

Vicariance, colonisation, and fast local speciation in Asia Minor and the Balkans as revealed from the phylogeny of spined loaches (Osteichthyes; Cobitidae)

Jörg Bohlen^{a,*}, Anabel Perdices^{b,c}, Ignacio Doadrio^b, Panos S. Economidis^d

^a Institute of Animal Physiology and Genetics, Laboratory of Fish Genetics, Rumburská 89, 27 721 Liběchov, Czech Republic

^b National Museum of Natural History, CSIC, Department of Biodiversity and Evolutionary Biology, C/ José Gutiérrez Abascal, 2, 28006 Madrid, Spain

^c University of Lisbon, Faculty of Sciences, Department of Animal Biology, Centro de Biologia Ambiental, Bloco C-2, 3° Piso, Campo Grande, 1749-016 Lisbon, Portugal

^d Aristotle University, Laboratory of Ichthyology, P.O. Box 134, Thessaloniki 54006, Greece

Received 21 September 2005; revised 12 December 2005; accepted 13 December 2005

Available online 24 January 2006

Abstract

We reconstruct the phylogeny of the morphologically diagnosable subgenera *Bicanestrinia*, *Beysehiria*, and *Cobitis* sensu stricto of the genus *Cobitis* from Asia Minor and the Balkans. We used the complete cytochrome *b* gene of 65 specimens in order to infer their evolutionary history in this zoogeographically interesting area. Our phylogeographic analysis did not evidence the previously suggested monophyly of the *Bicanestrinia* subgenus but revealed five monophyletic lineages in the area: the lineages *Bicanestrinia* I–IV including all species of *Bicanestrinia* plus the lineage *Cobitis* s. str. The monotypic subgenus *Beysehiria* from Lake Beyşehir in Anatolia was closely related to the syntopic population of *C. turcica* and nested inside the lineage *Bicanestrinia* III. The strictly allopatric distribution of the four lineages of *Bicanestrinia* suggests that vicariance has played a major role in the diversification of *Bicanestrinia*. All analysed species of *Cobitis* s. str. from Asia Minor and Balkans were closely related to *Cobitis* s. str. from Central Europe, the Danube basin and the Caucasus, indicating at least two colonisation events into Asia Minor and the Balkans. A third, recent colonisation event led to the presence of *C. strumicae*, generally restricted to the Aegean Sea drainage, in the Danube basin. Besides the evidences of vicariance and colonisation events in the phylogenetic history of the genus *Cobitis* in Asia Minor and the Balkans, our analysis suggested also a rapid morphological evolution of *C. bilseli* in a lacustrine environment. Application of *Cobitis* mitochondrial cytochrome *b* clocks of 0.68% sequence divergence per million years (MY) suggest that the split between the five major lineages happened approximately 12.4–17.6 MYA, and according to the lack of basal resolution of this monophyletic group probably the split of all lineages happened within a narrow time window. © 2005 Elsevier Inc. All rights reserved.

Keywords: Zoogeography; Freshwater fishes; *Cobitis*; Europe; Anatolia

1. Introduction

Freshwater organisms are bound to hydrogeographic systems and their distribution is linked to the hydrogeographic history of the inhabited area. Therefore, the geological events might have differently affected the evolutionary history of freshwater organisms and terrestrial animals and

plants. One of the most important areas for the colonisation of Europe by terrestrial animals and plants are Asia Minor and the Balkans, having served first as a bridge for African mammals during Early Miocene, and second as refuges and source areas for its recolonisation after Pleistocene glaciations (Hewitt, 1999; Rögl, 1998; Rokas et al., 2003). However, their impact on the colonisation history in strictly freshwater fauna is poorly studied.

The geological history of Asia Minor and the Balkans during Cenozoic was greatly influenced by the collision of the African and Arabian plates with the Eurasian plate,

* Corresponding author. Fax: +420 315 639510.
E-mail address: bohlen@iapg.cas.cz (J. Bohlen).

promoting the closure of Tethys Sea and the upfolding of the Alpidic mountain chain along the southern margin of Eurasia (Neveeskaja et al., 1987; Rögl, 1998, 1999). The most important consequences for Asia Minor and the Balkans were the fusion of the seven Anatolian subplates, the upfolding of the Balkan, Caucasus, Pindus, and Taurus mountains and the uplift of the Central Anatolian highlands (Hrbek and Meyer, 2003; Kosswig, 1964). The complex geological history has also caused multiple connections, redirections, and truncations of water systems and their faunas in the whole area and led to multiple events of vicariance and local isolations (Economidis and Bănărescu, 1991; Hrbek et al., 2002). These geological processes have promoted speciation of freshwater fishes favouring the presence of local endemics (Hrbek et al., 2002; Kosswig, 1964). However, most of the endemic taxa are restricted to small local areas and only very few have a wide distribution. One of the few exceptions is provided by spined loaches of the subgenus *Bicanestrinia* (genus *Cobitis*; Cobitidae), which are endemic to Asia Minor and the Balkans, occurring in the whole area from western Iran to western Greece.

