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A molecular phylogenetic framework for the evolution of parasitic strategies in cymothoid isopods (Crustacea)

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Abstract

The parasitic isopods belonging to the family Cymothoidae attach under the scales, in the gills or on the tongue of their fish hosts, exhibiting distinctive life-histories and morphological modifications. According to conventional views, the three parasitic types (scale-, gill-, and mouth-dwellers) correspond to three distinct lineages. In this study, we have used fragments of two mitochondrial genes (large ribosomal DNA subunit, 16S rRNA, and cytochrome oxidase I) and two species for each of the three parasitic habits to present a preliminary hypothesis on the evolutionary history of the family. Our molecular data support the monophyly of the family but suggest that – contrary to what was previously believed – the more specialized mouth- and gill-inhabiting species are not necessarily derived from scale-dwelling ones.

Key words: Ecological adaptation – host–parasite interaction – mitochondrial DNA – phylogeny – Cymothoidae – *Ceratothoa* – *Anilocra* – *Nerocila*

Introduction

Cymothoid isopods represent one of the most derived lineages of isopods (Brusca and Wilson 1991; Dreyer and Wägele 2001; Brandt and Poore 2003), and currently include 42 genera and over 325 described species (N. Bruce, personal communication), most parasitizing teleost fish, particularly in warm temperate and tropical seas. Some species attach externally, under the scales or at the base of fins, while other more specialized forms inhabit the gill chamber or the buccal cavity, exhibiting very distinctive eco-morphological adaptations (i.e. modifications of the appendages) and peculiar life-history traits (e.g. protandrous hermaphroditism). Brusca (1981) initially proposed that the externally (scale/fin) attaching forms would represent a distinct lineage from the internal (gill/mouth) forms, whose specialized adaptations reflect a more derived status. A decade later, a thorough revision by Bruce (1990) identified three subfamilies, putatively corresponding to three different evolutionary lineages: the basal Anilocrinae (external scale parasites, sometimes burrowing underneath the skin of the host), and the more derived Livonecinae (gill-dwellers), and Cymothoinae (mouth-dwellers, sometimes also known as 'tongue-biters'). However, careful field observations within the Anilocrinae have shown that some degree of behavioural flexibility can be observed even within a genus (Bruce 1987). In general, the distinctive phenotypic adaptations of cymothoids, strongly constrained by the type of parasitic strategy, may hinder the choice of reliable morphological characters suitable for phylogenetic reconstruction (Horton 2000). As a result, there still remains a high degree of uncertainty as to which evolutionary scenario can best explain the current diversity observed in this family, and a molecular perspective is needed.

Despite the power and effectiveness of molecular markers in phylogenetic analysis, their use is seldom strictly confined to the recovery of a pattern of phylogenetic relationships among a set of studied taxa (Avise 2004). Rather, phylogenetic trees may serve as crucial frameworks to test hypotheses of monophyly/paraphyly of ecologically divergent lineages, and to discover how often a given adaptation has arisen during the evolutionary history of a taxon.

In recent years, a number of studies have successfully focused on the positioning of phenotypically measurable adaptive traits on the main branches of the phylogenetic tree of several groups of organisms. These results have proven momentous in the reconstruction of the evolutionary history of several high-profile taxa (e.g. Milinkovitch et al. 1994; Springer et al. 2001), and allowed for major reinterpretations of the adaptive transformations that led to the present-day ecological diversity (Cunningham et al. 1992; Milinkovitch 1995; Teeling et al. 2002; Danforth et al. 2003).

Here, we have employed fragments of two mitochondrial DNA genes to test for (1) the reliability of the conventional view that the three parasitic types (scale-, gill- and mouth-dwellers) actually correspond to three distinct evolutionary lineages (subfamilies), and (2) whether present-day scale-dwelling species can be seen as ancestral to the more specialized gill- and mouth-inhabiting forms.

Materials and Methods

Sampling

Table 1 reports the cymothoid species sampled for this study as well as information on their life-history and host species. Our taxon sampling includes two representatives for each of the three parasitic habits found in cymothoids (see Table 1 and Introduction for details). For *Anilocra physodes* Linnaeus 1758 we sampled two populations.

