

Phylogeography of Ponto-Caspian crustaceans: a benthic–planktonic comparison

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Abstract

The Black, Azov, Caspian and Aral Seas, remnants of the intracontinental Paratethys basin, are home to a spectacular diversity of crustaceans. This study examines the past history of the Ponto-Caspian fauna through comparative phylogeographical studies on both benthic and planktonic taxa, based on an examination of nucleotide diversity in the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene. The COI data reveal a striking example of phylogeographical concordance. All species analysed, three amphipods and three cladocerans, are characterized by two monophyletic clades corresponding to the Black and Caspian regions. However, this phylogeographical partition is, on average, four times deeper for the benthic amphipods than for the planktonic cladocerans. Based on standard molecular clocks, the Black and Caspian lineages of benthic crustaceans diverged at varied intervals from 1 to 8 million years ago. By contrast, planktonic lineages are more recent with their divergence occurring in the last million years. Levels of intraspecific polymorphisms are variable and generally lower in planktonic than benthic taxa. The mechanisms responsible for the high diversity of crustaceans in the Ponto-Caspian region are discussed on the basis of these results.

Keywords: COI, crustaceans, mitochondrial DNA, Ponto-Caspian, speciation, species flocks, vicariance

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Introduction

The Ponto-Caspian biogeographical region includes the Black, Azov, Caspian and Aral Seas. As with other ancient lakes (Brooks 1950), many groups of aquatic organisms including crustaceans, molluscs and fishes have radiated in this region (Mordukhai-Boltovskoi 1979; Dumont 1998). The crustaceans are particularly diverse, with separate radiations of cladocerans, copepods, cumaceans, amphipods, mysids and decapods, leading Zenkevich (1963) to propose that the Caspian should be called the 'Crustacean Sea'.

The origin and age of this rich fauna has been intensively debated, but little is known about the mechanisms of speciation responsible for this high level of endemism. Diversification has been linked to shifting salinity regimes and to the fragmentation of basins within the region (e.g. Zenkevich 1963; Dumont 2000). Throughout its past, short episodes of abrupt salinity rises as a result of saline

infusions from neighbouring marine waters were followed by a slow return to brackish conditions. For example, during the late Miocene, the continental Paratethyan basin, a remnant of the Tethys Sea, evolved into several successive brackish basins such as the Sarmatian and Pannonian, the slightly brackish Pontian basin and the almost fresh Dacian basin (Fig. 1). Near the end of the Dacian period, 4–5 million years ago (Myr), a major reduction in water levels, which coincided with uplift, led to the separation of the Black and the Caspian basins. Since then, these two basins have been separated, barring short periods of contact. The Caspian basin subsequently experienced a drastic regression during the Kimmerian period (Fig. 1d), followed by a major transgression which resulted in an inflow of oceanic water, probably from the Persian Gulf (Fig. 1e; Zenkevich 1963). This oceanic influence had a devastating effect on the brackish water fauna of Pontian origin in the Caspian basin (Akchagylian, 3.4–1.6 Myr), but only a minor effect on the Black Sea basin which maintained its brackish water communities. However, during the Pleistocene, climatic and

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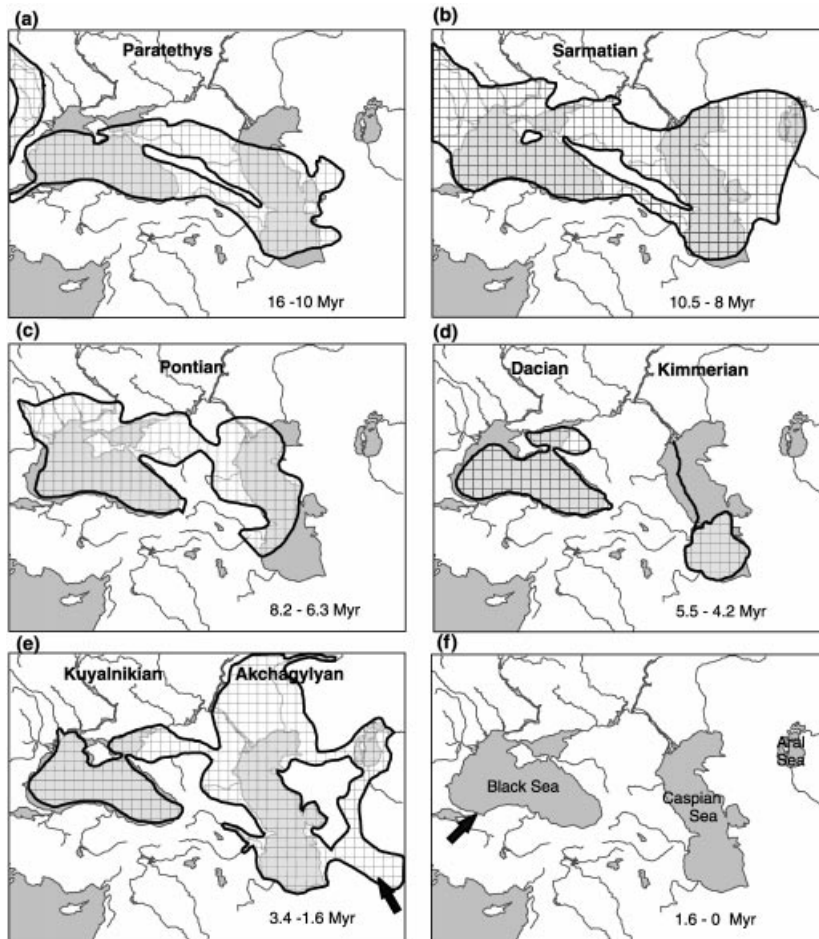


