Phylogeny and adaptive radiation in the Onychopoda (Crustacea, Cladocera): evidence from multiple gene sequences

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Abstract

Members of the order Cladocera show remarkable morphological and ecological diversity. One of the most spectacular adaptive radiations in this group has involved species of the suborder Onychopoda, which have adopted a novel feeding strategy, predation, and have colonized habitats with a broad range of salinities. In order to evaluate the origins and systematics of this group, we derived a molecular phylogeny for its three component families including nine of 10 recognized genera based on three mitochondrial (mt) gene sequences: cytochrome *c* oxidase subunit I (COI), the ribosomal small and large subunits (12S and 16S) and one nuclear gene sequence: the small ribosomal subunit (18S). Maximum-parsimony, maximum-likelihood and neighbour-joining phylogenetic analyses were largely congruent, supporting the monophyly of the suborder and each of its families. Comparative analyses of data based on total evidence and the conditional combination of the ribosomal genes produced relatively congruent patterns of phylogenetic affinity. By contrast, analyses of single gene results were inconsistent in recovering the monophyletic groups identified by the multigene analyses. Based on the reconstructed phylogeny, we discriminate among the existing hypotheses regarding the evolutionary history of the onychopods. We identify a prolonged episode of speciation from the Miocene to the Pleistocene with two pulses of diversification. We discuss our results with reference to the geological history of the Ponto-Caspian basin, the region which fostered the onychopod radiation.

Introduction

Members of the order Cladocera show remarkable morphological diversity (Fryer, 1987) and species comprising the suborder Onychopoda form one of the most morphologically distinctive groups of cladocerans. Their morphological radiation is linked to their predatory mode of feeding and their success in colonizing habitats with a wide range of salinities. This group includes 10 genera and about 33 species (Mordukhai-Boltovskoi, 1968; Rivier, 1998) which share a grasping mode of feeding in contrast to the filter feeding strategy employed by other cladocerans. They also have a novel reproductive system – their embryos are protected by a closed brood pouch, which secretes the nutrients necessary for their development (Egloff *et al.*, 1997; Rivier, 1998). It has been suggested that these characteristics facilitated the transition of an ancestral freshwater cladoceran to the ocean, a 'hostile' habitat with scarce and intermittent food resources (Aladin & Potts, 1995; Rivier, 1998). One argument supporting this hypothesis is the fact that the only other marine cladoceran aside from the onychopods, the filter feeding *Penilia avirostris* (Sididae), has independently acquired a similar brood pouch.

Members of the Onychopoda have traditionally been assigned to three families: Cercopagidae, Podonidae, and Polyphemidae. The Polyphemidae is least diverse, including a single freshwater genus with two recognized species, one of which is restricted to the Caspian Sea. The family Cercopagidae is slightly more diverse as it includes

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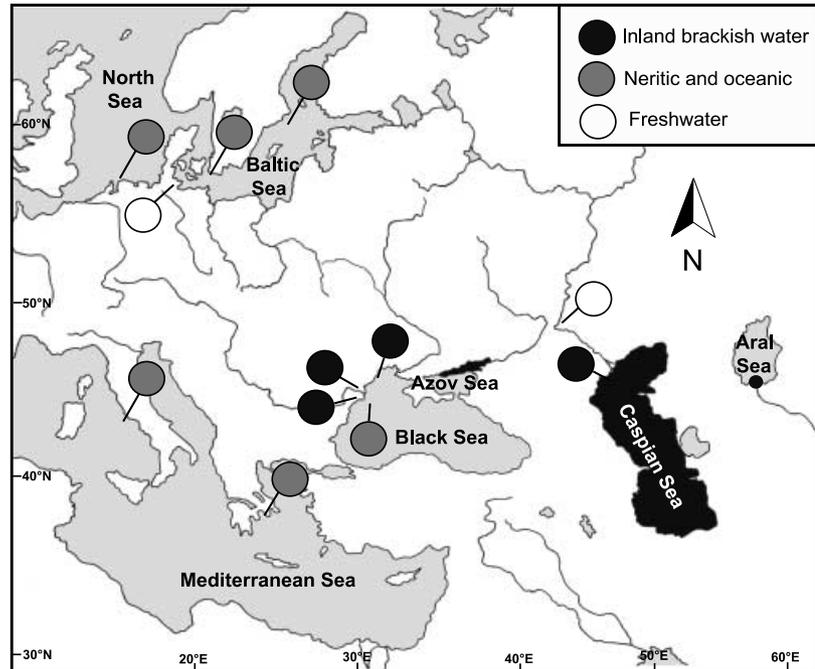Tel.: 519 824-4120; e-mail: mcristes@uoguelph.ca

Fig. 1 Collection sites for predatory cladocerans examined in this study and the natural distribution (black areas) of the endemic Ponto-Caspian onychopods.

two genera (*Cercopagis* and *Bythotrephes*) and about 14 described species (Rivier, 1998). All but one of these species were, until recently, restricted to the vicinity of the Black and Caspian Seas. The final family, the Podonidae, includes seven genera and 17 species. Three of these genera (*Caspievadne*, *Cornigerius* and *Podonevadne*) are restricted to the Ponto-Caspian basin, but the other four (*Evadne*, *Pleopis*, *Podon*, *Pseudevadne*) occur in the world's oceans (Onbe, 1999). The fact that most (72%) onychopod species are endemic to the Caspian, Azov and Aral Seas, or to the estuaries and lagoons of the Black Sea (Fig. 1) suggests that the Ponto-Caspian basin fostered much of the radiation within this group (Zenkevitch, 1963; Mordukhai-Boltovskoi, 1965; Dumont, 1998b). The onychopods are not the only group which diversified in this area. Similar radiations have occurred in many other Ponto-Caspian lineages, such as cumaceans, mysids, copepods, amphipods, decapods, mollusks and fishes (Mordukhai-Boltovskoi, 1979; Dumont, 1998a). However, the timing of these radiations and the extent of their diversification outside the Ponto-Caspian area remain uncertain. Unlike other ancient lakes, where adaptive radiations were restricted to benthic lineages, the Ponto-Caspian area fostered several episodes of radiation in planktonic lineages such as *Mysis* (Väinölä, 1995), the cyclopoid copepods (Monchenko, 1998) and the onychopods (Mordukhai-Boltovskoi, 1965). Most of these groups are euryhaline, which suggests that fluctuations in salinities, coupled with the fragmentation of waterbodies within the Ponto-Caspian basin, were

important in triggering their diversification (Zenkevitch, 1963; Dumont, 1998b).