DNA sequencing

Genomic DNA was extracted from the single legs of ethanol-preserved specimens. We either used the DNeasy Tissue Kit from Qiagen (Valencia, CA, USA) following the manufacturers protocol or the standard Chelex extraction. We designed a cymothoid-specific primer pair (16S-cym-for: 5′-AGCCCTGTTCAATGGGATTA-3′; 16S-cym-rev: 5′-TCCCTGGGGTAGTTTCATCTT-3′) to amplify a 493-bp (base pair) fragment of the large ribosomal DNA subunit, 16S rRNA

Table 1. Cymothoid species sampled in the present study and sample size (*n*). Parasitic habits are identified as follows: S (species attaching on scales), M (species dwelling in the mouth) and G (species attaching in the gill chamber)

Species[1]	*n*	Parasitic habit	Reference	Host in this case	GenBank Accession number	
					16S	COI
Anilocra physodes Linnaeus, 1758	6	S	this study	*Symphodus tinca* (Linnaeus, 1758), Labridae	EF455808–9	EF455817–18
Nerocila bivittata Risso, 1816	1	S	this study	*Sarpa salpa* (Linnaeus, 1758), Sparidae	EF455810	EF455819
Ceratothoa collaris Schiœdte and Meinert, 1883	1	M	this study	*Lithognathus mormyrus* (Linnaeus, 1758), Sparidae	EF455807	EF455816
Ceratothoa italica Schiœdte and Meinert, 1883	3	M	this study	*Lithognathus mormyrus* (Linnaeus, 1758), Sparidae	EF455804–6	EF455813–15
Olencira praegustator Latrobe, 1802	–	G	Wetzer (2002)	–	AF259547	AF260844
Elthusa vulgaris Stimpson, 1857	–	G	Wetzer (2002)	–	AF259546	AF255790
Cirolana rugicauda Heller, 1861	–	–	Wetzer (2002)	–	AF259544	AF255788
Crenoicus buntiae Wilson & Ho, 1996	–	–	Wetzer (2002)	–	AF259532	AF255776
Pentidotea resecata Stimpson, 1857	–	–	Wetzer (2002)	–	AF259538	AF255782

[1]For *A. physodes* two different populations were analysed. These were from Anzio (AZ; central western Italy, Tyrrhenian Sea; three specimens) and Foce Varano (FV; south eastern Italy, Adriatic Sea; three specimens). *N. bivittata* was also collected in Anzio, and *C. italica* (haplotypes coded SB1-3 in Fig. 1) and *C. collaris* were collected in Sabaudia, central Tyrrhenian Sea.

gene (16S). PCR amplifications of a 455-bp fragment of the cytochrome oxidase I (COI) gene were carried out using the primers reported in Folmer et al. (1994). Double-stranded PCR conditions for both genes were 2 min at 94°C, followed by 35 cycles of 1 min at 95°C, 30 s at 50°C and 1 min at 72°C with a final elongation of 2 min at 72°C. PCR fragments were purified with a PCR purification kit (Qiagen) or using exonuclease and shrimp alkaline phosphotase. Sequences were determined with automated sequencers: ABI 3100 using an ABI BigDye PRISM kit (Applied Biosystems, Foster City, CA, USA), and a Beckman CEQ8000, (High Wycombe, UK) following the manufacturer's instructions. To promote accuracy, strands were sequenced in both directions for each individual. Sequences were edited using Sequencher 4.5 (Gene Code Corporation©, Ann Arbor, MI, USA). COI sequences were easily aligned by eye, following the reading frame; 16S sequences were aligned in CLUSTAL X (Thompson et al. 1997) with the following gap penalties: gap opening = 10; gap extension = 0.10 (parameters selected after multiple runs at increasing stringency). Sequences have been submitted to GenBank; accession numbers are reported in Table 1.