Fig. 1 Successive stages in the evolution and fragmentation of the Paratethys basin from middle Miocene (a), through the late Miocene (b) and (c), the Pliocene (d) and (e) and Pleistocene (f). Filled areas represent the shorelines for each geological interval after Jones & Simmons (1997) and Zenkevitch (1963). Arrows indicate reconnections of the Ponto-Caspian basin with the world ocean followed by inflow of oceanic water.

hydrologic changes produced dramatic transformations in the Black Sea basin. The global rise in sea level during the interglacials, along with tectonic movements, enabled repeated incursions of Mediterranean water into the Black Sea. As a result, the resident fauna experienced a high rate of extinctions being replaced by marine forms (Wall & Dale 1974). The few survivors retreated into the estuarine, lacustrine or riverine habitats along the margins of the Black Sea. In contrast, organisms in the Caspian Sea were less affected by hydrologic fluctuations during the Quaternary.

Most authors (e.g. Băcescu 1940; Bănărescu 1991; Dumont 2000) have concluded that the primary Miocene basins, the Sarmatian and Pontian Lakes, played a decisive role in initializing the radiation of most Ponto-Caspian lineages and terms such as 'Sarmatian relicts' or 'Pontian relicts' are common in the literature when referring to the origins of the local fauna. In fact, the fossil record links the Pontian basin with the radiation of two molluscan genera, *Hypanis* and *Didacna*, which now have a Ponto-Caspian range. It is generally believed that taxa of Miocene origin survived in the succeeding Pliocene and Pleistocene basins of the Black

and Caspian Seas. However, because both basins experienced at least one major bout of extinction during episodes of high salinity, coupled with several opportunities for subsequent faunal exchange, there is much confusion regarding the evolutionary history of the fauna.

The present distribution of the Ponto-Caspian fauna provides important biogeographical clues. Among the 450 recognized Ponto-Caspian species, 209 are confined to the Caspian Sea, 20 are restricted to the Sea of Azov or to low salinity habitats along the margins of the Black Sea and a few are endemic to the Aral Sea (Mordukhai-Boltovskoi 1979). The remaining species (almost 40%) are shared between at least two of these basins. The low incidence of endemics in the Black-Azov basin has led some authors to suggest that much of the Black Sea fauna penetrated the basin just 10 000 years ago (Mordukhai-Boltovskoi 1964, 1979). This hypothesis of recent dispersal has been challenged by studies on molluscs which have established that many Black Sea species are Pliocene relicts, supporting the continuity of a 4–5-Myr-old fauna in the Black Sea basin and therefore a vicariant origin for the Black/Caspian lineages (Grigoriev & Gozhik 1978). The lack of Ponto-Caspian

species from Pliocene deposits in the bottom sediments of the Caspian Sea has sometimes been interpreted as evidence for a mass extinction, implying a discontinuity of the fauna in the Caspian Sea and a subsequent repopulation by lineages from the Black Sea (Grigorovich *et al.* 2002). In general, the role of freshwater or slightly brackish coastal habitats along the basin margins as refugia is considered less important than the exchange of lineages prompted by transient contacts between basins.

These considerations suggest that the high diversity of endemics in the Ponto-Caspian region reflects not only the isolation of these basins from the world oceans, but also a complex process of immigration and speciation. The surrounding rivers, estuaries and lagoons likely acted as evolutionary reservoirs by serving as refuges during periods of mass extinction in the main basins. However, at least for the pelagic flocks of Ponto-Caspian crustaceans, such as mysids and onychopod cladocerans, intralacustrine sympatric speciation has been invoked as the most probable mechanism of speciation (Väinölä 1995; Cristescu & Hebert 2002).

Phylogeographical studies provide the opportunity to gain new insights into the relationship between geological history and biotic diversity (Avice 2000; Arbogast & Kenagy 2001). Moreover, the opposing models, the origin of species via late Miocene vicariance events (e.g. Băcescu 1940; Bănărescu 1970) vs. late Pliocene (Grigorovich *et al.* 2002) through Holocene dispersal events (Mordukhai-Boltovskoi 1964) produce predictions that can be tested by determining the extent of genetic divergence between lineages from different geographical areas. For example, the dispersalist hypothesis predicts high genetic similarity between the Black and Caspian faunas, whereas the vicariant hypothesis predicts deeper divergence between lineages from the two basins. The present study investigates mtDNA diversity in three benthic and three planktonic crustaceans occurring in both the Caspian and Black Seas, reconstructs the phylogeny of these lineages and examines the concordance of their phylogeographical architectures to test the vicariance vs. dispersalist hypotheses.