According to recent phylogenetic studies, the genus *Cobitis* is represented in Europe by five major mitochondrial lineages (six if the Iberian *C. calderoni* is counted as major lineage): the most distinct is the ‘Siberian lineage’ that represents the sister lineage to a group formed by the ‘Adriatic lineage’ and the subgenera *Iberocobitis*, *Bicanestrinia*, and *Cobitis* s. str. without resolved phylogenetic relationships (Ludwig et al., 2001; Perdices and Doadrio, 2001). In previous phylogenetic studies, the *Bicanestrinia* lineage appeared as a monophyletic group (Ludwig et al., 2001; Perdices and Doadrio, 2001) that occurs with some species of *Cobitis* s. str. in Asia Minor and the Balkans. *Cobitis* s. str. is the most widespread subgenus of *Cobitis* ranging from western France to the Volga in Europe and from Mongolia to Japan in Asia (Bănărescu, 1990). Within Asia Minor and the Balkans, *Cobitis* s. str. inhabits Central Greece and the Turkish Aegean basin. Besides these five mitochondrial lineages, the monotypic subgenus *Beysehiria* was recently described as an endemic lineage from the endorheic Lake Beysehir in Anatolia (Erkakan et al., 1999), but not yet studied genetically. Therefore, the spined loaches of the genus *Cobitis* include a local endemic (= *Beysehiria*), a whole-range endemic (= *Bicanestrinia*) and a Eurasian group (= *Cobitis* s. str.) in Asia Minor and the Balkans offering a great model to study the impact of vicariance, colonisation, and local isolation in freshwater fish speciation in the whole of the area. It further enables to show the phylogenetic connections of the faunas of Asia Minor and the Balkans with those of surrounding areas. Moreover, the geological complexity of Asia Minor and the Balkans made the phylogenetic relationships of these freshwater fishes an important database for the biogeographical reconstruction of the area. We use the complete mitochondrial cytochrome *b* gene to reconstruct the phylogeny of the subgenera *Beysehiria*, *Bicanestrinia*, and *Cobitis* s. str. from

Asia Minor, the Balkans and surrounding areas with special emphasis on the geographic structure. This reconstruction should enable us to draw conclusions on the origin of the subgenera of the spined loaches and the major evolutionary mechanisms shaping their biogeographical patterns.

2. Materials and methods

2.1. Taxon sampling and laboratory protocols

An overview about the geographic origin of the samples and GenBank accession numbers can be found in Table 1 and Fig. 1. A total of 65 specimens of spined loaches were analysed. The material for this study includes 10 species of *Bicanestrinia*: *C. arachthosensis*, *C. elazigensis*, *C. hellenica*, *C. levantina*, *C. linea*, *C. meridionalis*, *C. punctilineata*, *C. strumicae*, *C. trichonica*, and *C. turcica*; the only species of *Beysehiria* (*C. bilseli*) and three species of *Cobitis* s. str. from the Aegean region: *C. fahirae*, *C. stephanidisi*, and *C. vardarensis*. From neighbouring areas, we included three species and one undescribed species of *Cobitis* s. str.: *C. elongatoides*, *C. taenia*, *C. tanaitica*, and *C. sp.*; four species of the ‘Adriatic lineage’ (*C. bilineata*, *C. elongata*, *C. ohridana*, and *C. zanandreae*) and one species, *C. melanoleuca*, of the ‘Siberian lineage’. Some more distantly related European loach fishes served as outgroup taxa (*Sabanejewia balcanica*, *S. larvata*, and *Misgurnus fossilis*).

DNA was extracted from fin or muscle tissue with chloroform-phenol standard methods (Palumbi et al., 1991; Sambrook et al., 1989). Amplification protocols were performed as previously described (Perdices and Doadrio, 2001). The entire cytochrome *b* gene (1140 bp) was PCR amplified using the primers GluDG.L (5’ TGA CT TGA AR AACCA YCGTTG 3’; Palumbi, 1996) and H16460 (5’ CGAYC TTCGG ATTAA CAAGA CCG 3’; <http://nmg.si.edu/bermlab.htm>). The purified PCR band was used as template in a cycle sequencing reaction using the dRhodamine terminator cycle sequencing kit (PE Applied Biosystems). In some cases, the internal primer Cb3H (5’ GGCAA ATAGG AARTA TCATT C 3’; Palumbi, 1996) was also used for sequencing.

2.2. Phylogenetic analysis

Sequences were aligned and revised with Sequencher ver. 4.0 (Gene Codes). No length variation was found in any of the mtDNA genes analysed. Nucleotide composition was examined for variable sites, and the χ^2 homogeneity test of base frequencies was done in PAUP* v. 4.0b10 (Swofford, 2002) for all positions. Nucleotide saturation was analysed by plotting uncorrected p distances at 1st, 2nd, and 3rd codon position against absolute distance values. Relations between genotypes were resolved by distance methods with Sequencher 6.1 (<http://nmg.si.edu>).

