

The “Crustacean Seas” — an evolutionary perspective on the Ponto–Caspian peracarids

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Abstract: A spectacular adaptive radiation of crustaceans has occurred in the Black, Caspian, and Aral seas. This study tests several evolutionary scenarios based on the extent of genetic differentiation and the phylogenetic relationships among endemic mysids and gammarid amphipods from the Black and Caspian seas. Molecular phylogenies for these taxa were based on two mitochondrial genes: cytochrome *c* oxidase subunit I and the large ribosomal RNA subunit (16S), and one nuclear gene, the large ribosomal RNA subunit (28S). The results support the monophyly of the Ponto–Caspian gammarids (genera *Dikerogammarus*, *Echinogammarus*, *Obesogammarus*, and *Pontogammarus*), suggesting their origin from one colonization event. By contrast, several colonization events preceded the radiation of the Ponto–Caspian mysids (genera *Limnomysis* and *Paramysis*). Levels of intraspecific divergence were variable, with mysids showing either no geographic structure or deep genetic splits reflecting a long history of reproductive isolation between populations in marine settings and those in fresh waters. These findings suggest that the diversity of the Ponto–Caspian crustaceans has been underestimated and that species regarded as euryhaline are often composed of distinct evolutionary groups whose taxonomic status should be reevaluated.

Résumé : Il s’est produit une extraordinaire radiation des crustacés dans la mer Noire, la mer Caspienne et la mer d’Aral. Nous évaluons plusieurs scénarios évolutifs basés sur l’étendue de la différenciation génétique et les relations phylogénétiques chez les mysidés et les amphipodes gammaridés endémiques des mers Noire et Caspienne. Les phylogénies moléculaires de ces taxons se basent sur deux gènes mitochondriaux, la sous-unité I de la cytochrome *c* oxydase et la grande sous-unité d’ARN ribosomique (16S), ainsi qu’un gène nucléaire, la grande sous-unité d’ARN ribosomique (28S). Nos résultats appuient l’hypothèse de la monophylie des gammaridés Ponto–Casiens (genres *Dikerogammarus*, *Echinogammarus*, *Obesogammarus* et *Pontogammarus*), ce qui laisse croire qu’ils sont issus d’un même épisode de colonisation. En revanche, plusieurs épisodes de colonisation ont précédé la radiation des mysidés Ponto–Casiens (genres *Limnomysis* et *Paramysis*). Les niveaux de divergence interspécifique sont variables : chez les mysidés, ou bien il n’y a pas de structure géographique, ou alors il y a d’importantes divergences génétiques qui reflètent une longue période d’isolement génétique entre les populations des milieux marins et celles d’eau douce. Ces résultats indiquent que la diversité des crustacés ponto-casiens a été sous-estimée et que les espèces considérées comme euryhalines sont souvent composées de groupes évolutifs distincts dont le statut taxonomique a besoin d’être réévalué.

[Traduit par la Rédaction]

Introduction

Many groups of organisms including fishes, mollusks, turbellarians, and crustaceans have radiated in the Ponto–Caspian biogeographic region, which includes the Black, Azov, Caspian, and Aral seas. However, the endemism in varied crustacean lineages (e.g., cladocerans, copepods, cumaceans, decapods, amphipods, and mysids) is significantly higher than in most other animal groups. For example, more than 70 endemic amphipods and approximately 28 mysid species have been described from the Ponto–Caspian region (Cărăușu 1943; Băcescu 1954; Barnard and Barnard 1983). In fact, the Ponto–Caspian mysid fauna is the most diverse fresh–brackish water mysid fauna in the world (Băcescu 1954; Mauchline 1980). Since most of these

endemics are confined to the Caspian Sea, this region has been considered the center for their radiation (Zenkevitch 1963). As a result of this diversity, the Caspian Sea has been nicknamed the “Crustacean Sea”. However, its sibling seas, the Black and Azov seas, also support a substantial number of endemics in their less saline estuaries, peripheral lakes, and rivers. In fact, almost 50% of Ponto–Caspian taxa are shared between these basins and their evolutionary origin is particularly ambiguous.

The four basins in the Ponto–Caspian region formed some 6 million years (Mys) ago following disintegration of the Paratethys Sea, an arm of the more extensive Tethys Sea (Dumont 1998). The shared occurrence of species in the Black and Caspian seas could be explained by either these late Miocene – early Pliocene vicariance events that sepa-

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rated the basins or more recent (late Pleistocene) dispersal from the Caspian Sea into the Black Sea. This matter is unresolved. Several authors have suggested that most Ponto–Caspian lineages originated long before the present basin configuration, possibly during the late Miocene (Băcescu 1940; Barnard and Barnard 1983; Dumont 2000), while others argued for a more recent Pliocene or Pleistocene origin (Mordukhai-Boltovskoi 1964; Grigorovich et al. 2003).

The Ponto–Caspian mysids have been partitioned into nine genera: *Acanthomysis*, *Caspiomysis*, *Diamysis*, *Hemimysis*, *Katamysis*, *Limnomysis*, *Mysis*, *Paramysis*, and *Schistomysis* (Băcescu 1940, 1954). Väinölä (1995) showed that the pelagic flock of Caspian *Mysis* (*Mysis microphthalmus*, *Mysis amblyops*, and *Mysis caspia*) reflects a recent intralacustrine radiation, but their relationships with the other endemic mysid genera have not been investigated. The present study addresses this gap in knowledge by examining the genera *Paramysis* and *Limnomysis*. Not only is *Paramysis* the most speciose of the nine Ponto–Caspian mysid genera, but it also contains species such as *Paramysis kroyeri*, which occurs in salinities ranging from fresh–brackish coastal lakes (salinity < 4‰) to the intertidal and subtidal zones of the Black Sea (salinity = 18‰).

The Ponto–Caspian amphipods belong largely to the families Gammaridae, Pontogammaridae, and Corophiidae, which are each represented by several speciose genera (Cărăușu 1943; Barnard and Barnard 1983; Martin and Davis 2001). The morphological radiation of Ponto–Caspian amphipods has been linked to their fossorial adaptations because most species are burrowers. Although a general evolutionary trend towards increased specialization for burrowing is evident, the phylogenetic affinities among Ponto–Caspian gammarids are unclear. It has been suggested that the nonfossorial genus *Echinogammarus* is primitive because its gnathopods resemble those of the *Gammarus* group (Barnard and Barnard 1983). *Dikerogammarus* is thought to be derived from the Ponto–Caspian *Echinogammarus* lineage, since its first gnathopod lacks the palmar spination typical of *Echinogammarus* while retaining the *Gammarus* type of first antenna (Barnard and Barnard 1983). Other genera such as *Obesogammarus* and *Pontogammarus* share additional derived characters such as setae on the bases of pereopods 5–7 and setae on the posterior margins of pereopods 3 and 4. While *Obesogammarus* has the primitive condition of setal tufts, *Pontogammarus* has a single row of coalesced setae. Within *Pontogammarus*, a further subdivision has been proposed by Karaman and Barnard (1997), who suggested the removal of *Pontogammarus maeoticus* from the genus because of the presence of a plesiomorphic longer inner ramus on uropod 3 establishing the genus *Euxinia*. We employ the original nomenclature. Although generally accepted, the morphological phylogeny for this group is based on shared derived states (synapomorphies). It seems likely that the assemblage of primitive features and shared derived characters has been altered by convergent evolution. Furthermore, most diagnosable characters are affected by phenotypic plasticity.