Hypotheses of onychopod phylogeny

Most past efforts to understand the phylogenetic relationships of cladocerans have focused on higher taxonomic levels (Fryer, 1987), examining the monophyly of the order and its component suborders. Martin & Cash-Clark (1995) proposed the first morphologically based phylogeny for the onychopod families. They suggested that the family Polyphemidae was basal, with the Cercopagidae and Podonidae as sister groups derived from it (Fig. 2a). By contrast, Rivier (1998) provided evidence for the monophyly of the Polyphemide and Cercopagidae based on shared structures in their eye, head shield and valve morphology (Fig. 2b). The uncertainty in morphologically based phylogenies for this group derives from the fact that their reconstruction cannot be made on the basis of unique synapomorphies. In fact it seems likely that the assemblage of primitive features and shared derived characters has been altered by convergent adaptations linked to a predatory lifestyle and the occupancy of shared environments. Moreover, most taxa show phenotypic plasticity which affects diagnosable characters and makes taxon delimitation difficult. A possible solution to the uncertain phylogeny of this group lies in the application of molecular or combined molecular–morphological approaches.
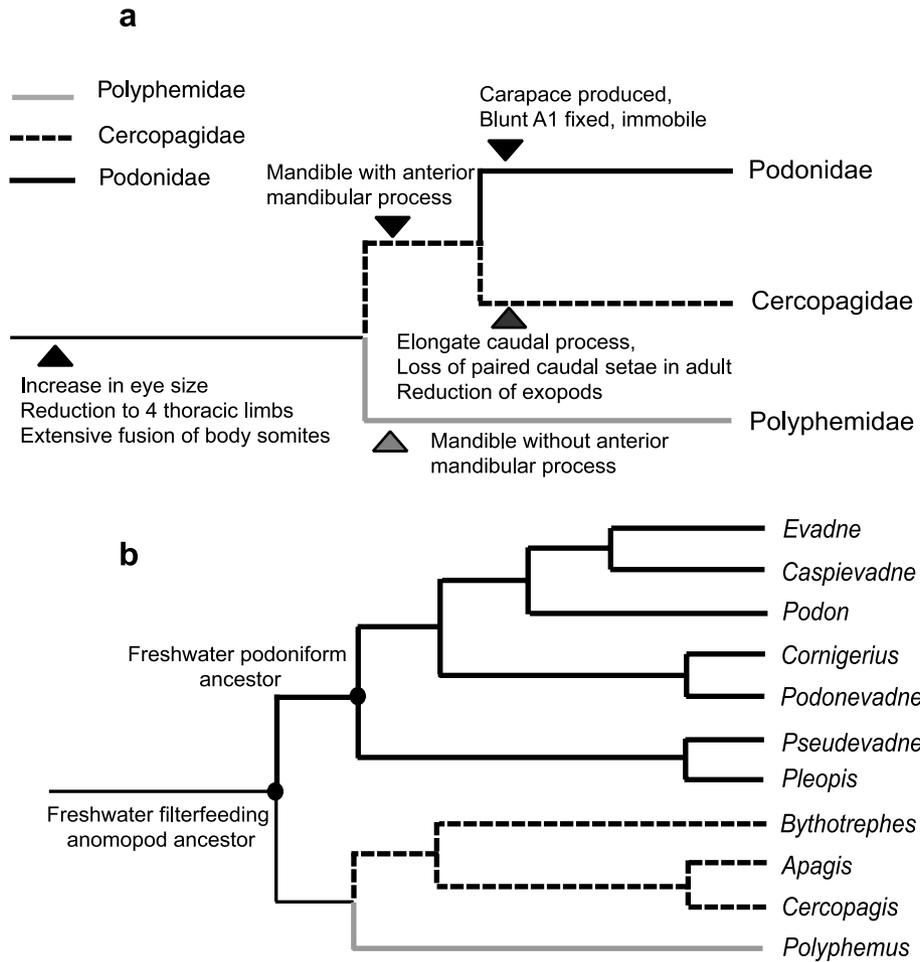
a

b

Fig. 2 Morphologically based hypotheses of onychopod phylogeny. (a) Onychopod families (Martin & Cash-Clark, 1995). (b) Cladogram inferred from the evolutionary tree proposed by Rivier (1998).