Phylogenetic trees were reconstructed using maximum parsimony (MP) and Bayesian criteria. In MP analysis,

Table 1

Map No.	Species	River, Drainage, Country	Indiv.No.	Accession No.
Bicanestrinia				
1	<i>C. arachthosensis</i>	R. Arachthos, Arachthos, GR	162	AF263088*
			199	AY191581
2	<i>C. hellenica</i>	R. Louros, Louros, GR	156	AY191583
3	<i>C. hellenica</i>	R. Thiamis, Thiamis, GR	96	AF263087*
			219	AY191582
4	<i>C. trichonica</i>	L. Trichonis, Trichonis, GR	1178	AF263085*
			1179	AF263086*
5	<i>C. meridionalis</i>	L. Prespa, Prespa, GR	117	AF263083*
			120	AF263084*
6	<i>C. punctilineata</i>	R. Strymon, Strymon, GR	1486	AY191579
7	<i>C. punctilineata</i>	R. Angitis, Strymon, GR	1488	AY191580
8	<i>C. strumicae</i>	R. Strymon, Strymon, GR	1428	AY191578
9	<i>C. strumicae</i>	R. Kompsiates, Kompsiates, GR	1609	AY191573
10	<i>C. strumicae</i>	R. Macropotamus, Filiouris, GR	1659	AY191574
11	<i>C. strumicae</i>	R. Maritza (= Evros), Maritza, BG	18	DQ217372
12	<i>C. strumicae</i>	R. Erithropotamus, Evros, GR	1758	AY191576
13	<i>C. strumicae</i>	R. Maritza (= Evros), Maritza, BG	1799	AY191577
14	<i>C. strumicae</i>	R. Maritza (= Evros), Maritza, GR	1717	AY191575
15	<i>C. strumicae</i>	R. Ergene, Maritza, TR	228	DQ217373
16	<i>C. strumicae</i>	R. Vit, Danube, BG	357	DQ217374
17	<i>C. turcica</i>	Kirk Göz spring, Kirk Göz, TR	239	DQ217375
18	<i>C. turcica</i>	Creek at Antalya, Aksu, TR	220	DQ217376
19	<i>C. turcica</i>	L. Beysehir, L. Beysehir, TR	364	DQ217377
			222	DQ217378
			223	DQ217379
20	<i>C. turcica</i>	L. Sögüt Gölü, L. Sögüt Gölü, TR	227	DQ217380
21	<i>C. turcica</i>	Creek at Düger, L. Burdur, TR	226	DQ217381
22	<i>C. turcica</i>	Channal at Ovaciftlik, Sultansazligi Swamps, TR	376	DQ217382
23	<i>C. turcica</i>	Gemic spring, L. Aci Gölü, TR	28	DQ217383
24	<i>C. elazigensis</i>	R. Muhrad Nehri, Euphates, TR	370	DQ217384
			372	DQ217385
25	<i>C. cf. levantina</i>	Channal, R. Orontes, TR	380	DQ217386
			382	DQ217387
26	<i>C. linea</i>	Creek at Doroodzan, R. Kor, IR	4618	DQ217388
			4619	DQ217389
			4620	DQ217390
Beysehiria				
27	<i>C. bilseli</i>	L. Beysehir, L. Beysehir, TR	21	DQ217391
			362	DQ217392
			363	DQ217393
Cobitis s. str.				
28	<i>C. cf. fahirae</i>	Ditch at Bergama, Bakir, TR	159	DQ217394
			373	DQ217395
29	<i>C. spec.</i>	R. Rioni, Rioni, GE	244	DQ217396
30	<i>C. vardarensis</i>	R. Gallikos, Gallikos, GR	323	AF263079*
			2087	AY191568
31	<i>C. vardarensis</i>	R. Vardar, Vardar, GR	2086	AY191569
32	<i>C. vardarensis</i>	R. Agiaki, Vardar, GR	370	AF263080*
33	<i>C. vardarensis</i>	R. Aliakmon, Aliakmon, GR	2096	AY191570
34	<i>C. stephanidisi</i>	Velestino spring, Velestino, GR	743	AY191571
			744	AY191572
(35)	<i>C. taenia</i>	R. Weser, Haaren Creek, D	1	AF263077*
			2	AF263078*
36	<i>C. taenia</i>	R. Vistula, Vistula, PL	113	AY191565
37	<i>C. elongatoides</i>	R. Timis, Danube, RO	95	AF263081*
			98	AF263082*
38	<i>C. elongatoides</i>	R. Ialomitza, Danube, RO	52	AY191566
39	<i>C. elongatoides</i>	R. Moldova, Danube, RO	182	AY191567
40	<i>C. tanaitica</i>	L. Sinoe, L. Sinoe, RO	1	DQ217397
			3	DQ217398