Despite more than a century of taxonomic investigations, the mechanisms of speciation along with the factors responsible for the speciation of the Ponto–Caspian crustaceans remain controversial. There is, for example, no information on whether species flocks descended from a single ancestor or

arose through the recurrent colonization of each basin. Similarly, the timing of speciation events is poorly understood.

The present study examines the extent of genetic differentiation and phylogenetic relationship among six species of mysids and eight species of gammarid amphipods from the Black and Caspian seas. Additionally, taking these crustaceans as an example, this study explores the tempo and the mode of speciation responsible for the high species richness in the Ponto–Caspian area.

Materials and methods

Taxon sampling

Fourteen Ponto–Caspian crustacean species, including six mysids and eight amphipods, were analyzed in this study (Table 1). Samples were collected in 1999–2001 from brackish lagoons and estuaries, brackish inland seas, and freshwater ponds and lakes along the northwestern Black Sea coast and in the Caspian Sea (Fig. 1). The samples were sorted and preserved in 90% ethanol shortly after collection. The amphipod *Gammarus lacustris* and the mysids *Tenagomysis australis* and *Mysis relicta* were used as outgroups in the first phase of phylogenetic analyses. To test the monophyly of the Ponto–Caspian gammaroids, we included 16 additional taxa belonging to four gammaridean families (Acanthogammaridae, Gammaridae, Hyaellidae, and Crangonyctidae). These more inclusive phylogenies were rooted using the isopod *Glyptidotea lichtensteini*. Most amphipod taxa were represented by several populations in the phylogenetic analyses of the cytochrome *c* oxidase subunit I (COI) gene, with samples from both the Black and Caspian seas. A phylogeographic survey was also conducted for the mysids *Limnomysis benedeni* and *Paramysis kroyeri* at sites within the Black Sea region.

DNA extraction, amplification, and sequencing

Phylogenies were constructed using two mitochondrial genes: COI and 16S ribosomal RNA (rRNA), and one nuclear gene, 28S rRNA. The primer pairs LCOI490 (5'-GGT-CAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3') (Folmer et al. 1994), 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr (5'-CCGGTCTGAACATCAGATCAGT-3') (Palumbi 1996), and 28Sa (5'-TTGGCGACCCGCAATTTAAGCAT-3') and 28Sb (5'-CCTGAGGGAACTTCGGAGGGAAC-3') (Taylor et al. 1999) were used to amplify a 658-base pair (bp) fragment of the COI gene, an approximately 500-bp fragment of the 16S gene, and a 1400-bp fragment of the 28S gene. Total DNA was extracted by grinding one or several appendages in 50–100 µL of a proteinase K extraction buffer (Schwenk et al. 1998).

Each polymerase chain reaction (PCR) had a total volume of 50 µL and contained 1–2 µL of DNA template, 5 µL of 10× PCR buffer, 2.0 mmol/L MgCl₂, 0.2 mmol/L each dNTP, 5 pmol of each primer, and 1 unit of *Taq* DNA polymerase. The PCR conditions for COI consisted of one cycle of 94 °C (60 s) followed by five cycles of 94 °C (60 s), 45 °C (90 s), and 72 °C (60 s), 35 cycles of 94 °C (60 s), 51 °C (90 s), 72 °C (60 s), and 72 °C (3 min). The PCR conditions for 16S involved two cycles of 94 °C (30 s), 60 °C (45 s), and 72 °C (45 s) and five cycles of 93 °C (30 s),

Table 1. Species included in the molecular analyses, their geographic distribution, site code, site description, collection date, and GenBank accession Nos.

Taxon ^a	Geographic distribution	Site code ^b	Site description	Collection date	GenBank accession No. COI	16S/28S ^c
Order Mysidacea						
Family Mysidae						
Subfamily Mysinae						
Tribe Mysini						
<i>Limnomysis benedeni</i>	Black and Caspian seas	B5 B9	Agighiol, Danube Delta, Romania Burnas Lake, Ukraine	July 1999 July 1999	AY529017 ^d AY529018 ^d AY529019 ^d	
		B8 B3	Pokrov Lake, Ukraine Leahova Mica, Danube Delta, Romania	July 1999 Aug. 2001	AY529020 ^d AY529021 ^d	
		B2	Golovita Lake, Danube Delta, Romania	Aug. 2001	AY529022 ^d	
		BA	Ditch De Gyster, Netherlands	June 2001	AY529023 ^d AY529024 ^d	
		BA B04	River Ktoine, Netherlands Sf. Gheorghe, Black Sea, Romania	June 1999 Aug. 2001	AY529025 ^d AY529026 ^d	AY529054 ^d
<i>Mesopodopsis slabberi</i>	B, eastern Atlantic Ocean, (41°N– 58°N) and Baltic and Mediter- ranean seas	NA			AY529027 ^d	AY529055 ^d
<i>Mysis relicta</i>	Circumarctic and Baltic and North seas	NE	River Rhine, Netherlands	June 1999	AY529028 ^d	
<i>Neomysis integer</i>	Eastern North Atlantic Ocean (44°N–71°N) and Baltic and Mediterranean seas					
<i>Paramysis baeri</i>	Black and Caspian seas	C5	North Caspian Sea, Russia	Aug. 2000	AY529029 ^d AY529030 ^d	AY529056 ^d
<i>Paramysis intermedia</i>	Black and Caspian seas	B2	Golovita, Danube Delta, Romania	Aug. 2001	AY529031 ^d AY529032 ^d	AY529057 ^d
<i>Paramysis kessleri</i>	Black and Caspian seas	B2	Golovita, Danube Delta, Romania	Aug. 2001	AY529033 ^d AY529034 ^d	AY529058 ^d
<i>Paramysis kroyeri</i>	Black Sea	B11	Dniestr Liman, Ukraine	July 1999	AY529035 ^d AY529036 ^d	AY529059 ^d
<i>Paramesopodopsis rufa</i>	Pacific and Indian oceans	B4	Sf. Gheorghe, Black Sea, Romania Jarman et al., unpublished data	Aug. 2001	AY529037 ^d AF052393 ^e	AY529060 ^d
Tribe Leptomysini						
<i>Tenagomysis australis</i>	Pacific and Indian oceans		Jarman et al., unpublished data		AF052394 ^e	
Order Amphipoda						
Suborder Gammaridea						
Family Pontogammaridae						
<i>Obesogammarus crassus</i>	Black and Caspian seas	B5 B7 C8	Agighiol, Razim Lake, Romania Kahul Lake, Ukraine Bandar-e Anzali, Iran	Sept. 2001 Aug. 1999 Aug. 2000	AY189478 ^f AY189481 ^f AY189482 ^f	AY529061 ^d

Table 1 (continued).