Phylogenetic analyses

We retrieved from GenBank orthologue 16S and COI sequences of *Cirolana rugicauda* Heller, 1861, *Crenoicus buntiae* Wilson & Ho, 1996, and *Pentidotea resecata* Stimpson, 1857 (Accession numbers reported in Table 1). These were aligned to sequences obtained for this study and used as outgroups in all phylogenetic searches. Outgroup species were selected at increasing levels of taxonomic separation. *C. rugicauda* belongs to the Cirolanidae, a family placed in the same suborder as cymothoids (Cymothoida) (Brusca and Wilson 1991; Dreyer and Wägele 2001; Wetzer 2002; Brandt and Poore 2003). *C. buntiae* and *P. resecata* are more distantly related to cymothoids, belonging to different suborders (suborder Phreatoicidea, family Phreatoicidae, and suborder Valvifera, family Idoteidae, respectively).

To detect saturation, the absolute numbers of transitions (Ti) and transversions (Tv) were plotted against the uncorrected *p* genetic distances. For the COI data set, these plots were made for all positions and for third codon positions separately. The combined (16S + COI) data set were analysed by maximum parsimony (MP; heuristic searches, ACCTRAN character-state optimization, 100 random stepwise additions, TBR branch-swapping algorithm) (Farris 1970), maximum likelihood (ML; heuristic searches, 100 random stepwise additions, TBR branch swapping algorithm) (Felsenstein 1981), Neighbour-Joining (NJ) (Saitou and Nei 1987) and Bayesian methods (Rannala and Yang 1996; Mau and Newton 1997; Larget and Simon 1999; Mau et al. 1999; Huelsenbeck 2000). MP, ML and NJ analyses were performed using PAUP* 4.0β10 (Swofford 2003); Bayesian analysis was carried out using MRBAYES (Huelsenbeck 2000). MP searches were run, giving equal weight to all substitutions or down weighting Ti three times Tv (Tv3 × Ti). We ran the ML analyses on PAUP* 4.0β10 after having determined the best model of DNA substitutions (GTR + Γ model; variable rates, shape parameter $\alpha = 0.546$) that fit our data using MODELTEST (Posada and Crandall 1998). NJ analyses were carried out on ML distances (D_{ML}) calculated with the same parameters used for ML analyses. For the Bayesian approach, we employed the same model of sequence evolution as in the ML searches, allowing site-specific rate variation partitioned by gene and, for COI, by codon positions. MRBAYES was run for two-million generations (one cold and three heated Markov chains) with a sampling frequency of 100 generations. To determine the appropriate 'burn-in', we plotted the likelihood scores of sampled trees against generation-time. 'Burn-in' corresponded to the first 10% of the sampled trees; posterior probability values for each node were calculated based on the remaining 90% of the sampled trees. The robustness of the phylogenetic hypotheses was tested by bootstrap replicates (1000 replicates for MP and NJ and 100 replicates for ML) (Felsenstein 1985).

Alternative phylogenetic hypotheses were tested using the approximately unbiased tree selection test (AU, Shimodaira 2002) in the software package CONSEL (Shimodaira and Hasegawa 2001). We always compared tree topologies simultaneously (Shimodaira and Hasegawa 1999). For comparison, we also performed the Shimodaira and Hasegawa (SH) test (1999), as implemented in PAUP* 4.0β10, with the resampling estimated log-likelihood technique.

Results

We sequenced 494 bp from 16S rRNA gene and 455 bp from COI gene, totalling 949 bp for each individual included in the study. Multiple individuals of the same population of *A. physodes* had identical haplotypes, while in the case of *Ceratothoa italica* we found three haplotypes differing by a few substitutions at both genes (7 for 16S and 1–4 for COI). We found few indels in the alignment of the COI gene; these indels were limited to comparisons between outgroups and the ingroup. We observed no stop codons in the COI sequences. As expected, the number of indels was higher in the 16S alignment. We coded indels as gaps, and treated them as either missing data or fifth base. The removal or inclusion of gaps in the MP analyses did not result in statistically significant

Table 2. Summary of topological tests. AU and SH indicate the results of the approximately unbiased (Shimodaira 2002) and the Shimodaira and Hasegawa (1999) tests. S, G and M, respectively, identify the species attaching to scales, gills and mouth. Relationship between letters and species is given in Table 1. A tree topology is significantly different from the best tree at $p \leq 0.05$