Materials and methods

Six Ponto-Caspian crustacean species with different life history strategies, including three planktonic cladocerans and three benthic amphipods, were collected in 1998–2001 from 28 habitats spanning the Black and Caspian Seas (Fig. 2; Table 1). Cladocerans were collected from 14 sites, while amphipods were collected from 17 sites. Samples were subsequently sorted and preserved in 90% ethanol. Cladocerans were identified using Rivier's (1998) key, while amphipods were determined using Cărăușu (1943), Cărăușu *et al.* (1955) and Barnard & Barnard (1983). For the

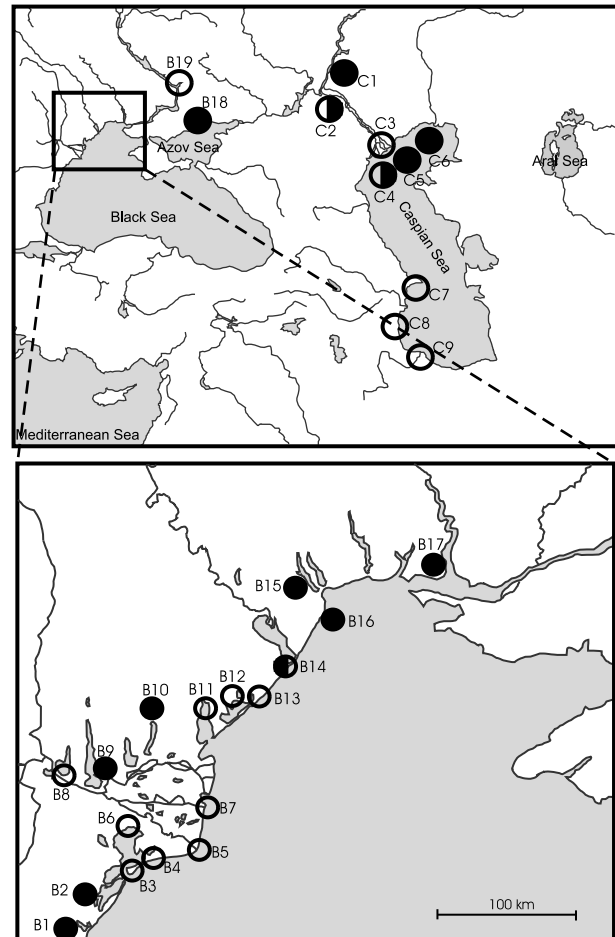


Fig. 2 Collection sites for cladocerans (●) and amphipods (○) examined in this study.

amphipod data set the Ponto-Caspian gammaroidean *Echinogammarus ischnus* was used as an outgroup, while the cladocerans were rooted with *Polyphemus pediculus* (GenBank Accession no. AY075048; Cristescu & Hebert 2002).

Sequence diversity was examined in the mitochondrial (mt) cytochrome *c* oxidase subunit 1 (COI) gene, a fast-evolving protein coding gene which often shows significant levels of genetic diversity at the intraspecific level (Cox & Hebert 2001). Whenever possible total DNA was extracted using the proteinase K method from three individuals from each habitat for the six species analysed. The primer pair LCOI490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') was used to amplify a 658 base pair (bp) fragment of COI (Folmer *et al.* 1994). The 50 μ L PCR reactions contained 1–2 μ L of DNA template, 5 μ L of 10 \times PCR buffer, 2 mM MgCl₂, 0.2 mM of each dNTP, 0.3 μ M of each primer and 1 unit of *Taq* polymerase. The polymerase chain reaction (PCR) conditions consisted of 1 min at 94 °C, followed by

Table 1 Species included in the molecular analyses, sampling locations (B, Black Sea; C, Caspian Sea) and collection dates; taxonomy follows Martin & Davis (2001)

Taxon	Location	Site/GenBank Accession no.	Collection date/author	
Class Branchiopoda				
Order Cladocera				
Family Cercopagidae				
<i>Cercopagis pengoi</i>	B	AF 320013	Cristescu <i>et al.</i> 2000	
	C-01	Volga River, Volzhskiy	August 2000	
	C-02	Volga River, Volgograd	August 2000	
	C	AF 320014	Cristescu <i>et al.</i> 2000	
	C-03	North Caspian Sea	August 2000	
	Family Podonidae			
	<i>Podonevadne trigona</i>	B-10	Kitai Lake	July 1999
		B-16	Chernomorka	September 2001
B-17		Bug Liman	September 2001	
B-18		Berdiansk	August 1999	
C-04		North Caspian Sea	August 2000	
C-05		North Caspian Sea	August 2000	
C-06		North Caspian Sea	August 2000	
<i>Cornigerius maeoticus</i>	B-01	Siutghiol Lake	July 1999	
	B-02	Razelm Lake, Histria	July 1999	
	B-09	Jalpuh Lake	July 1999	
	B-10	Kitai Lake	July 1999	
	B-14	Dniester Liman	July 1999	
	B-15	Brezan Liman	September 2001	
	B-16	Chernomorka	September 2001	
	C-01	Volga River, Volgograd	August 2000	
	C-02	Volga River, Volzhskiy	August 2000	
	C-04	North Caspian Sea	August 2000	
	C-05	North Caspian Sea	August 2000	
Class Malacostraca				
Order Amphipoda				
Suborder Gammaridea				
Family Pontogammaridae				
<i>Pontogammarus maeoticus</i>	B-03	Razelm Lake, Portita	August 1998	
	B-05	Black Sea, Sf. Gheorghe	August 2001	
	B-07	Black Sea, Sulina	September 2001	
	B-11	Sasyk Lake	August 1998	
	B-12	Burnas Lake	August 1998	
	B-13	Lebediovca	August 1998	
	B-14	Dniester Liman	August 1998	
	C-07	Baku	August 2000	
	C-08	Lankaran	August 1998	
	C-09	Bandar-e Anzali	August 1998	
<i>Pontogammarus crassus</i>	B-04	Leahova Mica	August 1998	
	B-06	Agighiol	September 2001	
	B-08	Kahul Lake	August 1998	
	C-09	Bandar-e Anzali	August 2000	
<i>Pontogammarus robustoides</i>	B-05	Danube River, Sf. Gheorghe	September 2001	
	B-06	Agighiol	August 1998	
	B-07	Danube River, Sulina	August 2001	
	B-08	Kakul Lake	August 1998	
	B-19	Dnieper River	September 2001	
	C-02	Volga River	July 2000	
	C-03	Volga Delta	July 2000	
	C-04	North Caspian Sea	August 2000	