Taxon ^e	Geographic distribution	Site code ^b	Site description	Collection date	COI	GenBank accession No.
<i>Pontogammarus (Euximia) maeoticus</i>	Black and Caspian seas	B11	Dniester Liman, Black Sea, Ukraine	Aug. 1999	AY189490 ^f	AY529061 ^d
		B13	Chernomorka, Ukraine	Sept. 2001	AY529038 ^d	
		B10	Lebediovca, Black Sea, Ukraine	Aug. 1999	AY189483 ^f	
		B6	Sulina, Black Sea, Romania	Aug. 1999	AY189494 ^f	AY529062 ^d
		C6	Baku, Azerbaijan	Aug. 2000	AY189500 ^f	AY529063 ^d
		C7	Lankaran, Azerbaijan	Aug. 2000	AY189504 ^f	
		C8	Bandar-e Anzali, Iran	Aug. 2000	AY189503 ^f	
<i>Pontogammarus obesus</i>	Black and Caspian seas	B1	Istria, Sinoie Lake, Romania	Aug. 1999	AY529039 ^d	AY529064 ^d
		C1	Volgograd Reservoir, Russia	Aug. 2000	AY529040 ^d	AY529065 ^d
					AY529041 ^d	
<i>Pontogammarus robustoides</i>	Black and Caspian seas	B5	Agighiol, Razim Lake, Romania	Aug. 1999	AY529042 ^d	
		B7	Kahul Lake, Ukraine	Aug. 1999	AY529043 ^d	
		B16	Krasnaya, Dnister River	Sept. 2001	AY529044 ^d	AY529066 ^d
		BA	Pregora River, Baltic Sea	Feb. 2001	AY529045 ^d	
		C3	Volga Delta, Astrakhan, Russia	Aug. 2000	AY529046 ^d	
		C1	Volgograd Reservoir, Russia	Aug. 2000	AY529047 ^d	AY529067 ^d
		B4	Sf. Gheorghie, Danube River, Romania	Aug. 2001	AY529048 ^d	
<i>Dikergammarus villosus</i>	Black Sea	B5	Agighiol, Razim Lake, Romania	Aug. 1999	AY529048 ^d	
		B6	Sulina, Danube River, Romania	Aug. 1999	AY529048 ^d	AY529068 ^d
<i>Dikergammarus haemobaphes</i>	Black and Caspian seas	C2	Volga River, Astrakhan, Russia	Aug. 2000	AY529049 ^d	AY529069 ^d
Family Gammaridae		C4	North Caspian Sea, Russia	Aug. 2000	AY529049 ^d	
<i>Echinogammarus ischnus</i>	Black and Caspian seas	B5	Agighiol, Razim Lake, Romania	Aug. 1999	AY326120 ^g	AY529070 ^d
		B15	Kozarovichi, Irpen River	Feb. 1999	AY326122 ^g	
		B14	Novodniestrovsk, Middle Dniestr River	Sept. 2001	AY326124 ^g	
		C3	Stream Bystraya, Volga River Delta	Sept. 2000	AY326125 ^g	AY529071 ^d
		C3	Priamaya Bolida, Volga River Delta	Aug. 2000	AY326126 ^g	
<i>Echinogammarus trichatus</i>	Black and Caspian seas	B12	Krasnaya Kosa, Dniestr Liman, Ukraine	Aug. 2001	AY529050 ^d	
<i>Gammarus lacustris</i>		B5	Agighiol, Razim Lake, Romania	Aug. 2001		AY529072 ^d
<i>Gammarus duebeni</i>		B1	Istria, Sinoie Lake, Romania	Aug. 1999	AY529051 ^d	
Family Acanthogammaridae	Northeast Atlantic Ocean	NA	Blue Pond, Manitoba, Canada	Sept. 2000	AY529052 ^d	AY529073 ^d
			Ironside et al., unpublished data		AF448520 ^h	
<i>Acanthogammarus maculosus</i>	Lake Baikal	BL	Lake Baikal (Vainölä et al. 2001)		AY061799 ⁱ	
<i>Acanthogammarus flavus</i>	Lake Baikal	BL	Lake Baikal (Vainölä et al. 2001)		AY061800 ⁱ	
<i>Eulimnogammarus cyaneus</i>	Lake Baikal	BL	Lake Baikal (Vainölä et al. 2001)		AY061801 ⁱ	

Table 1 (concluded).

Taxon ^a	Geographic distribution	Site code ^b	Site description	Collection date	COI	GenBank accession No. 16S/28S ^c
Family Gammaracanthidae						
<i>Gammaracanthus lacustris</i>	Boreal	NE	Finnish Lakes (Väinölä et al. 2001)		AY061796 ⁱ	
<i>Gammaracanthus caspius</i>	Caspian Sea	C	Central Caspian Sea (Väinölä et al. 2001)		AY061797 ⁱ	
<i>Gammaracanthus aestuariarum</i>	Circumarctic	NE	Western White Sea (Väinölä et al. 2001)		AY061798 ⁱ	
Family Hyalellidae						
<i>Hyalella montezuma</i>	North America	NA	Witt et al. 2003		AY152807 ^j	
<i>Hyalella</i> sp.	North America	NA	Witt et al. 2003		AY152783 ^j	
<i>Hyalella</i> sp.	North America	NA	Witt et al. 2003		AY152752 ^j	
Family Crangonyctidae						
<i>Crangonyx</i> sp.	North America	NA			AY529053 ^d	
Order Isopoda						
Suborder Valvifera						
Family Idoteidae						
<i>Glyptidotea lichtensteini</i>			Wetzer 2001		AF255781 ^k	

^aClassification after Bousfield (1977), Mauchline (1980), and Martin and Davis (2001).

^bB, Black Sea; BA, Baltic Sea; C, Caspian Sea; BL, Baikal Lake; NE, north Europe; NA, North America.

^c16S for mysids and 28S for amphipods.

^dGenBank accession Nos. AY529017–AY529073 represent taxa sequenced during this project.

^eGenBank accession Nos. AF052393–AF052394 correspond to specimens to S. Jarman, S. Nicol, N. Elliott, and A. McMinn (Institute of Antarctic and Southern Ocean Studies, University of Tasmania, G.P.O. Box 252-77, Hobart, TAS 7001, Australia, unpublished data).

^fGenBank accession Nos. AY189478–AY189504 correspond to specimens in Cristescu et al. (2003).

^gGenBank accession Nos. AY326120–AY326126 correspond to specimens in Cristescu et al. (2004).