Table 1 (continued)

Map No.	Species	River, Drainage, Country	Indiv.No.	Accession No.
Adriatic lineage				
(41)	<i>C. zanandreae</i>	Lago di Fondi, Fondi, I	11	AF263089*
(42)	<i>C. bilineata</i>	R. Reno, Reno, I	66	AF263091*
(43)	<i>C. bilineata</i>	R. Esino, Esino, I	126	AF263090*
44	<i>C. ohridana</i>	R. Aoos (= Vjose), Aoos, GR	200	AY191563
			201	AY191564
45	<i>C. elongata</i>	R. Nera, Danube, RO	114	AF263057*
Siberian lineage				
46	<i>C. melanoleuca</i>	R. Sinukha, Kuban, RUS	1187	AF263068*
Outgroup				
Mf	<i>Misgurnus fossilis</i>	R. Dye, Danube, CZ		AF263097*
Sr	<i>Sabanejewia romanica</i>	R. Bratia, Danube, RO		AF263095*
Sb	<i>S. balcanica</i>	R. Timis, Danube, RO		AF263093*

Country codes: BG, Bulgaria; CZ, Czech Republic; D, Germany; GE, Georgia; GR, Greece; I, Italy; IR, Iran; PL, Poland; RO, Romania; RUS, Russia; TR, Turkey. *Sequences recovered from GenBank (Perdices and Doadrio, 2001). Map numbers in brackets are outside the range of Fig. 1.

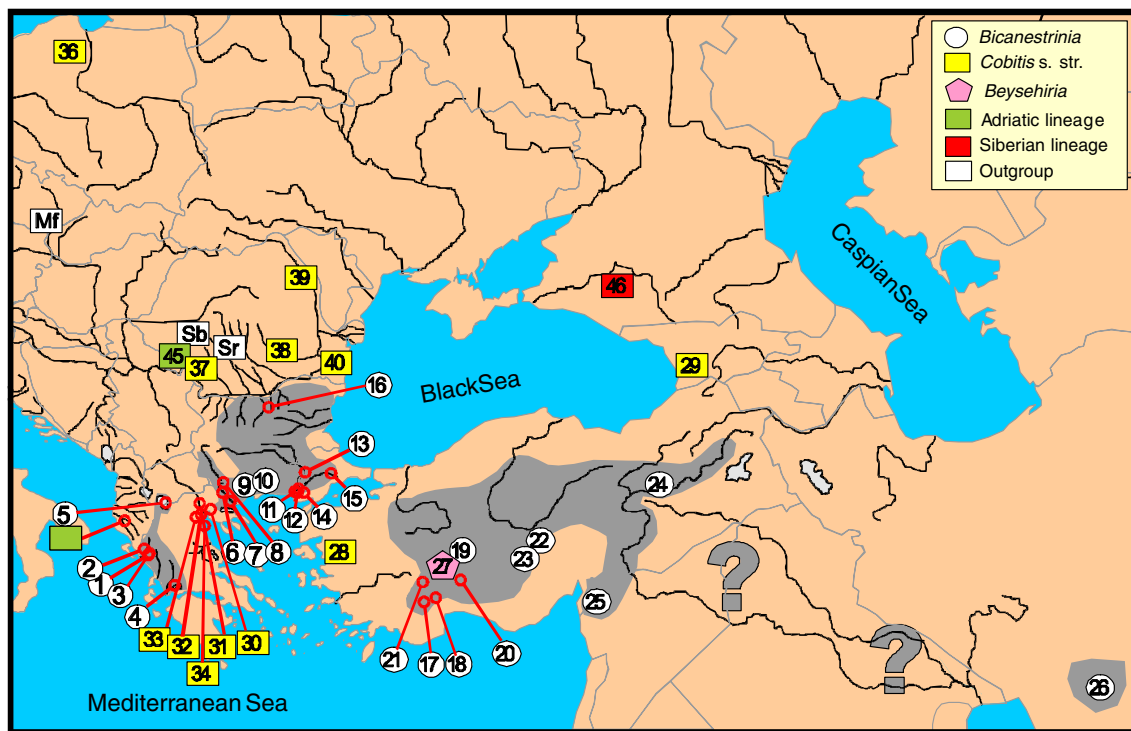


Fig. 1. Map of Europe and Asia Minor showing the known distribution area of *Bicanestrinia* loaches (dark shaded area) and the localities of samples included into the present study. Numbers in the symbols represent the sample number and correspond to the numbers given in Table 1. Question marks stand where no information about distribution of bicanestrine species is available.

