogenetic analysis resulted in a monophyletic Hexapoda with moderately strong bootstrap support of 90%, while in the original phylogenetic tree (Koenemann et al. 2010), proturans and diplurans were placed as a sister group of Xenocarida (clade Remipedia + Cephalocarida). All the remaining clades were recovered, but with slightly higher bootstrap supports.

Morphological analysis

For transmission electron microscopy, several specimens of settled and metamorphosing tantulus larvae of A. pertzovi were fixed in 4% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS). The samples were then washed three times for 15 min in PBS and stored at 4°C before being postfixed in buffered 2% OsO4, dehydrated using ethanol and embedded in Araldite resin. Ultrathin sections were cut with a diamond knife and stained for 40 min with saturated uranyl acetate and for 5 min with lead citrate. The sections were examined and photographed with a JEOL JEM 100B and JEOL-1011 80 kV.

A settled tantulus larva of Serratotantulus chertoprudae Savchenko and Kolbasov 2009, cypris larva of lepadid barnacle Conchoepera virgatum (Spengler, 1790) and y-cypris larva of Hansenocaris toci Kolbasov and Heeg, 2003 were processed for scanning electron microscopy. They were postfixed in 2% OsO4, dehydrated in a graded ethanol series and acetone, and critical point dried in CO2. The specimens were then sputter-coated with platinum-palladium alloy and examined in a JEOL JSM-6380LA microscope at operating voltages of 15–20 kV.

For comparison with the Rhizocephala (Figs 1i, 2e,f and 3e–g), we use TEM pictures scanned from photographic originals kindly provided by the Prof. Jens T. Heeg, who had survived after preparation of his marvellous studies (Heeg 1985; Bresciani and Heeg 2001).
plan should encompass a cephalon, a six-segmented thorax with appendages, a four-segmented abdomen without legs and a telson with furcal rami, but the elements in the tantulocaridan abdomen may be nothing more than annulations rather than true somites (Huys et al. 1994). Another, more convincing similarity is the position of the female gonopore on the first thoracic segment in both the Thecostraca and the Tantulocarida (Boxshall and Lincoln 1987; Grygier 1987; Boxshall and Huys 1989; Boxshall 1991; Huys et al. 1993). Unfortunately, we do not know the sexual stage of the Facetotecta, whence the gonopore position in that taxon remains unknown.

Cuticle ornamentation
Sexual stages of the Tantulocarida have a univalved carapace (Fig. 5a–c) ornamented with a pattern of cuticular ridges and different types of pores/pits that closely resembles that seen in the Facetotecta (Fig. 5d). A similar patterning can be seen in the cuticle of some rizocephalans nauplii (Høeg et al. 2004) and in a few cirripede cyprids (Elfimov 1995; Moyse et al. 1995). The extent to which such patterns are useful in deep phylogeny is debatable, as they are likely highly adaptive to the ecology of the species concerned.

Larval stages
Both the Thecostraca and the Tantulocarida have a special larval instar inserted between the naupliar and adult phases. Called cyp ridoid in the Thecostraca and tantulus in the Tantulocarida, these two instars exhibit some similarities that might suggest homology. They both accomplish the change from life in the pelagic to attachment on the potential host animal (Fig. 1a,f). Both can swim using six pairs of natatory thoracopods and eventually attach using cephalic glandular secretions. Unlike the thecostracan...
cypridoid larvae, all stages of the Tantulocarida lack compound eyes. But this is hardly surprising considering the meiobenthic conditions of their environment. Parasitism in Thecostraca seems to have little correlation with presence/absence of compound eyes. They exist in the assumedly parasitic Facetotecta, but are reduced in the parasitic Ascothoracida. Cyprids of the parasitic Rhizocephala normally lack compound eyes, but they are spectacularly present in a few species (Glenner et al. 2010). A significant difference to all thecostracans is the presence in tantulocaridans of a parthenogenetic stage in the life cycle, but this could easily be an autapomorphy of the taxon.