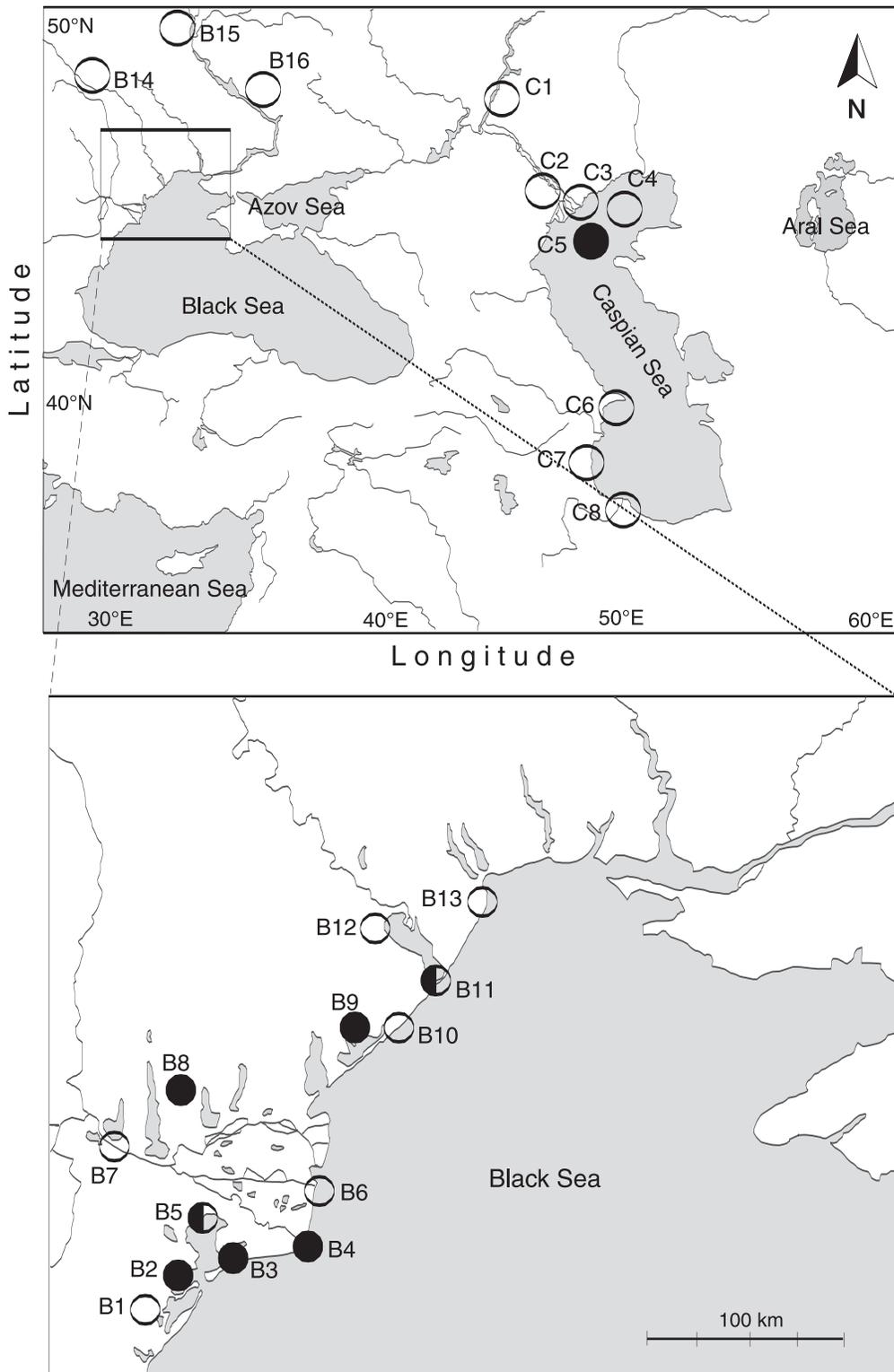
^hGenBank accession No. AF448520 corresponds to specimen in J.E. Ironside, A.M. Dunn, D. Rollinson, and J.E. Smith (Biological Sciences, University of Leeds, Miall Building, Leeds, West Yorkshire LS2 9JT, UK, unpublished data).

ⁱGenBank accession Nos. AY061796–AY061801 correspond to specimens in Väinölä et al. (2001).

^jGenBank accession Nos. AY152752–AY152807 correspond to specimens in Witt et al. (2003).

^kGenBank accession No. AF255781 corresponds to specimen in Wetzer (2001).

Fig. 1. Collection sites for mysids (solid circles) and amphipods (open circles) examined in this study.



55 °C (45 s), and 72 °C (45 s) followed by 29 cycles of 93 °C (30 s), 50 °C (1 min), and 72 °C (3 min). The temperature profiles for 28S consisted of 35 cycles at 93 °C (30 s), 50 °C (30 s), and 72 °C (1 min). Amplified PCR products were gel purified (2% agarose) using the Qiaex kit

(QIAGEN Inc., Valencia, Calif.) and sequenced in one or both directions (for the 28S fragment, both strands were sequenced) using an ABI 377 automated sequencer (ABI, Foster City, California) and the Big Dye terminator 3 sequencing kit (ABI). All sequences obtained during this study

Table 2. Number of sites available (TS), variable sites (VS), cladistically informative sites (IS), base frequencies, transition to transversion ratio (Ti/Tv), and χ^2 test of homogeneity of base frequencies across ingroup taxa for each data set and each partition.

Data set	Best fit model	TS	VS	IS	Base composition (%)				Ti/Tv	χ^2
					A	C	G	T		
Amphipoda										
COI	TVM + I + G	643	297	284	25	21	18	36	1.3	64.36, df = 108, $p = 0.99$
COI + 28S	GTR + I + G	2034	451	294	24	23	27	26	1.5	6.20, df = 36, $p = 0.99$
Mysidacea										
COI	TVM + I + G	633	354	291	22	18	24	36	1.1	235.66, df = 81, $p < 0.01$
COI + 16S	HKI + I + G	1121	527	339	25	17	23	35	1.1	85.34, df = 30, $p < 0.01$

Note: COI, cytochrome *c* oxidase subunit I; 28S and 16S, ribosomal RNA subunits; TMV, transversal model; I, invariable sites; G, gamma; GTR, general time-reversible model; HKY, Hasegawa–Kishino–Yano model.

have been deposited in GenBank under accession Nos. AY529017–AY529073 (Table 1).

Phylogenetic analyses

Sequences were aligned using the SeqApp 1.9 sequence editor and Sequencher (Gene Codes Corporation, Ann Arbor, Michigan). The estimates of sequence divergence in the amphipod data set were corrected using Tamura and Nei's (1993) method. LogDet distances (Lake 1994; Gu and Li 1996) were used to correct for inequalities of base composition in the mysid data set. COI amino acid distances were estimated using a Poisson correction. Molecular phylogenies were based on nucleotide data. Since most trees had congruent topologies regardless of the inclusion or exclusion of the third codon position of the COI gene, we only present trees based on the complete data set while discussing the few cases of incongruence. Phylogenies were inferred in PAUP* 4.0b.10 (Swofford 2001) and MRBAYES 3.0 (Huelsenbeck and Ronquist 2001) using four analytical approaches: maximum parsimony (MP), maximum likelihood (ML), Bayesian inference of phylogeny (BA), and neighbor joining (NJ). MP trees were estimated using a branch-and-bound or a heuristic search algorithm with 100 replicates, with sequences added at random and tree bisection–reconnection branch swapping. ML analysis employed the best-fit model of nucleotide substitution estimated by MODELTEST (Posada and Crandall 1998), a heuristic search consisting of 10 replicates with taxa added randomly and tree bisection–reconnection branch swapping. The stability of phylogenetic hypotheses was assessed with bootstrap analyses (1000 replicates for MP and NJ and 100 replicates for ML). A Bayesian phylogenetic analysis (e.g., Huelsenbeck et al. 2001) was conducted using the model of nucleotide substitution estimated by MODELTEST. One out of every 100 trees was sampled for 500 000 generations and the strict consensus tree was computed with 1000 trees excluded as the burnin. We employed the incongruence length difference test (Farris et al. 1995) to examine the significance of conflict among data sets. The test was performed in PAUP* with the partition homogeneity test and with 1000 random bipartitions analyzed by tree bisection–reconnection branch swapping on 100 random sequence addition replicates. The homogeneity of base composition across taxa was assessed using the goodness-of-fit (χ^2) test implemented in PAUP*. The timing of invasions and radiations is based on COI clock calibration of 1.4%–

2.2% sequence divergence per million years (Knowlton et al. 1993; Knowlton and Weigt 1998).