Topology	AU	SH
$MP^1_{unweighted}$	0.502	0.887
$MP^2_{unweighted}$	0.579	0.887
$MP_{Tv3\times Ti}$	0.347	0.856
NJ	0.347	0.856
ML	(best)	(best)
Bayesian	0.529	0.927
Ceratothoa collaris sister to *Nerocila bivittata*	0.347	0.856
Ceratothoa collaris sister to *Anilocra physodes*	0.121	0.709
Ceratothoa collaris sister to *Olencira praegustator*	< 0.001*	0.000*
Ceratothoa collaris sister to *Elthusa vulgaris*	< 0.001*	0.001*
Ceratothoa italica sister to *Nerocila bivittata*	0.439	0.798
Ceratothoa italica sister to *Anilocra physodes*	0.331	0.847
Ceratothoa italica sister to *Olencira praegustator*	< 0.001*	0.000*
Ceratothoa italica sister to *Elthusa vulgaris*	0.002*	0.001*
M + G monophyletic	< 0.001*	0.038*
S basal	< 0.001*	0.038*

MP, maximum parsimony; NJ, neighbour-joining; ML, maximum likelihood.
[1]Gaps treated as missing data.
[2]Gaps treated as 5th base.
*$p \leq 0.05$.

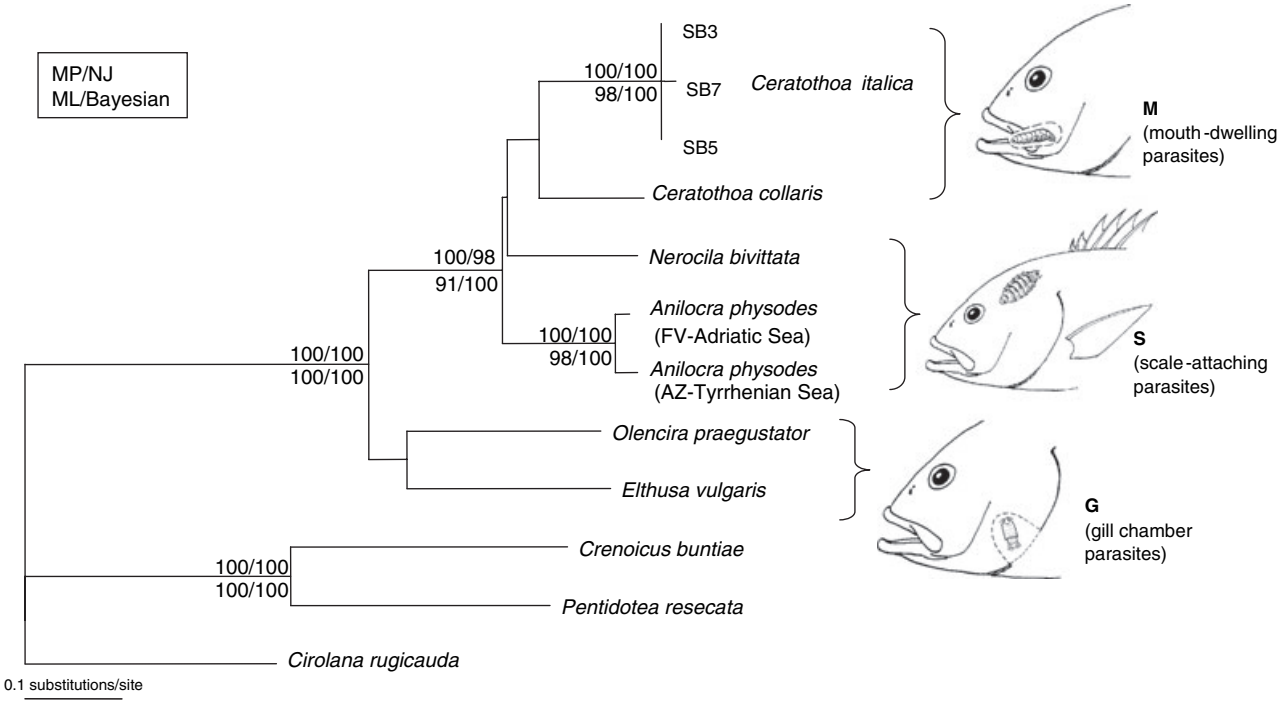

Fig. 1. Maximum likelihood (ML) estimate of the phylogenetic relationships among Cymothoid taxa obtained based on mitochondrial 16S and cytochrome oxidase I sequences. Numbers at nodes are statistical support for unweighted maximum parsimony (MP), neighbour-joining (NJ), ML and Bayesian analysis (posterior probabilities are as percentages). Only statistical supports of ≥50% are shown. Location codes are as in Table 1. A schematic representation of the three parasitic types (S, scale-; G, gill- and M, mouth-dwellers) is given next to the tree branches