five cycles of 60 s at 94 °C, 90 s at 45 °C, 60 s at 72 °C; followed by 35 cycles of 60 s at 94 °C, 90 s at 51 °C, 60 s at 72 °C; followed by 5 min at 72 °C. PCR products were gel purified using the Qiaex kit (Qiagen Inc.) and sequenced in one direction using an ABI 377 automated sequencer and the Big Dye terminator 3 sequencing kit (30 cycles and 55 °C annealing temperature).

Sequences were aligned using the SeqApp 1.9 sequence editor, and the alignments were unambiguous as they contained no nonsense codons and no insertions or deletions. All sequences obtained during this study have been submitted to GenBank under Accession nos: AY189478–AY189523 (Table 2).

Phylogeographical analyses were based upon COI nucleotide sequences. Intraspecific, uncorrected and corrected pairwise distances using the Tamura–Nei model, which incorporates parameters for unequal nucleotide frequencies, different rates of transition and transversion substitutions and different rates of transition substitutions between purines and pyrimidines (Tamura & Nei 1993), were calculated in PAUP* 4.0b 10 (Swofford 2001). Standard indices of genetic variation, haplotype diversity (h) and nucleotide diversity (π), were calculated for each lineage and both geographical regions using DNASP 3.53 (Rozas & Rozas 1999). Phenograms were constructed in PAUP* using neighbour joining (NJ) analysis. Molecular phylogenies were inferred in PAUP using maximum likelihood (ML) analysis and employed a heuristic search option (10 replicates, one tree held per replicate, sequences added at random, branch swapping by nearest neighbour interchanges). Clade support was estimated with bootstrap analyses (1000 replicates for neighbour-joining (NJ) trees and 100 replicates for ML trees; Felsenstein 1985). The constancy of rates of molecular evolution was tested using a log-likelihood ratio test (Huelsenbeck & Crandall 1997) conducted on data sets reduced to 33 lineages of cladocerans and 43 lineages of amphipods. Finally, the homogeneity of base frequencies across taxa was assessed using the χ^2 test in PAUP.

As no mitochondrial clocks are available for cladocerans or amphipods, we used the snapping shrimp mitochondrial COI clock of 1.4% to 2.2% sequence divergence between pairs of lineages per Myr (Knowlton *et al.* 1993; Knowlton & Weigt 1998) to date the timing of population isolation. Hence, these estimates are provisional.

Results

Sequence diversity

Intraspecific haplotype diversities and nucleotide diversities were generally higher for the amphipods than for the cladocerans. We found between three and seven unique haplotypes for cladocerans and between five and 22

haplotypes for the amphipods (Table 3). For example, 85% of the *P. maeoticus* individuals represented unique haplotypes, while only 8–35% of the cladoceran individuals were genetically distinctive. Overall, and particularly for amphipods, much of the genetic variation was restricted to third position sites (Table 2). Base composition of the COI fragment was moderately A + T rich (mean AT content = 59% for cladocerans and 62% for amphipods) as is typical of mtDNA and it was homogeneous across lineages in each group ($P > 0.99$). Likelihood ratio tests suggested that both amphipod and cladoceran data sets were consistent with a constant rate of evolution among the taxa included in the study (Table 4).

Phylogenetic relationships

For the cladoceran data set, the ML tree had a log-likelihood score of –1666.3. This analysis supported the monophyly of the genus *Cercopagis* and the monophyly of the two podonid genera, *Cornigerius* and *Podonevadne* with the exception of *Podonevadne* haplotypes from Kitai Lake (habitat B10). After screening 10 individuals from this habitat (six *Podonevadne*, four *Cornigerius*), two haplotypes were identified. One haplotype (*Pa*) was confined to *Podonevadne*, but the other haplotype (*Cd*), which clusters with *Cornigerius* lineages, was shared by three *Podonevadne* and by four *Cornigerius* (Fig. 3a,b). The presence of this shared haplotype suggests that the sympatric populations of *Cornigerius* and *Podonevadne* in Kitai Lake are hybridizing.

For the amphipod data set, the ML analysis produced a tree with a log-likelihood score of –2621.9 that supported the monophyly of all three *Pontogammarus* species as well as the monophyly of the Black/Caspian clades (Fig. 4b).

Phylogeographical structure

Phylogenetic analyses recovered two main phylogroups (corresponding to the Black and Caspian geographical regions) in all six species (Figs 3 and 4). Only recently established populations of *Cornigerius* (haplotype *a*) and *Cercopagis* (haplotype *b*) in reservoirs along the Volga River failed to conform to this phylogeographical pattern. Although these populations occupied sites in the Caspian drainage system, they showed a strong affinity to the Black Sea lineages (Fig. 3a,b).