Results

Patterns of divergence

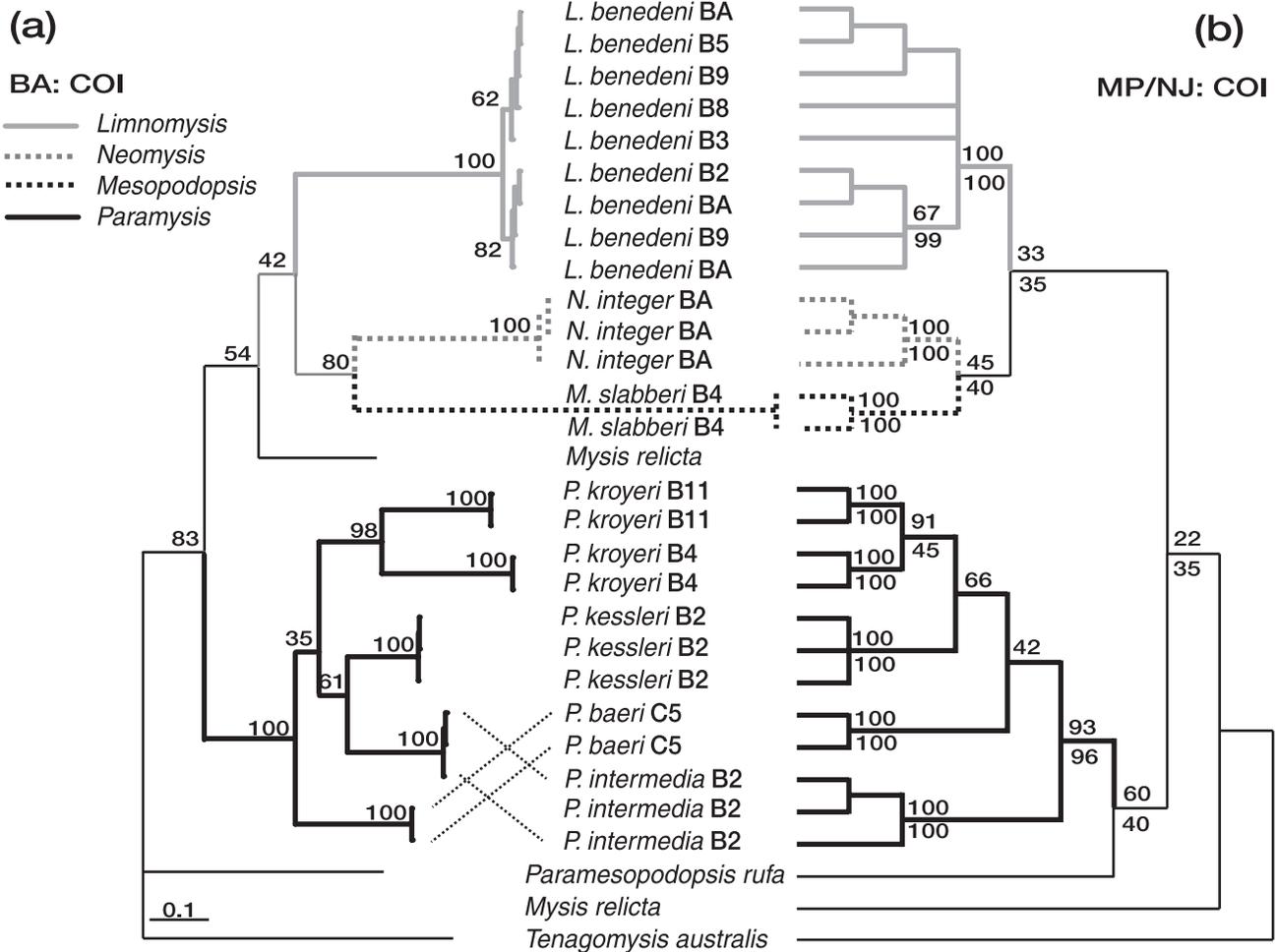
Pairwise sequence divergences were highly variable in both data sets and for all three genes. As expected, higher divergences were found for the fast-evolving COI gene than for the rRNA genes. For amphipods, Tamura–Nei nucleotide distances at COI ranged from 17% to 28% for comparisons between species, while divergences between “conspecific” populations from the Black and Caspian seas ranged from 2% to 7%. The mysids showed even higher divergences at COI with distances ranging from 19% to 49% (the lower values reflected intrageneric comparisons). Despite the very high nucleotide distances at COI in *Paramysis*, Poisson-corrected amino acid distances ranged from just 0% to 2%. Base frequencies were unequal, with the mitochondrial genes possessing a higher A–T content than the nuclear gene (Table 2). Base composition for the COI gene was homogenous across the amphipod taxa ($p = 0.99$) but was very heterogeneous among the mysids ($p < 0.001$) (Table 2). Among the three genes analyzed, the nuclear 28S gene had the highest transition to transversion ratio (1.6). The results of the partition homogeneity tests were not significant, as p values were greater than 0.05 in both groups (amphipods: $p = 0.48$; mysids: $p = 0.45$), suggesting congruence in phylogenetic signal among the genes. However, we analyzed the data sets both separately and in combination (Eernisse and Kluge 1993), enabling separate analysis for COI with a more extensive data set. The second round of analyses included a combination of COI and 16S for the mysids and a combination of COI and 28S for the amphipods. To test the monophyly of the Ponto–Caspian gammaroids, we introduced multiple outgroups in a third round of analyses.

Phylogenetic analyses

Mysids

The parsimony analyses of the COI data resulted in four equally parsimonious trees (tree length = 1066, consistency index = 0.55, homoplasy index = 0.45, retention index = 0.81). The topology of the strict consensus MP tree was similar to that of the NJ tree. The tree estimated in the Bayesian phylogenetic analyses differed from the MP and NJ phylo-

Fig. 2. (a) Strict consensus of 19 000 trees estimated in the Bayesian phylogenetic analysis (BA) for the cytochrome *c* oxidase subunit I (COI) gene for mysids. The numbers above the nodes indicate the Bayesian posterior probabilities. (b) Strict consensus of the four most parsimonious trees (tree length = 1066, consistency index = 0.55, homoplasy index = 0.45, retention index = 0.81) obtained using the maximum parsimony (MP) criterion for the COI gene. The numbers above the nodes indicate MP bootstrap support (1000 replicates), and the numbers below the nodes indicate neighbor-joining (NJ) bootstrap percentages (1000 replicates) for the nodes supported by both methods.