Ages and rates of diversification in the onychopods

Given the apparent importance of the Ponto-Caspian basin as the site of onychopod evolution, it is worth noting the major geological and climatic events which shaped this basin and influenced its ecology. During the late Miocene, some 8–10 million years (myr) ago, the Paratethys Sea, a remnant of the marine Tethys Sea, evolved into the brackish Sarmatian Lake, and later into the slightly brackish Pontic Lake (Zenkevitch, 1963; Jones & Simmons, 1997). By the end of the Pontic period (5–4 myr ago), uplifts fragmented this lake into the Black and Caspian Seas. Since then, these two basins have largely been separated, barring periods of ephemeral contact. One opportunity for faunal exchange between the basins occurred during the late Pliocene (2–3 myr ago) when the vast Akchagyl Lake had connections with both the Kuyalnits (Black Sea) and the Persian Gulf. During the Pleistocene, climatic and hydrologic changes produced dramatic transformations of the Black Sea basin. The global rise in sea level following each glaciation led to repeated incursions of saline Mediterranean water into the brackish Black Sea. This inflow increased the salinity of the Black Sea, creating a pycnocline in which a surface layer derived from river inflows was separated from the more saline, denser water of Mediterranean origin. This stable stratification of water masses created a transition from oxic to anoxic conditions at the sediment–water interface. Palaeontological studies on mollusks and pelagic microfossils provide evidence that much extinction of the local, brackish water biota occurred about 7000–3000 years ago as a result of the latest flood of saline Mediterranean waters (Wall & Dale, 1974). The survivors of this extinction event are currently confined to low salinity estuaries, lagoons and rivers along the margins of the Black and Azov Seas (Banarescu, 1991). By contrast, the Caspian basin maintained its endemic fauna throughout the Pleistocene. Despite drastic climatic fluctuations and ephemeral periods of contact with the Black Sea (Chepalyga, 1985),

the salinity of this basin has remained relatively steady over this interval maintaining its characteristic north – south gradient.

A major challenge in reconstructing the history of life lies in establishing the relative age of lineages lacking fossil records. Despite the absence of palaeontological data for most of the groups which diversified in the Ponto-Caspian, there has been a tendency to link local radiations to Miocene environments. The term 'Sarmatian relicts' has been used by many authors when referring to the Ponto-Caspian endemics, suggesting that most of them originated in the Sarmatian Lake some 8–10 myr ago (Motas, 1977). However, other authors suggest that most of the Ponto-Caspian taxa radiated 5–6 myr ago, during the Pontian (Mordukhai-Boltovskoi, 1979; Banarescu, 1991). The clearest evidence for such diversification relates to the species flock of limnocardiids, a molluscan family with an excellent fossil record which radiated during the Pontian (Banarescu, 1991). Interestingly, the Pontian Lake had ecological conditions similar to those of the modern Caspian Sea, whereas the Sarmatian Sea was more saline, resembling conditions in the Black Sea today. Focusing specifically on the onychopods, Rivier (1998) proposed that much of the speciation in this group (including the radiation of the marine podonids) occurred very recently, during the last 10 000–20 000 years of the Pleistocene. Dumont (2000) similarly suggested that the marine podonids radiated within the Ponto-Caspian area and were only released into the world's oceans during the Holocene, when the Black Sea established contact with the Mediterranean. By contrast, based on their analysis of 12S sequence diversity in onychopods, Richter *et al.* (2001) suggested that marine podonids first radiated in the world's oceans, entering the Ponto-Caspian basin, just 2–3 myr ago, during the Akchagylian transgression. Consequently, these authors place the major speciation events of the Ponto-Caspian podonids during the upper Pliocene and the radiation of the Cercopagidae and Polyphemidae during the Pleistocene.

Our work examines the tempo of morphological and genetic changes in the predatory cladocerans based on a molecular phylogenetic approach in an attempt to reconcile the competing hypotheses regarding their evolution. In contrast to the earlier molecular study on this group, which relied on the analysis of a single mitochondrial gene (Richter *et al.*, 2001), the present results are based on a study of both a nuclear gene (18S) and three mitochondrial genes (COI, 12S, 16S).

Materials and methods

Taxon sampling

Fourteen onychopod species, including representatives from nine of the 10 recognized genera, were examined (Table 1). The habitats sampled included marine waters, brackish estuaries, brackish inland seas, and freshwater ponds and lakes (Fig. 1). Samples were collected using a 100 or 200 μm mesh net and were subsequently sorted and preserved in 90% ethanol. Taxa were identified using Rivier's (1998) and Negrea's (1983) keys. As multiple outgroups are preferable for polarizing characters, we chose two anomopod cladocerans, *Daphnia pulex* and *Bosmina coregoni,* as outgroup species in our phylogenetic analyses. Our outgroup choice was based on previous morphological and recent molecular analysis of cladoceran phylogeny (Martin & Cash-Clark, 1995; Negrea *et al.*, 1999) which suggest that the anomopods are the sister group to the onychopods.

DNA extraction, amplification and sequencing

Phylogenies were constructed using three mitochondrial genes – cytochrome *c* oxidase subunit I (COI), 12S, 16S ribosomal DNA (rDNA), and one nuclear gene – 18S rDNA. The primer pairs LCOI490 and HCO2198 (Folmer *et al.*, 1994), 12CR3 and 12s-5c (Richter *et al.*, 2001), 16Sar and 16Sbr (Palumbi, 1996), were used to amplify a 658 base pair (bp) fragment of the COI gene, a 565-bp fragment of the 12S gene, and a 570-bp fragment of the 16S gene, respectively. For the nuclear 18S gene fragment, the primers 9F and 2004R (Crease & Colbourne, 1998) were used to amplify a 2000-bp fragment which was subsequently partially sequenced using the amplification primer 2004R. The sequenced 18S fragment was approximately 800 bp in length. Total DNA was extracted from three to five individuals from each habitat for 13 of the onychopod species using proteinase K methods. Amplification and sequencing were performed as described in Cristescu *et al.* (2001), with the only difference being a change in the temperature profiles for the 16S and 18S polymerase chain reaction (PCR) amplification. The PCR for 16S consisted of two cycles of 94 °C (30 s), 60 °C (45 s), 72 °C (45 s); five cycles of 93 °C (30 s), 55 °C (45 s), 72 °C (45 s); followed by 29 cycles of 93° C (30 s) 50° C (1 min) and 72° C (1 min). The temperature profiles for 18S consisted of 35 cycles of 93 °C (30 s), 50 °C (30 s) and 72 °C (3 min). We performed sequencing in both directions only when ambiguous sites were encountered. Two onychopod taxa were incompletely represented in the four partitions. For *Evadne spinifera* we obtained an aberrant COI sequence (possibly a pseudogene), whereas for *E. anonyx* we only used the 12S sequence from GenBank (Richter *et al.*, 2001). All sequences obtained during this study have been submitted to GenBank under accession numbers: AY075047–AY075093 (Table 1).