The nucleotide divergences (Tamura–Nei) between Black and Caspian populations of the three cladoceran species were only slightly variable (from 1.4% to 1.6%). By contrast, the amphipods showed a variable level of genetic divergence (from 2.3% to 11.1%) with the deepest geographical split between Black and Caspian populations of *Pontogammarus maeoticus*.

Table 2 Variable sites of the 639/642 bp fragment of the COI gene; sites shown in bold type represent nonsynonymous substitutions (B, Black Sea; C, Caspian Sea)

COI Haplotype	Site	GenBank Accession no.	Nucleotide position
			51 93 175 207 213 249 270 325 411 423 456 459 520
<i>Cornigerius maeoticus</i>			
a	B	AY189510	A T T A T A G C T T G G T
b	B	AY189511	A T T A T A G C A T G G T
c	B	AY189512	A T T A T A G T T T G G T
d	B	AY189513	G T T A T A G C T T G G T
e	C	AY189514	A C C G C C A C T T A A C
f	C	AY189515	A C C G C C A C T T A A T
g	C	AY189516	A C C G C C A C T C A A T
			66 177 195 210 249 294 300 360 366 387 438 471 477 517 520 537 597 600
<i>Podonevadne trigona</i>			
a	B	AY189517	T A A T C A A A G A A C T G G A
b	B	AY189518	T A G T C A A A G A A C T G G A
c	C	AY189519	G A A A C G G G A G G C T G G A
d	C	AY189520	T A A T A G G A A G A G C C A G T
e	C	AY189521	T G A T A G G A A G A C T G A A
f	C	AY189522	T G A T A G G A A G A A T T G A A
			37 81 104 195 206 207 306 324 351 366 393 417 447 471 477 483 546 558 609 620
<i>Pontogammarus crassus</i>			
a	B	AY189478	A A C G A T A T C G C A T G G C T
b	B	AY189479	A A C G A T A T C G C A T G G C A
c	B	AY189480	A A C G A A T C G C A T G G G C A
d	B	AY189481	A G C G A T A T A G C A T G G C T
e	C	AY189482	G A T A A T G C A A T G C A A A T T
			12 15 19 21 30 39 45 69 78 81 84 90 99 108 162 168 177 186 189 192 193 204 216 219 222 225 228 231 243 247 252 276 297 300 303 309 318 321 324 331 332
<i>Pontogammarus maeoticus</i>			
a	B	AY189483	C T C A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
b	B	AY189484	C T C A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
c	B	AY189485	C T C A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
d	B	AY189486	C T C A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
e	B	AY189487	C T C A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
f	B	AY189488	C T C A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
g	B	AY189489	C T C A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
h	B	AY189490	C T C A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
i	B	AY189491	C T C A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
j	B	AY189492	C T C A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
k	B	AY189493	C T T A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
l	B	AY189494	C T C A T A T T T A C A C C G G C A G G A T T T G T G C T A T C G G C A A G T A A G
m	B	AY189495	C T C A T A T C T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G
n	B	AY189496	C T C A T A T T T A C A C C G G T A G G A T T T G T G C T A T C G G C A A G T A A G

Table 2 *Continued*

COI Haplotype	Site	GenBank Accession no.	Nucleotide position
o	B	AY189497	C T C A T A T T T A C A C A G T A G G G G T T T G T G C T A T C G G C A A G T A A G
p	B	AY189498	C T C A T A T T T A C A G G G T A G G A C T T G T G C T A T C G G C A C G T A A G
q	B	AY189499	T T C A T A T T T A C A C G G T A C G A T T T G T G C T G T C G G C A A G T A A G
r	C	AY189500	C C T G C G C T C G T A T G A T G A A A C C C A C A C C G T T A C A T G C T C T C
s	C	AY189501	C C T G C G C T C G T A T G A T G A A A C C C A C A C C G T T A C A T G C T C T C
t	C	AY189502	T C T G C G C T C G T A T G A T G A A A C C C A C A C C G T T A C A T G C T C T C
u	C	AY189503	C C T G C G C T C G T A T G A T G A A A C C C A C A C C G T T A C A T G C T C T C
v	C	AY189504	C C T G C G C T C G T A T G A T G A A A C C C A C A C C G T T A C A T G C T C T C
			340
a	B	AY189483	C C T G G T C G G T C A A T C A T T A T A T A C G A A A T C T A T G A T T G
b	B	AY189484	C T G G T C G G T C A A T C A T T A T A T A C G A A A T C T A T G A T T G
c	B	AY189485	C T G G T C G G T C A A T T G T A T A C G A A A T C T A T G A T T G
d	B	AY189486	C T G G T C G G T C A A T T A T A T A C G A A A T C T A T G A T T G
e	B	AY189487	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
f	B	AY189488	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
g	B	AY189489	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
h	B	AY189490	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
i	B	AY189491	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
j	B	AY189492	C T T G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
k	B	AY189493	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
l	B	AY189494	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
m	B	AY189495	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
n	B	AY189496	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
o	B	AY189497	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
p	B	AY189498	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
q	B	AY189499	C T G G T C G G T C A T T A T A T A C G A A A T C T A T G A T T G
r	C	AY189500	A C A C C G A A T T G G T A C C C A T A C G G C T C A C C G T T H T C H G T A H G
s	C	AY189501	A C A C C G A A T T G G T A C C C A T A C G G C T C A C C G T T H T C H G T A H G
t	C	AY189502	A C A C C G A A C T A G T A C C C A T A C G G C T C A C T A C T H T C H A T A T G
u	C	AY189503	A C A C C G A A C T A G T A C C C A T A C G G C T C A C T A T H T C H A T A T G
v	C	AY189504	A C A C C G A A C T A G T A C C C A T A C G G C T C A C T G T H T C H A T A T G
			345
			351
			354
			357
			363
			390
			393
			399
			409
			414
			438
			444
			447
			453
			456
			459
			462
			480
			483
			489
			492
			498
			510
			516
			525
			531
			534
			543
			546
			552
			561
			570
			585
			587
			594
			603
			606
			609
			615
			636
			639
<i>Pontogammarus robustoides</i>			12
a	B	AY189505	C G C T C G T T T T T A G C G C G T T A C G C A G C C C
b	B	AY189506	C G C T C G T T T T T A G C G C G T T A C G C A G C C C
c	B	AY189507	C G C T C G T T T T T A G C G C G T T A C G C A G C C C
d	B	AY189523	C G C T C G T T T T T A A T G C G T T A C G C A G T C C
e	C	AY189509	T A T G T A C C C C G G T T T A C C G C A T G A C G
f	C	AY189508	T A T T C A C C C C G G T T T A C C G C A T A A C G