Lattice organs

These chemosensory organs occur in the carapace of all or almost all cypridoid larvae in the Thecostraca and are considered an important synapomorphy for the taxon (Elfmö 1986; Jensen et al. 1994; Høeg and Kolbasov 2002; Høeg et al. 2009). Ontogenetically, they develop from conventional setae in the nauplius (Rybakov et al. 2003) and are in the cyprid present as two anterior and three posterior pairs of highly modified structures lying almost flush with the cuticular surface (Fig. 1g). In the Tantulocarida, a thorough search of the dorsal surface in both tantulus larva and free-swimming male individuals has failed to reveal any lattice organs. The sensory apparatus of tantulocaridans consists of conventional setae situated in pores and distributed over the dorsal surface of the cephalon in the tantulus (Fig. 1b,c) and on the cephalothorax in males (Fig. 5b,c), (Savchenko and Kolbasov 2009; Kolbasov and Savchenko 2010; Petrunina and Kolbasov 2012). Accepting an in-group position of the Tantulocarida within the Thecostraca would therefore entail that these otherwise virtually omnipresent organs have been secondarily lost.

Cirripedia and Tantulocarida

The cirripedes and the tantulocaridans seem to deviate extensively in most aspects of their morphology, but we will discuss two potential homologies.

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Cement glands

Both the cirripede cypridoid larva, called the cyprid, and the tantulus larva terminate their pelagic life by adhering to the substratum with secretion from multicellular cephalic glands, called the cement glands in cirripedes (Figs 1d,e,h,i and 3a). In cyprids (Walker 1971; Høeg 1985, 1987), the two cement glands are located ventrolaterally in the cephalon close to the antennules (Fig. 2e). A single, cuticle-lined duct leads from each gland to its exit on the attachment disc distally on the antennule of the same side (Figs 1i and 2f). A muscular sac located between the gland and the antennule controls the secretion process (Walker 1971; Høeg 1985). The cypris cement is a biochemically complex substance that is under intense study because thoracican cirripedes (stalked and acorn barnacles) are detrimental as fouling organism on ships and other man-made objects in the sea (Walker 1971; Okano et al. 1996).

Tantulocaridans have no antennules and their cement glands are connected with a special unpaired proboscis (Figs 2b,c and 3a,d), which is extruded when free-swimming, but retracted in settled specimens (Figs 2c and 3a). Four tubular ducts (secretion canals) traverse the proboscis (Figs 2b and 3a) and open on its terminal disk (Fig. 2c). This suggests that adhesive gland connects to the proboscis by means of two canals (ducts) instead of the single cement canal known from cirripede cyprids (Walker 1971; Høeg 1985, 1987). Furthermore, tantulocaridans seem not to have any muscular sac. The chemical nature of the tantulocaridan cement, released near the oral disc, remains unknown, but in TEM sections, it is not unlike that of the reticulated structure of cypris cement (Fig. 1e,i). Although morphological differences are many, it is not entirely inconceivable that the tantulus and cypris cement glands could be homologous. This, however, would only be consistent with a sister group relation to the

Fig. 3. Internal anatomy (a–c, e–g – TEM, d – SEM) of tantulus larva and parthenogenetic stage of Tantulocarida: cephalon and rootlet system (a–c – Arcticotantulus pertzovi, d – Serratotantulus chertoprudae) and cypris larva of Cirripedia Rhizocephala (original photographs provided by Pr. J.T. Høeg) (e – Peltogastrella sulcata, f – Lernaeodiscus porcellanae). (a) transverse section of cephalon of parthenogenetic stage through proboscis (prb) with glandular ducts (cgd) and gut. (b) transverse section through anterior part of oral disc (od) and rootet system (rtl). (c) transverse section of stylet (st), basal part. (d) tantulus larva, broken anterior part of cephalon, showing tip of stylet (st), gut wall (gw) and proboscis (prb) with four glandular ducts. (e) transverse section through wall of rootlet system, showing details of its ultrastructure: microcuticular projections (mp) and homogeneous layer (hl). (f, g) internal anatomy of kentrogon. (f) longitudinal section of settled kentrogon through penetrated stylet (st). (g) transverse section of stylet (st) in its basal part. Abbreviations: cgd - cement gland duct, gw - gut wall, hl - homogeneous layer, mp - microcuticular projections, od - oral disc, prb - proboscis, rtl - rootlet system, st - stylet. Scale bars in µm