Phylogenetic reconstruction

Sequences for the four genes were initially aligned using Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA). Secondary sequence structure models for 12S, 16S

Table 1 Species included in the molecular analyses, their habitat preferences (F – freshwater; M – neritic or oceanic; B – inland brackish water), and GenBank accession numbers.

| Taxon* | Habitat | GenBank accession no.† | | | |
		COI	12S	16S	18S
Family Polyphemidae					
Polyphemus pediculus	F	AY075048	AY009495	AY075066	AY075080
Family Cercopagidae					
Cercopagis pengoi	F–B	AF320014	AY009494	AY075067	AY075081
Bythotrephes longimanus	F	AF435130	AY009493	AY075069	AY075082
Family Podonidae					
Cornigerius maeoticus	F–B	AY075047	AY075058	AY075068	AY075083
Evadne anonyx	B		AY009499		
E. nordmanni	M	AY075049	AY009498	AY075070	AY075084
E. spinifera	M		AY075059	AY075071	AY075085
Pleopis polyphemoides	M	AY075050	AY075060	AY075072	AY075086
Podon leuckarti	M	AY075051	AY009496	AY075073	AY075087
P. intermedius	M	AY075052	AY009497	AY075074	AY075088
Pseudevadne tergestina	M	AY075053	AY075061	AY075075	AY075089
Podonevadne trigona	F–B	AY075054	AY075062	AY075076	AY075090
P. angusta	B	AY075055	AY075063	AY075077	AY075091
P. camptonyx	B	AY075056	AY075064	AY075078	AY075092
Family Daphniidae‡					
Daphnia pulex	F	AF117817	AF117817	AF117817	AF117817
Family Bosminidae‡					
Bosmina coregoni	F	AY075057	AY075065	AY075079	AY075093

*Classification after Rivier (1998). †GenBank accession no. AY075047 – AY075093 represent taxa sequenced during this project. GenBank accession no. AF117817; AF435130; AF320013 – AF320014; AY009493 – AY009499; correspond to specimens used in Crease (1999), Therriault *et al.* (2002); Cristescu *et al.* (2001); Richter *et al.* (2001), respectively. ‡Taxa used as outgroup.

(Taylor *et al.*, 1998) and 18S (Crease & Colbourne, 1998) were used as a guide to align hypervariable regions of the ribosomal genes. Sites that contained gaps and the ambiguous sections of the alignment (almost 430 sites from the ribosomal genes) were excluded from subsequent analyses. Molecular phylogenies were inferred using three analytical approaches. Maximum-parsimony (MP), maximum-likelihood (ML) and neighbour-joining (NJ) (Saitou & Nei, 1987) methods were employed using PAUP* 4.0b.8 (Swofford, 2001). MP trees were estimated using the branch-and-bound search with all characters having equal weight and gaps treated as 'missing', whereas ML trees were constructed using the HKY85 model and the heuristic search option with sequences added at random and tree bisection-reconnection branch swapping. All estimates of sequence divergence were corrected using Tamura and Nei's method (Tamura & Nei, 1993). We assessed the stability of phylogenetic hypotheses with both bootstrap analyses (Felsenstein, 1985) (1000 replicates for both NJ and MP and 100 for ML) and decay indices (Bremer, 1994) calculated using the AutoDecay program (version 4.0.2; Eriksson, 1999, Bergius foundation, Stockholm). The homogeneity of base composition across taxa was assessed using the goodness-of-fit (χ^2) test implemented in PAUP* (Swofford, 2001). The appropriateness of performing a total evidence analysis, a conditional data combination or separate analyses of all data partitions

was assessed using the incongruence length difference (ILD) test (Farris *et al.*, 1995) performed with the partition homogeneity test in PAUP* with 1000 random bipartitions analysed by TBR branch swapping on 10 random sequence-addition replicates. To test for constancy of rates of molecular evolution, we used a log-likelihood ratio test, performed in PAUP* to statistically estimate the validity of the molecular clock hypothesis (Huelsenbeck & Crandall, 1997). The linearized NJ tree was employed to examine the relative timing of the onychopod evolution under the assumption of a molecular clock (Takezaki *et al.*, 1995). As two taxa, *E. spinifera* and *E. anonyx*, were coded as missing in several data partitions, we performed the ILD measures and the likelihood ratio tests after removing these taxa from the data set.

Molecular clock, ages and rates of diversification in Onychopoda

The snapping shrimp mitochondrial COI clock (Knowlton & Weigt, 1998) of 1.4% sequence divergence per million years , the porcelain crab mitochondrial 16S clock (Stillman & Reeb 2001) of 0.53% sequence divergence per million years and the arthropod mitochondrial clock (Brower, 1994) of about 2.3% sequence divergence were used for dating major evolutionary events within the onychopod group.