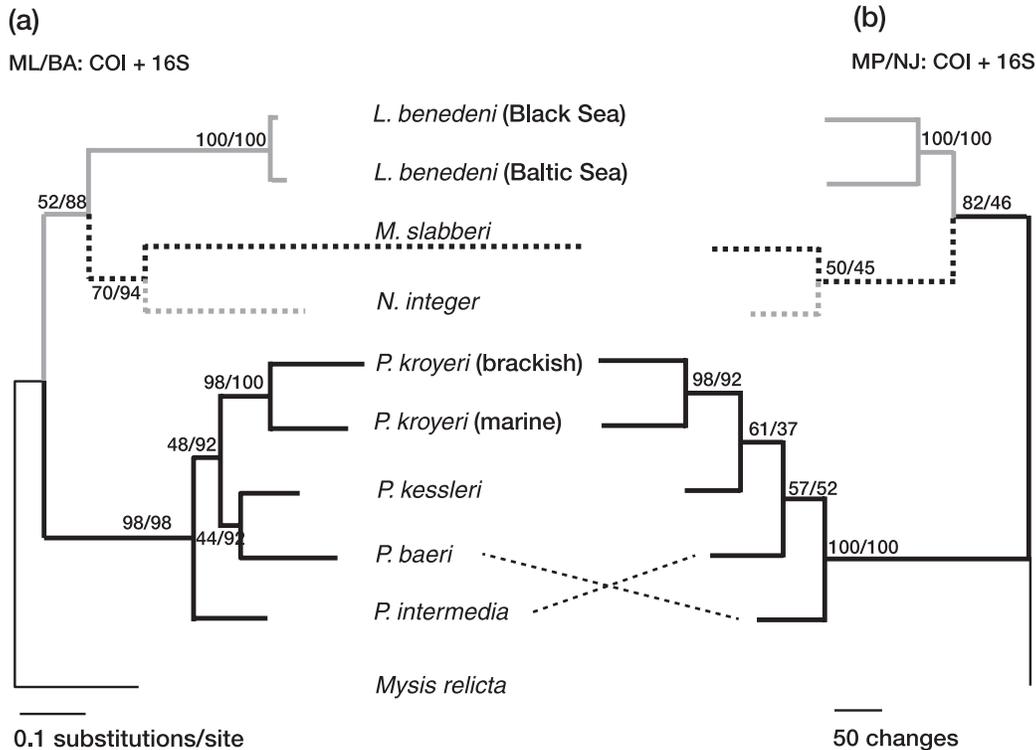
genies solely in the placement of *Mysis relicta*. By contrast, *Mysis relicta* was consistently placed as a sister group to *Paramysis* in all phylogenetic reconstructions based on only first and second codon positions (trees not shown). All analyses unambiguously supported the monophyly of the genus *Paramysis* and the affinity between *Mesopodopsis slabberi*, *Neomysis integer*, and *L. benedeni* (Fig. 2). Likewise, ML, BA, MP, and NJ analyses of the combined data set supported the monophyly of the five *Paramysis* species (Fig. 3). The rest of the genera clustered together with *L. benedeni* as sister taxon to the *Mesopodopsis slabberi* and *N. integer* group. Despite inconsistencies in the position of outgroup taxa, neither the data sets nor algorithms supported the monophyly of the Ponto-Caspian mysids with respect to the non-Ponto-Caspian taxa.

Amphipods

The strict consensus tree of the five most parsimonious trees (tree length = 900, consistency index = 0.51, homo-

plasy index = 0.49, retention index = 0.82) for the COI gene had a topology congruent with the trees estimated in both the Bayesian and NJ analyses (Figs. 4 and 5). All phylogenetic reconstructions supported the monophyly of the family Pontogammaridae as well as the monophyly of each of its genera (*Pontogammarus*, *Obesogammarus*, and *Dikerogammarus*). As predicted by morphologically based phylogenies, the genus *Echinogammarus* occupied a basal position. Within the family Pontogammaridae, *Dikerogammarus* was a sister taxon to the cluster formed by *Obesogammarus* and *Pontogammarus*. However, when the mitochondrial (COI) and nuclear (28S) genes were combined, the topology of the trees changed slightly with respect to the position of the genus *Dikerogammarus*, although statistical support for varying arrangements (bootstrap and Bayesian posterior probabilities) was weak. Additional phylogenetic analyses of the COI gene included multiple outgroups from four gammaroid families. The relationship between the ingroup taxa did not change in the BA and NJ analyses and there

Fig. 3. (a) Maximum likelihood (ML) phylogram ($-\ln$ likelihood = 6061.61) for the combined data sets (cytochrome *c* oxidase subunit I (COI) and 16S genes) for mysids. The numbers above the nodes indicate bootstrap support (100 replicates) followed by Bayesian posterior probabilities as percentages. (b) The most parsimonious tree (tree length = 1162, consistency index = 0.69, homoplasy index = 0.31, retention index = 0.48) obtained using the maximum parsimony (MP) criterion of the combined data sets (COI and 16S). The numbers above the nodes indicate MP bootstrap support followed by neighbor-joining (NJ) bootstrap percentages for the nodes supported by both methods (1000 replicates). BA, Bayesian phylogenetic analysis.



was increased statistical support for most of the clades with strong support for the monophyly of the Ponto–Caspian gammaroids, including the genus *Echinogammarus* (Fig. 6).

Discussion

Phylogenetic inferences

Ponto–Caspian mysids

All phylogenetic reconstructions in this study confirmed the monophyly of the genus *Paramysis*. However, these analyses did not fully resolve phylogenetic relationships among its component taxa (*Paramysis baeri*, *Paramysis kessleri*, *Paramysis kroyeri*, and *Paramysis intermedia*). Moreover, at a generic level, no single, completely resolved topology was favored by all analytical approaches. Certainly, MP and BA methods were not decisive owing to the low statistical support for deep nodes and the instability of the position of key taxa such as *Mysis relicta*. Although the relationship between the Ponto–Caspian genera *Limnomysis* and *Paramysis* and the related mysids (*Mysis relicta*, *N. integer*, and *Mesopodopsis slabberi*) was not resolved, the paraphyly of the Ponto–Caspian mysids (genera *Limnomysis* and *Paramysis*) was apparent, suggesting that there have been multiple invasions and radiations in the region.

The high COI divergences of 19%–27% within the *Paramysis* flock contrast with the very low allozyme distances (0.06) found by Väinölä (1995) for members of the Caspian

Mysis species flock. In fact, a surprisingly high level of sequence divergence (24%) was found between two adjacent brackish (B11) – marine (B4) populations of *Paramysis kroyeri*. This value is higher than the mean COI divergence value of 11.3% among congeneric animal species (Hebert et al. 2003) and far higher than the mean intraspecific divergence of 1%–2% often recorded in the phylogeographic literature (Avice 2000).

Ponto–Caspian gammaroids

This study supports the monophyly of the Ponto–Caspian gammaroids (genera *Dikerogammarus*, *Echinogammarus*, *Obesogammarus*, and *Pontogammarus*) as well as the monophyly of each individual genus. In addition, this study confirms the monophyly of the family Pontogammaridae while refuting the monophyly of the family Gammaridae. Since all reconstruction methods and all data sets support a close phylogenetic relationship (sister groups) between members of the family Pontogammaridae and the two *Echinogammarus* species, the assignment of the Ponto–Caspian *Echinogammarus* to the family Pontogammaridae warrants consideration. Furthermore, the genus *Pontogammarus* is paraphyletic when *Pontogammarus (Euxinia) maeoticus* is treated as a member of the genus *Euxinia*, indicating that its assignment to a new genus is not supported by molecular data. Barnard and Barnard (1983) suggested that the Ponto–Caspian gammaroids originated from a fresh–brackish water *Gammarus*-type ancestor and that the evolutionary path fol-

Fig. 4. (a) Strict consensus of 9400 trees estimated in the Bayesian phylogenetic analysis (BA) for the cytochrome *c* oxidase subunit I (COI) gene for amphipods. The numbers above the nodes indicate the Bayesian posterior probabilities followed by the neighbor-joining (NJ) bootstrap percentages (1000 replicates) for the nodes supported by both methods. (b) Strict consensus of the five most parsimonious trees (tree length = 900, consistency index = 0.51, homoplasy index = 0.82) obtained using the maximum parsimony (MP) criterion of the COI gene. The numbers above the nodes indicate bootstrap support greater than 50% (1000 replicates).