only minimal trees were retained and zero length branches were collapsed. Different weighting schemes were employed to adjust for transitional bias (8:1, 6:1, and 4:1 equal weights). In all cases, MP analysis was performed using heuristic searches with TBR branch swapping and 100 replicates of random addition of taxa. MP analysis was implemented in PAUP* with 1000 bootstrap replicates to assess support of branches. We used the program Model test 3.06 (Posada and Crandall, 1998) to find the best model of evolution that fits our data for Bayesian analysis. Bayesian inference was generated using MrBayes 3.0 (Huelsenbeck

and Ronquist, 2001) assuming the General Time Reversal model of evolution to run 1,000,000 generations of four simultaneous Monte Carlo Markov chains. The temperature parameter was set to 0.2 sampling trees every 100 generations. Log-likelihood stability was reached after 30,000 generations; the 300 first trees were excluded using the remaining trees to compute a 50% majority-rule consensus tree. Posterior-probability values were used to assess support of branches. We used the g1 statistic to determine if phylogenetic signal was significantly non-random in the complete dataset (Hillis and Huelsenbeck, 1992).

To examine whether a particular tree topology corresponded to a significantly better interpretation of the data than an alternative tree, we used the Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999), as implemented in PAUP*.

Nucleotide rate homogeneity of cytochrome *b* sequences across taxa was assessed using a χ^2 test of a ML log-likelihood ratio test of a clock-enforced and a non-enforced trees (Page and Holmes, 1998) performed in PAUP*. Under the assumption of constant rate of nucleotide substitution, we converted uncorrected *p* genetic distances calculated from the clock enforced tree to absolute time using a calibration calculated for the subgenus *Iberocobitis* assuming a sequence divergence (*p* distances) for the cytochrome *b* gene of 0.68% per million years based on the opening of the Strait of Gibraltar 5.3 million years ago (Doadrio and Perdices, 2005).

3. Results

Base frequencies were homogeneous across taxa and did not differ significantly among species ($\chi^2 = 54.36$, degrees of freedom (df) = 201, $p = 1.0$). There were 416 variable sites (353 parsimony informative) in the complete cytochrome *b* sequence (1140 bp) of 68 individuals. The majority of variable (27.8%) and informative (24.8%) sites are third positions. Model test selects the GTR+I+G model under the AIC criterion with base frequencies (A = 29.7%, C = 27.11%, G = 12.59%, T = 30.6%), Nst = 6, substitution rates: (A-C 1.12, A-G 21.66, A-T 1.70, C-G 0.09, C-T 17.96, G-T 1.0), gamma parameter = 1.1260 and invariable positions = 0.5266.

All phylogenetic reconstructions have well-supported and highly congruent topologies (Fig. 2). MP trees using 8:1, 6:1, 4:1 or equal weights were very similar, and we use the empirical Ts/Ti ratio (~8) for *Cobitis* to calculate MP trees. Our phylogenetic analyses identified the Siberian *C. melanoleuca* as the sister taxon to all other *Cobitis* species (96% bootstrap (bo) and 100 posterior probability (pp)), which are divided into six well-supported monophyletic mitochondrial groups (>87% bo and pp): *Bicanestrinia* I–IV and *Cobitis* s. str. V, and the Adriatic lineage (Fig. 2). Although all analyses recovered six monophyletic lineages with the Adriatic lineage always as the basal group, the phylogenetic relationships between *Bicanestrinia* I–IV and *Cobitis* s. str. V were not resolved. Only Bayesian analysis identified *Bicanestrinia* I and IV as sister lineages (71 pp). Saturation plots of 1st, 2nd, and 3rd codon positions against absolute distances were linear with third positions accumulating most rapidly (data available on request from authors) indicating that unresolved relationships did not result from nucleotide saturation.

We observed considerable haplotypic divergence (uncorrected *p*) between the Adriatic lineage, *Bicanestrinia* I–IV and *Cobitis* s. str. V (mean 11.6 ± 0.8 , range 10.3–12.4), and between *Bicanestrinia* I–IV and *Cobitis* s. str. V (mean $10.3\% \pm 1.3$; range 8.4–12.0%). However, the divergence

among species within each lineage is relatively low (mean $4.5\% \pm 2.9$) (Table 2). *Bicanestrinia* Lineage I or *Bicanestrinia* I contains the *Bicanestrinia* species from Eastern Turkey and Iran. *Bicanestrinia* II comprises all *Bicanestrinia* species inhabiting Western Greece (Adriatic and Ionian Seas). *Bicanestrinia* III includes all *Bicanestrinia* species inhabiting Eastern Greece and Bulgarian rivers draining into the Aegean basin including the population of *C. strumicae* from the Danubian basin. *Bicanestrinia* IV includes the *Bicanestrinia* species *C. turcica* from the Central Anatolian highlands and *C. bilseli* of the subgenus *Beysehiria*. The hypotheses of monophyly of *Beysehiria* and *C. turcica* are rejected (Shimodaira–Hasegawa test $p = 0.05$, $-\ln L = 215.1$ and 146.3, respectively). *Cobitis* s. str. or Lineage V includes all species of *Cobitis* s. str. from central Greece, western Turkey, western Caucasus, Central Europe, and the Danube basin. The *g*₁ statistic showed some phylogenetic signal for the complete dataset (*g*₁ = -0.5). Sequence divergence between individuals of the same species is generally low (mean 0.6%, range 0.0–2.0%). The exception is the specimens of *C. turcica* that differ by a mean sequence divergence of 4.8% (0.0–7.3%).