changes in tree topologies (see Table 2 and below). Patterns of sequence variation reflected the typical features of a mitochondrial genome. We observed an excess of As and Ts (A + T = 0.631; 0.675; 0.695 for 16S + COI, 16S and COI, respectively) and a bias against Gs (G = 0.194, 0.202 and 0.187 for the same partitions). The G frequency was the lowest in COI 3rd codon positions (G = 0.149). We found similar levels of sequence variation when we analysed the two genes together (59.6% of variable sites of which 37.93% were parsimony informative) or separately (variable sites 62.3% for 16S, 56.7% for COI; parsimony informative sites for the two genes were 32.18% and 44.17%, respectively); COI 3rd codon positions was by far the most variable partition (70.4% of variable sites of which 57.23% were parsimony informative). A visual inspection of saturation plots (not shown) suggests that saturation is not a problem in the combined data set within the Cymothoidae. Ti show slight signs of saturation in outgroups versus ingroup comparisons for 16S, while they are almost completely saturated in COI 3rd codon positions.

Fig. 1 shows the ML tree obtained on the combined data set using the GTR + Γ model of sequence evolution, and

summarizes the results of the other phylogenetic searches run. The ML tree is statistically indistinguishable from the NJ, Bayesian and multiple MP searches (the latter were run with various weighting schemes and treatment of gaps).

Mitochondrial DNA suggests monophyly of cymothoids, although no definitive conclusions can be drawn, due to our limited taxon sampling. A cluster grouping *Elthusa vulgaris* Stimpson 1857 and *Olencira praegustator* Latrobe 1802 branches off first in the cymothoid clade, but the sister taxon relationship between the two species is not supported. On the other hand, our data yielded a remarkable support for the placement of the scale-dwelling *A. physodes* and *Nerocila bivittata* Risso 1816 within the same branch as the mouth-dwelling *Ceratothoa*, and, quite surprisingly, we could not consistently recover a monophyletic *Ceratothoa* clade.

Given the obvious phylogenetic and taxonomic implications in the latter result, we tested a variety of competing hypotheses against our tree. In particular, we alternatively forced *C. italica* or *Ceratothoa collaris* Schiœdte and Meinert 1883 to be sister taxa to the other species included in the study. Tree topologies having either *C. italica* or *C. collaris* as sister species to *N. bivittata* and *A. physodes* were not statistically different from the unconstrained tree, whilst the hypotheses of the two *Ceratothoa* species being sister taxa to *O. praegustator* or *E. vulgaris* are statistically unlikely (Table 2). The results of the topological tests and the fact that the amount of genetic divergence we found between *C. italica* and *C. collaris* is remarkably high ($D_{ML} = 0.251 \pm 0.007$) and almost identical to the values we obtained for most of the intergeneric comparisons (*Nerocila* versus *Ceratothoa* $D_{ML} = 0.245 \pm 0.006$; *Anilocra* versus *Nerocila* $D_{ML} = 0.251 \pm 0.003$; *Anilocra* versus *Ceratothoa* $D_{ML} = 0.272 \pm 0.009$) raise the possibility of the genus requiring further taxonomic revision. It is worth noting here that the genus *Ceratothoa*, like most mouth-dwelling cymothoid genera, is a taxonomically difficult genus. Due to the nature of their habit, many morphological characters commonly used in isopod taxonomy (e.g. setation and external ridges etc.) have been lost or greatly reduced, and other important characters are highly variable or polymorphic. The body of *C. collaris* is morphologically rather more heavily-built than other members of the *Ceratothoa* (T. Horton, personal observation) and the species is quite variable having been divided into three 'forms' by Monod (1924a,b). We believe that the use of molecular data can greatly improve taxonomic revisions of the Cymothoidae, and we expect the present work to be a valuable first step towards this task.