Results

Sequence diversity

The magnitude of interspecific sequence divergence as well as that between the four genes was highly variable. A maximum pairwise nucleotide divergence of 16% for all genes combined was observed between the onychopod families. Within the Cercopagidae and the Podonidae, the mean level of genetic divergence among species was 14 and 8%, respectively. The least genetically diverged group was the Ponto-Caspian podonids which showed less than 1% sequence divergence. As expected, less divergence was found for the rDNA genes than for the faster evolving protein coding gene COI. Consequently, the number of parsimony informative sites varied among the genes from 46 informative sites in the 18S data set to 230 informative sites in the COI data set (Table 2). A total of 2162 characters, including 498 cladistically informative characters, were available after combining the data sets for 12S, 16S, 18S rDNA and COI. Each of the four genes displayed unequal base frequencies with 18S, the slowest evolving of the four genes, displaying the least bias. All mitochondrial genes (COI, 12S and 16S) had a higher A-T content (59.1, 62.5, 60.5%) than the nuclear 18S rDNA gene (48.7%). The estimated Transition / Transversion (Ti / Tv) ratio among all taxa for the complete data set was 1.95. Among the individual data partitions, the COI data set had the highest Ti / Tv (5.42). Base composition for each gene was homogenous across the 14 taxa ($0.99 < P < 1.00$) (Table 2). Partition homogeneity tests indicated the presence of conflicting phylogenetic signal when data for all four genes were included in the comparison ($P = 0.04$). Although there is not a generally accepted *P*-value for a significant result, most authors argue for combining data when *P*-values are greater than 0.05. No significant heterogeneity among genes remained after the removal of COI from the data set ($P = 0.41$). This result suggests that only the COI gene provides a different phylogenetic signal, probably because of its saturation at the third codon position. There is no

generally accepted protocol to handle data showing incongruence (Huelsenbeck *et al.*, 1996). In the present study we employed two common strategies: a total evidence approach and a conditional combination of all ribosomal genes (12S, 16S and 18S) vs. the protein coding gene, COI, followed by an independent analysis of each gene. Likelihood ratio tests for the molecular clock hypothesis suggested that the COI and 16S data partitions were consistent with a constant rate of evolution among the taxa included in the study. A molecular clock hypothesis was, however, rejected for the 12S and 18S data partitions (Table 3).

Molecular phylogenetic analyses

Maximum-parsimony analysis of total evidence using the branch-and-bound algorithm found a single most parsimonious tree of 1748 steps long (CI = 0.53, RI = 0.53) (Fig. 3a). This tree had a similar topology with the strict consensus of the two shortest MP trees (tree length 863, CI = 0.7, RI = 0.28) obtained for the conditional data combination of the three rDNA genes (Fig. 3b). Maximum-likelihood analysis of the total evidence, and the rDNA gene data produced trees with similar topologies to both the MP trees and NJ trees (Figs 4 and 5). The most likely parsimony trees of the total evidence (Fig. 4a) and the conditional combination (Fig. 4b) had log-likelihood scores of −10618.3 and −6259.2, respectively. All analyses of the total evidence and conditional combination unambiguously supported the monophyly of the taxa belonging to the family Cercopagidae and to the family Podonidae. *Polyphemus pediculus* occurred as a monophyletic entity.

Ages of Onychopods

Application of calibrations for the mitochondrial COI and 16S genes (Knowlton & Weigt, 1998; Stillman & Reeb, 2001), and the arthropod mitochondrial clock (Brower, 1994) to the linearized NJ trees of the corresponding gene fragments suggested that diversification of the onychopods has occurred over the past 10–20 myr. The diversification of the cercopagid genera and of the marine

Table 2 The total number of sites available (TS), the number of variable sites (VS), the number of cladistically informative sites (IS), base frequencies, transition / transversion ratios (Ti / Tv) and χ^2 test of homogeneity of base frequencies across ingroup taxa for each data partition.

| Data set | TS | VS | IS | Base composition (%) | | | | Ti / Tv | χ^2 |
				A	C	G	T		
CO1	630	271	230	25.65	20.28	20.59	33.48	5.42	16.93; $P = 0.99$
12S	390	184	121	33.18	20.08	17.46	29.27	1.75	9.32; $P = 0.99$
16S	446	146	101	29.99	16.21	23.25	30.55	1.80	4.75; $P = 1.00$
18S	696	116	46	24.67	24.16	27.13	24.04	1.29	1.45; $P = 1.00$
12S, 16S, 18S	1532	446	268	26.04	21.30	23.72	28.93	1.49	9.87; $P = 0.99$
Total evidence	2162	717	498	27.64	20.61	22.63	29.13	1.95	10.48; $P = 0.99$

Table 3 Likelihood ratio test for the molecular clock hypothesis $2\Delta = \log L_{\text{no clock}} - \log L_{\text{clock}} (\chi^2_{(14,0.05)} = 23.68; \chi^2_{(13,0.05)} = 22.36; \chi^2_{(12,0.05)} = 21.03)$.

			Likelihood ratio test		
Data sets	$-\log L_{\text{no clock}}$	$-\log L_{\text{clock}}$	2Δ	d.f. $= n-2$	Null hypothesis
CO1	4112.8	4121.8	18.0	12	Not rejected
12S	2208.2	2230.4	44.5	14	Rejected
16S	1982.8	1987.1	8.6	13	Not rejected
18S	1878.4	1892.6	28.4	13	Rejected
Total evidence	10618.2	10633.5	30.6	12	Rejected

Maximum-likelihood trees were reconstructed using the HKY85 model.

Fig. 3 (a) The most parsimonious tree obtained using the maximum parsimony (MP) criterion of the total evidence (COI, 12S, 16S and 18S) and (b) Strict consensus of two shortest trees obtained using the same (MP) criterion on sequence divergence from the rDNA genes (12S, 16S and 18S). The numbers above the branches indicate bootstrap support greater than 50% (1000 replications). The numbers below the branches show the decay indices. MP trees were estimated using the branch-and-bound search with all characters having equal weight and gaps treated as 'missing'.

podonids occurred during Middle or Late Miocene. However, the radiation of the Ponto-Caspian podonids took place much more recently, during the Late Pleistocene (Fig. 6).