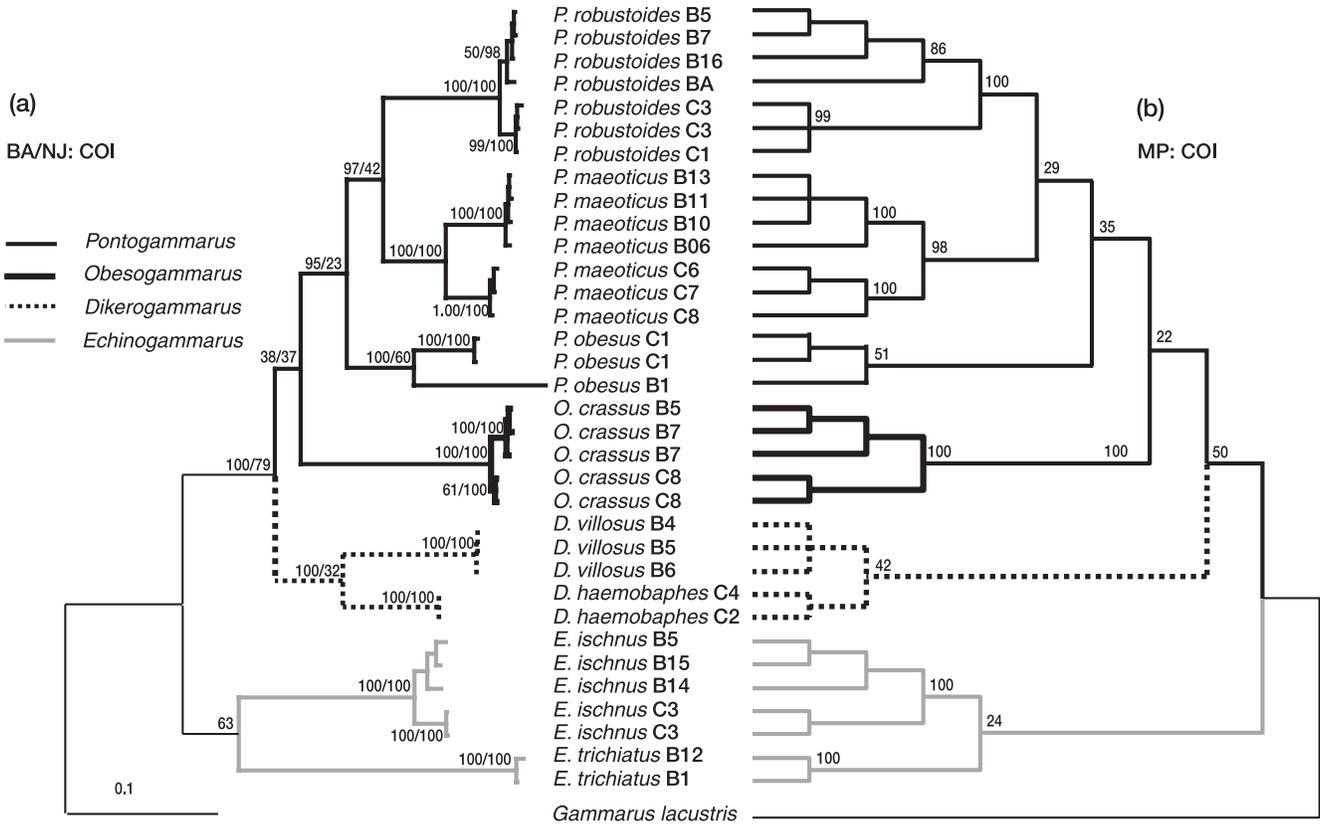


Fig. 5. (a) Maximum likelihood (ML) phylogram ($-\ln$ likelihood = 7616.56) for the total evidence (cytochrome *c* oxidase subunit I (COI) and 28S genes) for amphipods. The numbers above the nodes indicate bootstrap support (100 replicates) followed by the Bayesian posterior probabilities. (b) The most parsimonious tree (tree length = 1086, consistency index = 0.59, homoplasy index = 0.41, retention index = 0.51) obtained using the maximum parsimony (MP) criterion of the total evidence (COI and 28S). The numbers above the nodes indicate MP bootstrap support followed by neighbor-joining (NJ) bootstrap percentages for the nodes supported by both methods (1000 replicates). BA, Bayesian phylogenetic analysis.