Table 3 Variation in COI sequences for lineages of cladocerans and amphipods from the Black Sea (B) and the Caspian Sea (C). The estimated divergence time in million years (Myr) is based upon the calibrations of 1.4% and 2.2% sequence divergence per Myr (Knowlton & Weigt 1998; Knowlton *et al.* 1993)

Species	Nos. of localities	Nos. of haplotypes/sample size	Nos. of variable sites (total of 639/642 bp)	Haplotype diversity (h)	Nucleotide diversity (π)	Mean distance between B/C groups \pm SE (%)	Divergence time between B/C (Myr)	Haplotype diversity B/C (h)	Nucleotide diversity B/C (π)
Order Cladocera									
Family Cercopagidae									
<i>Cercopagis pengoi</i>	8	3/37	10	0.49	0.006	1.4 \pm 0.4	0.6–1.0	0.20/0.00	0.001/0.000
Family Podonidae									
<i>Podonevadne trigona</i>	7	6/17	18	0.74	0.009	1.4 \pm 0.4	0.6–1.0	0.25/0.80	0.000/0.008
<i>Cornigerius maeoticus</i>	11	7/24	13	0.78	0.005	1.6 \pm 0.5	0.7–1.1	0.69/0.83	0.001/0.002
Order Amphipoda									
Family Pontogammaridae									
<i>Pontogammarus crassus</i>	4	5/8	18	0.82	0.011	2.3 \pm 0.6	1.0–1.6	0.73/0.00	0.002/0.000
<i>P. maeoticus</i>	10	22/26	82	0.99	0.040	11.1 \pm 1.4	5.0–7.9	0.98/0.93	0.004/0.006
<i>P. robustoides</i>	8	6/12	26	0.85	0.017	3.4 \pm 0.7	1.5–2.4	0.75/0.50	0.003/0.002

Temporal processes

Based on the calibration for snapping shrimp isolated by closure of the Isthmus of Panama (Knowlton *et al.* 1993; Knowlton & Weigt 1998), we estimate that the divergence between Black and Caspian planktonic lineages occurred 1 Myr ago, while benthic lineages have more divergent isolation times. For example, Black and Caspian populations of *P. maeoticus* have been isolated for the past 5.0–7.9 Myr, while populations of *P. robustoides* and *P. crassus* last exchanged genes about 1.5–2.4 Myr and 1–1.6 Myr ago, respectively.

Discussion

Phylogeographical inferences from benthic and planktonic Ponto-Caspian taxa

A marked genetic divergence between Pontic and Caspian populations was found for all six crustacean species. Despite this concordance, the levels of genetic divergence between the two phylogroups varied greatly among the three amphipods, suggesting that faunal exchange between the basins occurred at different times from the late Miocene to Pleistocene. A late Miocene or early Pliocene isolation time was inferred for *P. maeoticus*, while a late Pliocene event was apparently responsible for the split of *P. robustoides*. Finally, an early Pleistocene separation time was estimated for *P. crassus*. In contrast to the marked Black/Caspian genetic split there was little genetic structure within each geographical region, suggesting a relatively high level of gene flow within single basins.

Cladocerans were also subdivided into Pontic and Caspian lineages. However, unlike the amphipods, cladocerans were characterized by a low and consistent level of intraspecific genetic divergence, suggesting their isolation since the middle Pleistocene (0.6–1.1 Myr ago). Cladocerans also showed low intraspecific haplotype and nucleotide diversities, suggestive of recent and severe bottlenecks. For example, only three haplotypes were detected among 37 individuals of *Cercopagis pengoi* from eight different localities. Higher haplotype diversity ($h = 0.80$) was present in the population of *Podonevadne trigona* from the Caspian Sea, and this diversity was associated with the co-occurrence of several divergent lineages ($\pi = 0.008$).