Discussion

Phylogenetic implications

The present study has confirmed the monophyly of the three onychopod families (Polyphemidae, Cercopagidae and Podonidae) by all tree reconstruction methods and all data sets except COI (which did not support the monophyly of the Cercopagidae). However, the homoplasious phylogenetic signals of the COI gene were not apparent in the total evidence trees. In fact, the support for the monophyly of the cercopagids in the total evidence trees was robust (100% bootstrap support for all algorithms). The results further indicate that the

families Cercopagidae and Podonidae are monophyletic, forming a sister group to the Polyphemidae. This result supports Martin & Cash-Clark's (1995) phylogeny based on morphology and Richter et al.'s (2001) molecular hypothesis based on the ribosomal 12S gene. This relationship among the families was recovered by ML and MP approaches in both the total evidence data set and the conditional data combination. NJ produced the same result when the total evidence was employed, but failed to recover it when only the rDNA genes were analysed. Within the podonids, we recovered two well supported clusters: one group includes the two marine genera *Podon* and *Pleopis,* whereas the other included the Ponto-Caspian podonids together with the marine genera *Evadne* and *Pseudevadne.* Within the latter, heterogeneous group, we found strong support (100% bootstrap and nine Bremer support) for the monophyly of the Ponto-Caspian podonids belonging to the genera *Podonevadne* and *Cornigerius.* In fact, the data reveal a
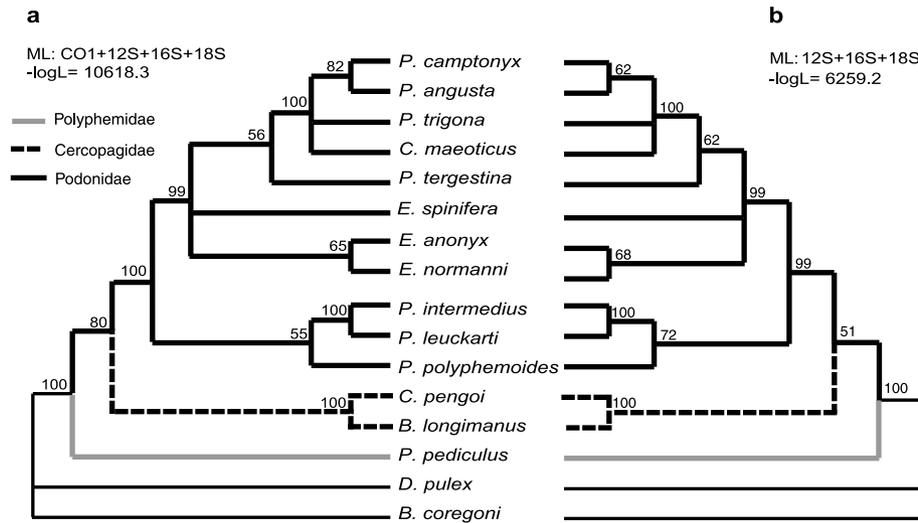
Fig. 4 Maximum-likelihood (ML) trees for (a) the total evidence (COI, 12S, 16S and 18S) and (b) the conditional data combination of rDNA genes (12S, 16S and 18S). The numbers above the branches indicate bootstrap support greater than 50% (100 replications). ML trees were constructed using the HKY85 model and the heuristic search option (with sequences added at random and tree bisection-reconnection branch swapping).
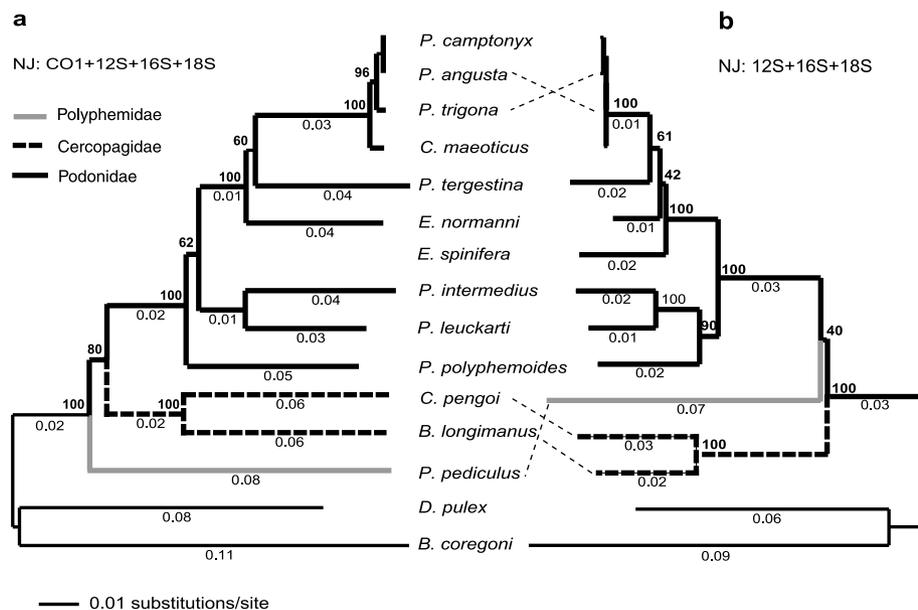

Fig. 5 Neighbour-Joining (NJ) trees (a) of the total evidence (COI, 12S, 16S and 18S genes) (b) of the rDNA genes (conditional data combination of 12S, 16S and 18S). The trees are rooted with the anomopod genera *Daphnia* and *Bosmina*. The number above the branches indicates the bootstrap support after 1000 replications, whereas the number below each branch shows the corrected distances based on Tamura-Nei corrected distance matrix.