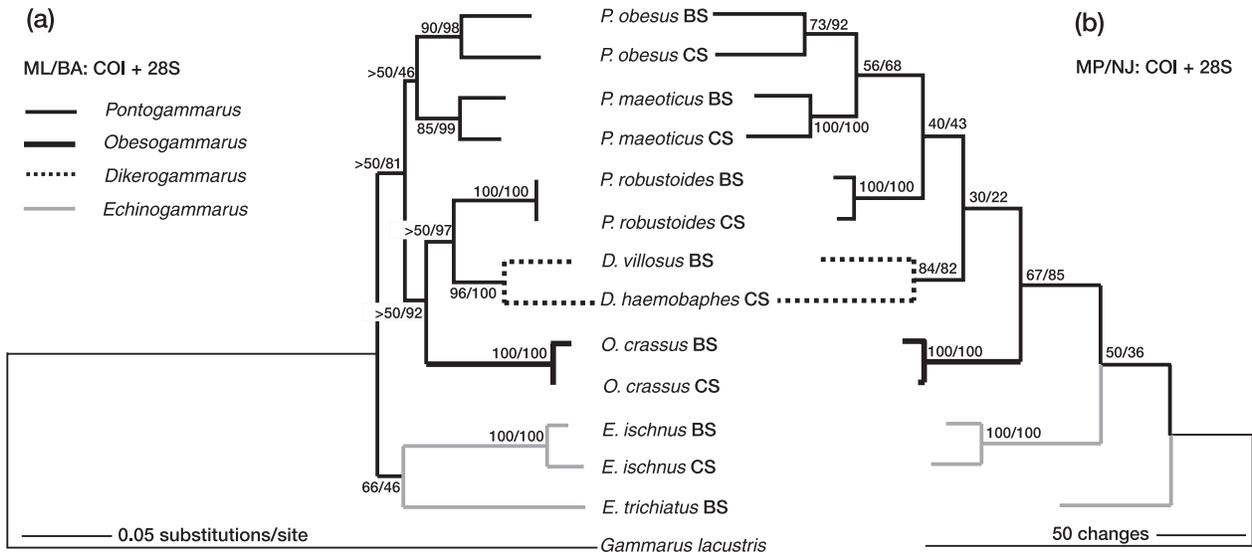
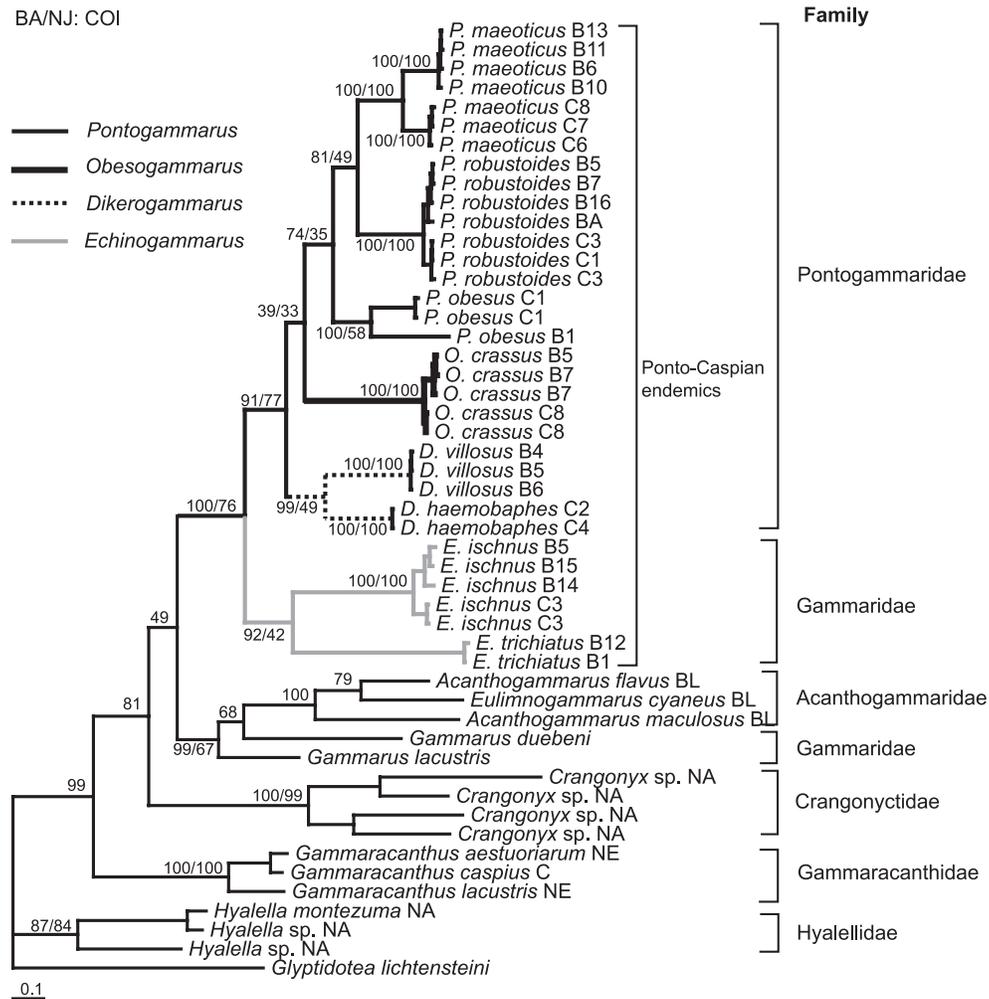


Fig. 6. Strict consensus tree estimated in the Bayesian phylogenetic analysis (BA) for the cytochrome *c* oxidase subunit I (COI) gene for amphipods. Numbers indicate the Bayesian posterior probabilities followed by the neighbor-joining (NJ) bootstrap percentages (1000 replicates) for the nodes supported by both methods.



lowed either the *Echinogammarus*–*Dikerogammarus* line or the Baikalian lineages leading to *Eulimnogammarus* and *Gmelina*. While this study did not examine *Gmelina*, our data strongly suggest that the Ponto–Caspian gammarids derive from an *Echinogammarus*-type ancestor. Certainly, the Baikalian group represented in our phylogeny by the genera *Acanthogammarus* and *Eulimnogammarus* has no direct evolutionary link with the Ponto–Caspian gammarids. Recent studies of sequence diversity in the nuclear 18S gene in Baikalian amphipods have revealed the presence of two main clades (Sherbakov et al. 1998). One consists of benthic, mostly “unarmed” taxa, while the other includes predominantly taxa with more ornamental complexity (Sherbakov et al. 1998; Sherbakov 1999). More relevant to the present study is the old age of the Baikalian generic lineages, which based on the COI calibration for the Caribbean crabs (Schubart et al. 1998) appear to be as old as the lake itself (approximately 20–30 Mys; Sherbakov et al. 1998; Sherbakov 1999). Similarly, our estimates based on the snapping shrimp COI clock (Knowlton et al. 1993; Knowlton and Weigt 1998) suggest that the age of the Ponto–Caspian lin-

eages is remarkably deep (approximately 8–16 Mys). It is likely that these lineages arose shortly after the origin of the basin (Paratethys and Sarmatian basins).

A consistent and marked genetic subdivision was detected at the “subspecific” level between populations of amphipods from the Black and Caspian seas reinforcing the role of geographic isolation and local refugia in lineage survival and evolution (Cristescu et al. 2003). For example, the level of COI divergence between populations of five amphipods (*Pontogammarus maeoticus*, *Pontogammarus robustoides*, *Pontogammarus obesus*, *Obesogammarus crassus*, and *Echinogammarus ischnus*) from the two basins varied from 2.3% to 11%, values that are significantly higher than those typical of intraspecific divergence (Avice 2000).

Geological and molecular reconciliation: age and sequence of invasions

When comparing the timing estimated by molecular results with geological evidence, we found that several cladogenesis events among amphipods and mysids appear concurrently and match various geological events. However,

these molecular clock estimates must be treated with the caution always accorded to these types of best estimates. Nevertheless, the COI divergences in this study support the conclusion that members of most peracarid species flocks diversified well in advance of the Pleistocene. While the close evolutionary relationship between members of the Caspian *Mysis* flock points to a recent (middle or late Pleistocene) intralacustrine origin (Väinölä 1995), and the extent of genetic divergence among the *Mysis relicta* species complex indicates a Pliocene radiation (Väinölä 1986; Väinölä et al. 1994; Dooh 2003), our COI data support a middle-late Miocene radiation for the *Paramysis* flock. Furthermore, it appears that the gammarid group radiated almost simultaneously. The timing provided by the COI clock corresponds to the extensive, brackish Paratethys and Sarmatian basins (16–10 and 10.5–8 Mys, respectively). These radiations have spanned a broad stretch of time, with estimates ranging from Miocene at the generic level to late Miocene throughout Pliocene at the congeneric level.

It appears that two major and contrasting evolutionary forces acted upon the gene pool of most Ponto–Caspian crustacean lineages. On one side, we observe an arresting morphological diversity between most members of species flocks (e.g., pontogammarids, paramysids, mysids, and onychopods), which is not necessarily associated with high genetic divergences, and on the other side, we detect sharp genetic subdivisions between morphologically uniform populations that inhabit ecologically distinctive habitats (Cristescu et al. 2003). A reassessment of the taxonomic status of the latter “species” is needed for a more inclusive view about the key evolutionary and ecological factors responsible for the plethora of endemics in the Ponto–Caspian region.

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