A log-likelihood test failed to reject the null hypothesis of evolutionary rate constancy across all *Cobitis* mtDNA haplotypes ($-\ln L = 8641.95$ and 8681.5; $\chi^2 = 79.26$, df = 66, $p = 0.1$). Therefore, mtDNA divergence can be used to calculate the divergence time of the analysed mtDNA lineages. According to these calculations, the divergence between the Siberian lineage and the rest of the lineages happened about 20–24 million years ago (MYA). The divergence between the Adriatic lineage and the Lineages I–V is dated back to 15–19.4 MYA, while the radiation of the *Bicanestrinia* I–IV and *Cobitis* s. str. V lineages happened about 12.4–17.6 MYA.

4. Discussion

4.1. Phylogenetic implications

Our phylogenetic analysis of the complete cytochrome *b* gene confirms *C. melanoleuca* as the sister lineage of all other *Cobitis* from Europe and Asia Minor (Perdices and Doadrio, 2001). Further on, our data resolved the phylogenetic position of the Adriatic lineage, showing that it is the sister lineage to all remaining species. However, our results do not resolve the monophyly proposed for the subgenus *Bicanestrinia* (Ludwig et al., 2001; Perdices and Doadrio, 2001). Our data identified four well-supported *Bicanestrinia* I–IV mtDNA lineages related to the *Cobitis* s. str. lineage V in the same monophyletic group (Fig. 2). This finding indicates a much higher diversification among the *Bicanestrinia* than formerly found with a restricted number of species analysed (Ludwig et al., 2001; Perdices and Doadrio, 2001). According to the lack of basal resolution combined with the absence of nucleotide substitution and the phylogenetic signal found in our data, the splitting between the four *Bicanestrinia* I–IV lineages and *Cobitis* s. str. V probably