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Fig. 4. Bayesian analysis of multiple sequence alignment modified from Run 5 in Koenemann et al. (2010), including two new 18S sequences of Tantulocarida, based on a prealignment using MUSCLE and manual secondary-structure optimization with alignment masking. Numbers on nodes represent posterior probability values. Black dots indicate tantulocarid branches, black triangles indicate out-groups noneuarthropod taxa (Onychophora and Tardigrada).

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Cirripedia, because the cypridoid larvae of the Facetotecta and the Ascothoracida lack a cement gland and attach with their antennules by purely mechanical means.

**Tantulocarida and Rhizocephala**

Within the Cirripedia, the Rhizocephala comprises a group of parasites with an advanced life cycle. Both the rhizocephalans and the tantulocaridans gain access to the interior of the host using a specialized stylet formed by the infesting larva (Delage 1884; Boxshall and Lincoln 1983; Høeg 1985, 1987), and both develop a nutrient absorbing system of rootlets inside the host. The full details of host invasion in both kentrogonid and aken-trogonid rhizocephalans have only recently come to light (Glenner et al. 2000; Glenner 2001; Høeg et al. 2005). However, according to the latest research on Akentrogonida are no longer considered to be the basic group inside Rhizocephala (Glenner and Hebsgaard 2006), and thus their advanced way of host penetration (without any stylet) is less comparable with Tantulocarida. Having settled on a host crab, the cyprid of more primitive kentrogonid rhizocephalan first moults into a kentrogon, which then produces a hollow cuticular stylet that penetrates the host integument and serves as a guide tube for injecting (Fig. 3f,g) highly simplified and slug or worm-shaped vermigon stage into the tissues (Delage 1884; Høeg 1985, 1987). After some time, the parasite starts growing out a system of rootlets, which have a highly specialized ultrastructure (Fig. 3e), and penetrates large parts of the host organism (Bresciani and Høeg 2001), and finally it produces the external reproductive body (Høeg et al. 2005). The Tantulocarida have no entirely endoparasitic phase. Their stylet is a solid structure produced by the tantulus and it serves only to puncture the host cuticle (Fig. 3c,d). Through this hole, the tantulus grows its rootlets that obviously serve for gaining nutrients just as their counterparts in the Rhizocephala (Fig. 3a,b). The tantulocaridan rootlets are covered with a thin cuticle, but other details of their ultrastructure remain uncertain. From this comparison, there is little basis for entertaining homologies between parasitism in tantulocaridans and rhizocephalans. The stylet differs in structure and serves different purposes. In addition, it is formed by the settling larva (tantulus) in tantulocaridans, but at a later instar (the kentrogon) in rhizocephalans. The mechanism of invading the host is wholly different and the root systems are formed by entirely different life cycle stages. Even if ultrastructural similarities should exist between the rootlets of tantulocaridans and rhizocephalans (Bresciani and Høeg 2001), they could probably be explained as the result of convergent evolution to parasitism.

**Conclusions**

Our molecular analysis from 18S rDNA sequence data points to a close relation between the Tantulocarida and the Thoecoscarida. The few available morphological characters are at least consistent with a sister group relation between these two taxa. Our analysis also suggests, but with much less support, a sister group relation between the Tantulocarida and the Cirripedia and thus that thecoscarids are paraphyletic in their present definition (Grygier 1987). There is no convincing morphological support for such a relation, and a common origin of tantulocaridan and rhizocephalian parasitism seems particularly unlikely on present evidence. We therefore conclude that the Tantulocarida and Thoecoscarida are very closely related, but that the precise phylogenetic position of the former must await future analyses.

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