Discussion

The preliminary nature of our study does not allow for a definitive and exhaustive description of the evolution of the ecological diversity in this family. We only had a few genera at our disposal, out of the 42 that have been hitherto described, which makes it impossible to fully address aspects of monophyly/paraphyly of each of the three major parasitic types (scale-, gill- and mouth-dwellers). However, it is worth stressing that it is particularly difficult to obtain specimens of this family either through fieldwork or sample-sharing; hence, we believe that this first attempt to illuminate cymothoid relationships using molecular markers certainly provides new insights into the evolutionary history of this group, and is likely to serve as a valuable platform for future investigations. Previous studies have identified three major lineages within the family Cymothoidae: the Anilocrinae, Livonecinae and Cymothoinae (Brusca 1981; Brusca and Wilson 1991). The separation of these lineages has until now been based largely on the character 'point of attachment of the parasite on the host' (i.e. external, gill cavity and buccal cavity). According to Brusca's hypothesis (1981), the externally attaching group should include the most primitive forms ancestral to the more specialized mouth-/gill- dwellers, while Bruce (1990) later suggested that cymothoid taxonomic arrangements – most of which still being based on the position on the host – were likely to reflect convergence due to similar life-styles rather than true phylogenetic affinities.

Our phylogenetic analyses – though, as already said, based on a small number of species – provide strong support to Bruce's (1990) warnings. In particular, we cannot support the hypothesis of a 'linear' evolutionary pathway that starts with externally attaching forms (Anilocrinae) and ends up with gill-mouth dwellers (Livonecinae + Cymothoinae). Indeed, forcing *N. bivittata* and *A. physodes* (both scale-dwellers) to be basal in our phylogeny produced topology is significantly worse than the unconstrained one (Table 2). Similarly, a phylogeny with mouth- and gill-dwellers constrained in a single monophyletic clade is statistically less likely than the one depicted in Fig. 1, which robustly – and rather surprisingly – place the gill-dwelling genera basal to the scale- and mouth-dwelling ones. These results, taken altogether, suggest a complex history, underlying the diversification of parasitic strategies in this group of specialized isopods, and indicate that gill and buccal parasitic habits evolved independently, rather than by a direct phylogenetic link, as had been hypothesized by earlier researchers (e.g. Brusca 1981).

Based on these preliminary molecular data, we are inclined to reject the view that the three traditional parasitic 'types' correspond to three true evolutionary lineages. Neither can we confirm the monophyletic status of the two scale-dwelling genera examined (i.e. *Nerocila* is not necessarily phylogenetically closer to *Anilocra* than it is to *Ceratothoa*, Fig. 1). It is evident that a more comprehensive survey of a greater number and variety of species will be necessary in order to provide more insights and greater support to this scenario. In addition, focusing on the data of pathogenicity, host-specificity, transmission-potential and life-history in general (Bull 1994) may prove useful to better understand the adaptive constraints and responses which might have played a role in the diversification of these parasitic types.

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Zusammenfassung

Molekular-phylogenetische Analysen zur Evolution parasitischen Strategien von Fischasseln (Isopoda, Cymothoidae)

Die parasitischen Isopoden aus der Familie der Cymothoidae heften sich an die Schuppen, Kiemen oder an die Zunge ihrer Fischwirte; dabei zeigen sie unterschiedlichen Lebenszyklen und morphologische Besonderheiten. Bisherigen Untersuchungen zufolge gehören die drei

Parasitentypen (Schuppen-, Kiemen- und Mundparasiten) zu drei unterschiedlichen phylogenetischen Linien. In der vorliegenden Untersuchung haben wir Fragmente von zwei mitochondrialen Genen (die groβe ribosomale DNA - Untereinheit, 16s rRNA und Cytochrome Oxidase I, COI) von je zwei Vertretern der drei Parasitentypen untersucht, um eine vorläufige Hypothese über die evolutionären Beziehungen innerhalb der Familie aufzustellen. Unsere molekularbiologischen Ergebnisse unterstützen die Monophylie dieser Familie. Sie unterstützen jedoch nicht die bisherige Annahme, dass die stärker spezialisierten maul- und kiemenparasitierenden Arten von den schuppenparasitierenden Arten abstammen.

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