The present phylogeographical data indicate a Black Sea origin for the recently established populations of *Cornigerius* and *Cercopagis* in the reservoirs along the Volga River (Caspian Sea drainage system). This result is not surprising, given the ecological similarity between these reservoirs along the Volga River and the marginal lakes (limans) along the Black Sea. Although there is a widespread belief that Ponto-Caspian endemics are very euryhaline, there is evidence that this variation reflects divergence between

COI data set	Likelihood ratio test				Null hypothesis
	$-\log L_{\text{no clock}}$	$-\log L_{\text{clock}}$	2Δ	d.f. = n-2	
Amphipods	2855.3	2875.9	41.2	41	Not rejected
Cladocerans	1589.9	1604.4	29.0	31	Not rejected

Table 4 Likelihood ratio test for the molecular clock hypothesis $2\Delta = \log L_{\text{no clock}} - \log L_{\text{clock}}$ ($\chi^2_{(31,0.05)} = 44.9$; $\chi^2_{(41,0.05)} = 56.9$). Maximum-likelihood trees were constructed using the HKY85 model and heuristic search option (10 replicates, one tree held per replicate, tree-bisection-reconnection branch-swapping algorithm)

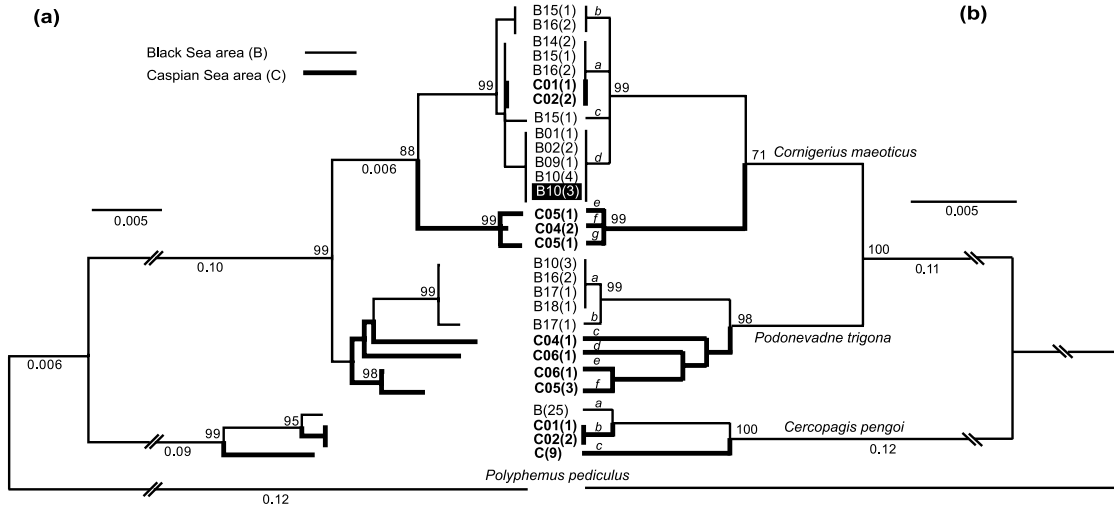


Fig. 3 Patterns of intraspecific divergence in COI for the onychopod cladocerans *Cornigerius maeoticus*, *Podonevadne trigona* and *Cercopagis pengoi*. (a) NJ tree based on uncorrected nucleotide distances. (b) Maximum likelihood phylogram ($-\ln$ likelihood = 1666.34). ML analysis employed the HKY85 model with molecular clock enforced and a heuristic search with a nearest-neighbour interchange branch-swapping algorithm. Numbers above nodes give the bootstrap support greater than 50%. Letters above the terminal branches identify unique haplotypes described in Table 2, while codes and bracketed numerals indicate the number of individuals with a specific haplotype encountered in each population. The highlighted Kitai Lake population (B10) identifies the haplotype of *P. trigona* which grouped closely with *C. maeoticus* lineages.

populations from the two major basins. For example, many crustaceans with broad distributions in the region are restricted to rivers or slightly brackish (< 4‰) lagoons and estuaries in the Azov-Black Sea basin, but occupy higher salinity (12‰–13‰) habitats in the Caspian Sea (Mordvkhai-Boltovskoi 1964; Zenkevich 1963; Barnard & Barnard 1983). Moreover, recent studies have identified physiological variation among ‘conspecific’ populations of Ponto-Caspian crustaceans (Saldatova 1986; Aladin 1995). These results suggest that many crustaceans consist of lineages showing marked regional variation in their salinity tolerance. In addition, morphological studies confirm the presence of intraspecific variation across the distributions of many Ponto-Caspian amphipods (Pjatakova & Tarasov 1996). This morphological and physiological evidence, when coupled with the present documentation of genetic divergence between the Black and Caspian populations, suggests that these populations form distinctive evolutionary groups with a long history of isolation.

Evolutionary patterns in planktonic and benthic Ponto-Caspian crustaceans

The consistent evidence for genetic divergence between Pontic and Caspian crustaceans is not unexpected, given the geological history of this region. The Black and Caspian basins have been separated since the Pliocene except during periods of major transgression when brief contacts allowed faunal exchange. For example, in the late Pliocene the Caspian basin (Akchagylian) was reconnected with both the Black Sea (Kuyalnikian) and the world oceans (Fig. 1e). During the Apsheronian transgression of the Pleistocene, a second transient connection between the Black and Caspian Sea was established. Throughout the Pleistocene, three of the interglacial periods were also extensive enough to enable the Black and Caspian basins to reconnect (e.g. Chepalyga 1985; Reid & Orlova 2002). The different divergence times obtained during this study (late Miocene, Pliocene and Pleistocene) supports a scenario of repeated