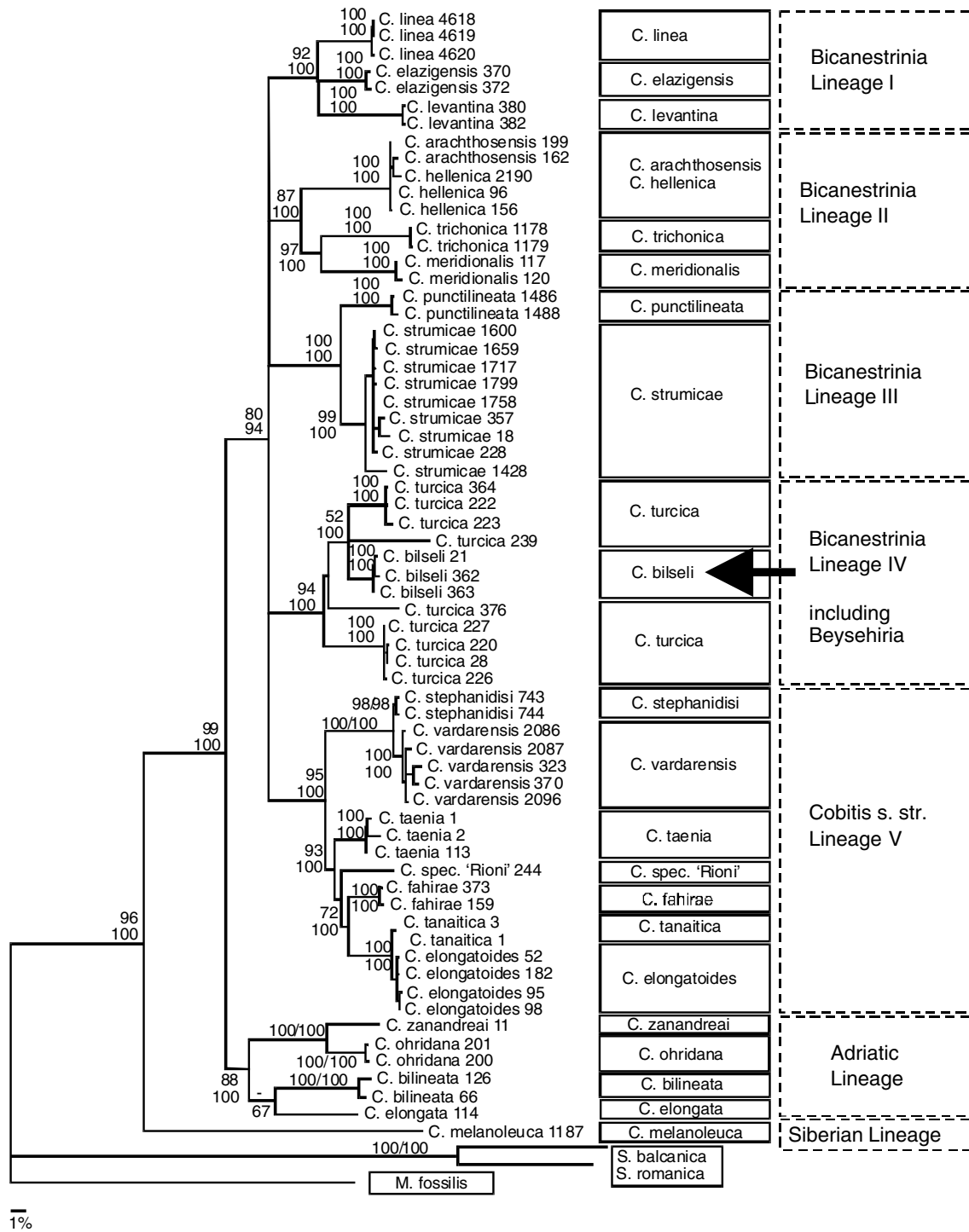


Fig. 2. Phylogenetic relationships based on the *cyt b* gene among spined loaches (genus *Cobitis*) from Asia Minor and the Balkans and some selected species from neighbouring areas. The phylogeny is the single tree recovered using weighted parsimony 8Ti:1Tv, and Bayesian methods. Upper values on the branches represent MP bootstrap and posterior probability values. Boxes indicate the phylogenetic lineages as found in this study, an arrow points on the only species of the subgenus *Beysehiria*.

happened within a narrow time window, making difficult to resolve their basal phylogenetic relationships. However, the likelihood of potential scenarios could be discussed related with the single morphological character that defines the *Bicanestrinia*: the derived character state of a duplication of the *lamina circularis* or ‘scale of Canestrini’, an ossified plate on the pectoral fin rays of males. The primitive condi-

tion of this character is a single *lamina circularis* (Bacescu, 1962) as observed in the Siberian and Adriatic *Cobitis* lineages as well as in other cobitid genera like *Misgurnus* and *Iksookimia*. Therefore, it is more parsimonious to assume that *Bicanestrinia* I to IV stemmed out from a common bicanestrine ancestor than to assume that *Cobitis* s. str. V stem out from a bicanestrine ancestor. It would require an

Table 2
Values below diagonal: mean uncorrected p distances \pm standard deviation and (range) between the identified mitochondrial lineages of *Cobitis*; values in the diagonal: mean uncorrected p distances \pm standard deviation and (range) within the identified mitochondrial lineages

	Bicanestrinia I Mesopotamia	Bicanestrinia II Greece	Bicanestrinia III Balkan	Bicanestrinia IV Anatolia	<i>Cobitis</i> s. str. V	Adriatic lineage	Siberian lineage
Bicanestrinia I	4.8 \pm 2.7 (0.0–7.3)						
Bicanestrinia II	9.7 \pm 0.5 (8.9–10.8)	5.7 \pm 3.9 (9.5–9.2)					
Bicanestrinia III	9.1 \pm 0.3 (8.8–10.0)	10.1 \pm 0.3 (9.5–11.1)	1.9 \pm 2.0 (0.0–5.6)				
Bicanestrinia IV	10.1 \pm 0.8 (9.0–11.8)	9.7 \pm 0.6 (8.7–11.4)	9.5 \pm 0.5 (8.9–11.9)	4.7 \pm 2.3 (0.0–7.3)			
<i>Cobitis</i> s. str. V	10.1 \pm 0.8 (9.0–11.8)	10.0 \pm 0.6 (8.6–11.2)	9.7 \pm 0.5 (8.4–11.2)	9.8 \pm 0.7 (8.5–12.0)	4.7 \pm 2.5 (0.0–7.6)		
Adriatic lineage	11.5 \pm 0.5 (10.7–12.3)	11.7 \pm 0.6 (10.4–12.9)	11.1 \pm 0.5 (10.3–13.2)	11.7 \pm 0.6 (10.3–13.2)	11.6 \pm 0.4 (10.7–12.4)	7.8 \pm 3.5 (0.2–10.4)	
Siberian lineage	14.2 \pm 0.7 (13.5–14.8)	15.2 \pm 0.4 (14.7–15.7)	14.2 \pm 0.5 (13.8–15.2)	14.5 \pm 0.3 (13.6–14.8)	14.4 \pm 0.3 (14.1–15.0)	15.7 \pm 0.4 (15.3–16.3)	—

additional evolutionary step, namely a secondary loss of the *lamina circularis* in the *Cobitis* s. str. lineage V. Even more evolutionary steps would have to be considered under the scenario of a multiple parallel duplication of the *lamina circularis*. We here consider the most parsimony solution as the most likely possibility, and we propose the *Cobitis* s. str. lineage V to be the sister group of the four *Bicanestrinia* I–IV lineages.