remarkably close evolutionary relationship among the members of this flock, but their marked divergence from the genera *Evadne* and *Pseudevadne*. It was also apparent that the *Evadne – Pseudevadne* group is paraphyletic incorporating two Ponto-Caspian species flocks: the brackish – freshwater *Podonevadne–Cornigerius* group and the more salt tolerant Caspian *Evadne* group which was represented in our phylogeny by a single species, *E. anonyx*. Although, the polyphyly of the Ponto-Caspian podonids was evident, the relationship between the two
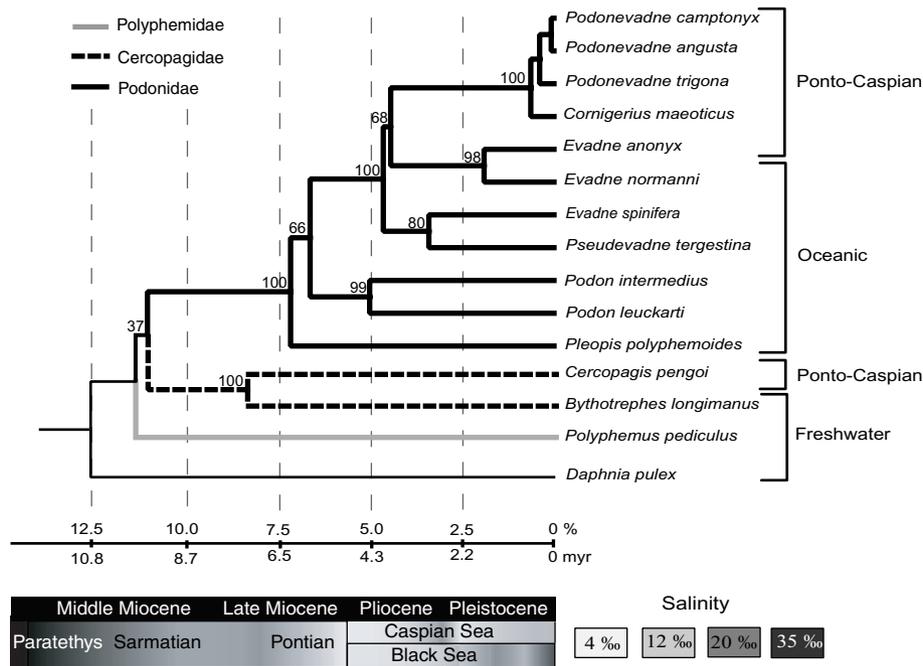
Fig. 6 Phylogenetic relationships among 13 onychopod species based on a NJ linearized tree of the mtDNA genes (COI, 12S, 16S). The numbers above the branches indicate the bootstrap support (1000 replicates). Genetic distances were corrected using the Tamura-Nei method. The arthropod mitochondrial clock (Brower, 1994) of 2.3% sequence divergence per million years was used to estimate divergence times. The grey scale represents the salinity gradient from 35‰ (black) to 2–4‰ (light grey) of the corresponding geological basins (after Jones & Simmons, 1997).

flocks with the marine *Evadne* was not clear. ML and NJ analyses suggest an affinity between *Pseudevadne tergestina* and *Podonevadne* – *Cornigerius* group, but the support for this group was weak. Although all methods of phylogenetic reconstruction employed suggested that *E. normanni* is the sister taxa of the Caspian *E. anonyx,* this conclusion was based only on the 12S gene and its support was weak.

We regard the total evidence hypotheses as the most robust as evidenced by the highest support for the major clades. The evidence from the conditional combination of the rDNA genes, which had a congruent pattern of phylogenetic affinity with the total evidence analysis, high bootstrap and Bremer support provides lower phylogenetic resolution within the shallow Ponto-Caspian groups. By contrast, analysis of the individual gene partitions yielded phylogenies which were inconsistent with the monophyletic groups and with the topology supported by the total evidence trees. Moreover, these conflicting taxonomic assemblages were only weakly supported. Our results strengthen arguments for the value of a total evidence approach (Hillis, 1987; Kluge, 1989; Remsen & DeSalle, 1998). It is apparent that combining data sets is a good option for intensifying phylogenetic signal, even when there is evidence of conflicts in the nature of this information.

Ages and rates of diversification in Onychopoda

Based upon the linearized NJ phylogeny for three mitochondrial genes and the arthropod mitochondrial clock (Fig. 6), we identified two relatively brief periods of onychopod diversification. The initial radiation of the marine podonids probably occurred during the late Miocene or early Pliocene. However, if the calibrations of Knowlton & Weigt (1998) and Stillman & Reeb (2001) are applied to the corrected nucleotide divergences of the COI and 16S fragments, respectively, this radiation is placed earlier in time, during the early and middle Miocene. Regardless of errors introduced by the calibration used or by the assumption of a constant rate of evolution among crustaceans, it is likely that the Paratethys and Sarmatian basins fostered this first diversification of the podonids. The two cercopagid genera, *Cercopagis* and *Bythotrephes,* last shared a common ancestor sometimes during upper or middle Miocene. The marine cladocerans most probably gained access to the oceans via the Mediterranean long before the Holocene, possibly during the late Miocene or the Pliocene when the Ponto-Caspian basin was connected with the world's oceans. This hypothesis is supported by the fact that intraspecific levels of genetic divergence in the COI gene of marine cladocerans are 100–250 times higher than the level expected if these taxa first