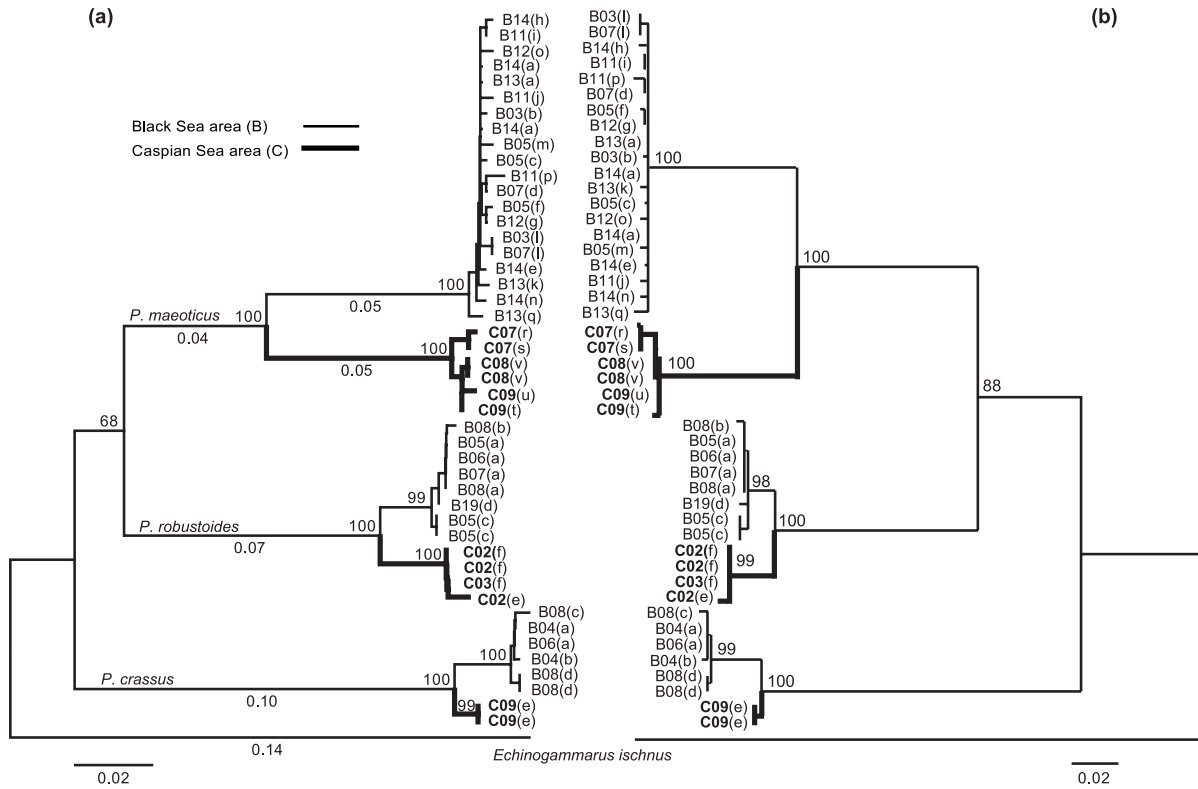


Fig. 4 Patterns of intraspecific divergence at COI for the amphipods *Pontogammarus maeoticus*, *Pontogammarus crassus* and *Pontogammarus robustoides*. (a) NJ tree for the COI gene based on Tamura–Nei nucleotide distances. (b) Phylogram of *Pontogammarus* species based on maximum likelihood (–Ln likelihood = 2621.98). The ML analysis employed the HKY85 model and a heuristic search with nearest-neighbour interchange branch-swapping algorithm. Codes identify sampling sites, while bracketed letters indicate unique haplotypes as described in Table 2. The numbers above the branches indicate bootstrap support greater than 50%.

colonization events throughout the history of the Ponto-Caspian basin, but also emphasizes the role of coastal refugia in allowing lineage survival during intermittent pulses of high salinity. At least for the species examined here, it seems that successful secondary invasions did not occur after the habitats within each geographical region were occupied. It is therefore probable that most of the crustaceans, which currently inhabit both basins, represent pairs of evolutionary significant units evolved under prolonged geographical isolation and exposed to different selective forces which has provoked their physiological differentiation. The role of vicariance in splitting gene pools is evident not only for the benthic amphipods with weak dispersal abilities, but also for the more vagile cladocerans. Therefore, we reject the Holocene (10 000 years ago) dispersalist hypothesis proposed by Mordukhai-Boltovskoi (1964) and argue for a relict origin of at least early Pleistocene age for the Ponto-Caspian fauna confined today to the Black Sea. Moreover, late Miocene and early Pliocene vicariant events left their genetic imprint on some species such as *P. maeoticus*.

Despite its complex history, the Ponto-Caspian basin is

an informative system for examining phylogeographical patterns and forces which drive diversification. Young clades such as the Caspian cladocerans or mysids provide phylogenetic evidence for sympatric diversification (Väinölä 1995; Cristescu & Hebert 2002), perhaps under strong divergent selection, whereas older clades, such as the amphipods, reveal the role of multiple colonization events and prolonged geographical isolation in taxon radiations. Moreover, the Ponto-Caspian basin has been unique among the other ancient lakes in fostering adaptive radiations in both benthic and planktonic taxa enabling an examination of phylogeographical patterns in lineages with contrasting life history strategies. Therefore, beyond establishing the colonization history of individual groups, phylogenetic analyses of Ponto-Caspian organisms are revealing key evolutionary and ecological factors that may well aid in explaining speciation in other ancient lakes.

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