colonized the oceans in the Holocene (M.E.A. Cristescu and P.D.N. Hebert unpublished work). Simultaneous with the diversification of the *Evadne* and *Pseudevadne* lineages, the lineage leading to the endemic Ponto-Caspian genera *Cornigerius* and *Podonevadne* diverged. This ancestral lineage survived the less saline periods (Pontic or Balakhan basins of the late Miocene, early Pliocene, respectively) and made a gradual transition to environments with lower salinity. The second major radiation, the diversification of the Ponto-Caspian endemic species belonging to the genera *Cornigerius* and *Podonevadne,* took place much more recently, during the Pleistocene. Members of this group are genetically almost indistinguishable. Only the genera *Cornigerius* and *Podonevadne* have a long enough history of isolation (1–1.5 myr) to enable their reliable discrimination. It appears that the radiation of the *Podonevadne* flock: *P. trigona, P. angusta* and *P. camptonyx* occurred very recently, possibly during the Holocene. Morover, analyses of COI diversity within these species revealed shared haplotypes between sympatric sister species suggesting that introgression occurs between these closely related taxa (M.E.A. Cristescu *et al.,* unpublished

work). We do not have molecular evidence regarding relatedness within the other endemic flock, the *Evadne* group (*E. anonyx, E. angusta* and *Caspievadne maximovit-schi*), which is confined to the middle and south Caspian Sea. Despite the morphological peculiarity of the genus *Caspievadne,* its similar limb structure to Caspian *Evadne* suggests their close evolutionary relationship (Fig. 7). Future genetic work is necessary to further explore relationships between these taxa with a view towards clarifying the factors involved in radiation. Nevertheless, the convergent evolution of body shapes in the two Caspian flocks (e.g. *C. maximovitschi* and *C. maeoticus*; *E. anonyx* and *P. camptonyx*; *E. prolongata* and *P. angusta*) suggest that these two groups evolved almost simultaneously, shaped by the same intralacustrine, evolutionary forces (Fig. 7). As the radiation in each group did not involve significant habitat or food specialization, we propose that disruptive selection by predators was the driving force in shaping the body plane diversity of onychopods.

In summary, our phylogenetic data reveal an onycho-pod radiation extending from the Miocene to the late Pleistocene in a chain of events intimately linked with
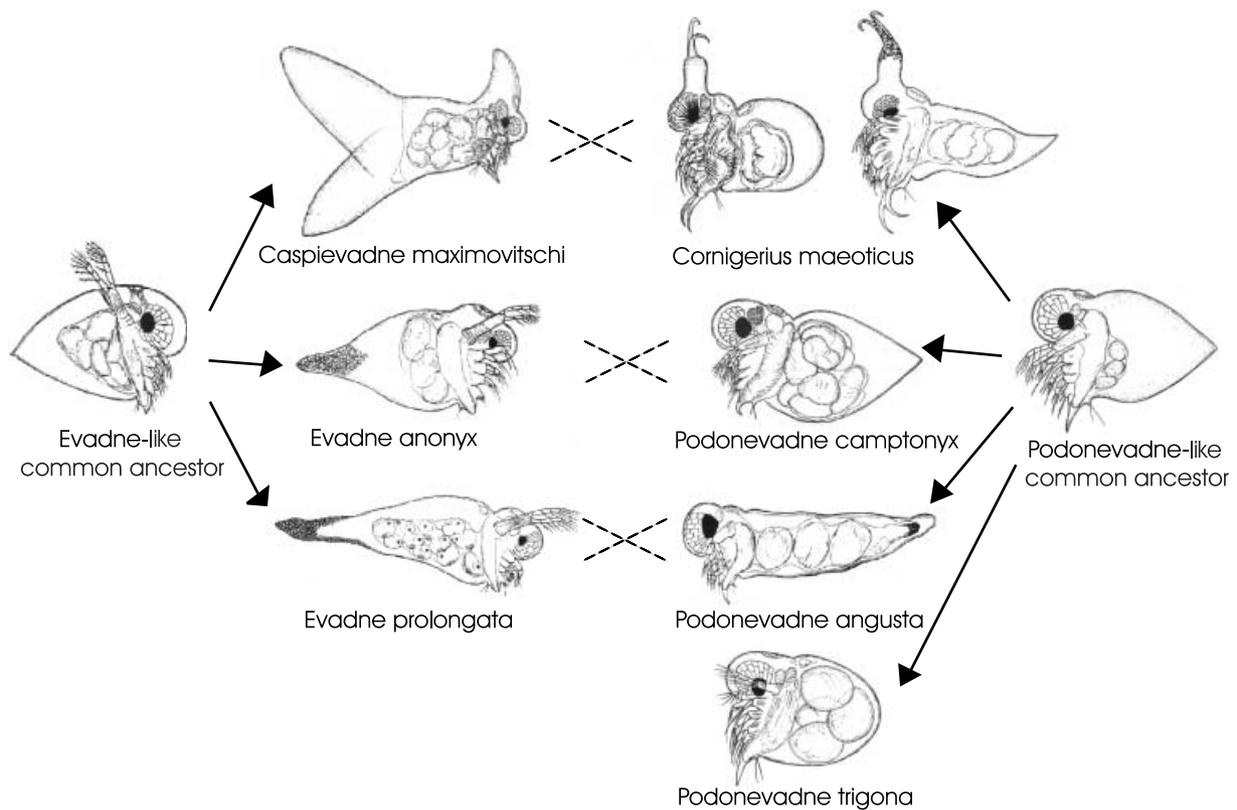

Fig. 7 Convergent evolution of body shape in two Ponto-Caspian podonid groups. The monophyly of the genera *Cornigerius* and *Pseudevadne* is supported by both limb morphology and genetic data, the monophyly of the genera *Caspievadne* and the Caspian *Evadne* is suggested only by their similar limb structure. Drawings after Rivier (1998).

the geological history of the Ponto-Caspian basin. This example resembles previously studied vertebrate radiations in which the Quaternary events were important in extending a radiation inaugurated earlier (Klicka & Zink, 1997; Avise, 2000). In this context, the identification of the evolutionary factors which directed the radiation in Ponto-Caspian lineages, and the reasons for these pulses of diversification appear more important than assigning a precise age to individual lineages.

Acknowledgments

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