According to our mtDNA data, most of the morphologically defined species of *Cobitis* turned out to be well differentiated. However, we found low genetic distance between *C. arachthosensis* and *C. hellenica* (mean 0.3 ± 0.2), which were already considered as two subspecies of the same species in their original description (Economidis and Nalbant, 1996). On the other hand, a remarkable genetic distinctiveness was observed within the bicanestrine specimens of *C. turcica* from southern and Central Anatolia. Similar high molecular diversity was observed in the cyprinodontid genus *Aphanius* across its Anatolian populations (Hrbek et al., 2002). Moreover, these authors found a correlation between the distribution of the monophyletic lineages identified for *Aphanius* with the different geologic and biogeographic units defined in Anatolia. Although the distribution of *C. turcica* is much more restricted than that of *Aphanius* and our sampling was more limited, we suggest a similar correlation of *C. turcica* and *Aphanius* upon a more detailed investigation.

4.2. Phylogenetic position of *Beysehira*

For the first time, the subgenus *Beysehira* was studied under a molecular perspective. The sister-relationship between its only species *C. bilseli* and the syntopic population of *C. turcica* strongly contradicts the results based on morphologic characters (Erkakan et al., 1999). *Beysehira* shows unique morphological characters among spined

loaches: the three-dimensional shape of the *lamina circularis*, the presence of skin prickles on the pectoral fins and sex-specific genital papillae (Erkakan et al., 1999). In addition, *C. bilseli* differs from the syntopic bicanestrine *C. turcica* in most diagnostic characters used for cobitid identification: the absence of duplication of the *lamina circularis*, the pigmentation pattern, the shape of the body scales and of the suborbital spine, the folding of the mental lobes and the exceptional large body size (Erkakan et al., 1999). To explain the enormous discrepancy between morphologic and genetic data one would have to consider either an extraordinarily rapid morphological evolution of *C. bilseli* not encompass with rapid genetic differentiation as it was found in other cases of sympatric lacustrine speciation, e.g., among cichlids in Lake Victoria and Lake Malawi (Albertson et al., 2003; Schlieven et al., 1994), among barbs in Lake Tana (Nagelkerke and Sibbing, 1997), and pupfishes in Laguna Chichancanab (Humphries and Miller, 1981; Humphries, 1984) or a mitochondrial introgression by the syntopic *C. turcica* into *C. bilseli*. Purely mitochondrial characters are unsuited to decide between these two competing hypotheses, therefore this topic has to await further investigation.

4.3. Biogeography

A single *lamina circularis* is present in most lineages of spined loach (Siberian lineage, Adriatic lineage, *Iberocobitis*, *Cobitis* s. str.), in the cobitid genera *Misgurnus* and *Ikso-okimia* as well as in the fossil *Cobitis centrochir* from Middle Miocene in Germany (Frickhinger, 1991), suggesting that the ancestor of current cobitids most likely exhibited one *lamina circularis*. Our phylogenetic analyses agreed with this hypothesis due to the most basal groups were lineages with one *lamina circularis*: *C. melanoleuca* and some of the species included in the Adriatic lineage (*C. bilineata*,

C. ohridana, and *C. zanandreae*). The mtDNA phylogeny supports an early Adriatic split, and the application of the molecular clock indicates that the age of this Adriatic split occurred between 15 and 19 MYA. These dates match the geologically based dates of the Alpidic orogenesis, which led to the upfolding of the Alps, Dinaric, Balkan, and Caucasus mountains (Rögl, 1999). Our mtDNA data suggest that the separation between *Bicanestrinia* I–IV and *Cobitis* s. str. V lineages occurred approximately 2–3 million years later (12–17 MYA), a time when the land connection between Central Europe and Anatolia broke (Rögl, 1998; Weisrock et al., 2001). Our phylogeny did not provide evidences about the geographical origin of the genus *Cobitis* but owing to the fact that the *Bicanestrinia* I–IV are geographically restricted to the Balkans and Anatolia region, this comparative restricted distribution suggests that the *Bicanestrinia* originated in these areas with a certain gene flow. The *Bicanestrinia* I–IV are distributed from Mesopotamia to the Adriatic basin with the exception of mountainous areas, the Vardar system, and the Turkish Aegean drainage (Fig. 3). In the later two areas occur species of the *Cobitis* s. str. V, a lineage otherwise occupying areas north of the Mediterranean. No overlap exists between the areas of distribution of the *Bicanestrinia* I–IV lineages. Under this scenario, vicariant events, namely the uplift of mountain ridges due to the continuous pressure of the African plate during the Miocene (Macey et al., 1998; Rögl, 1998; Weisrock et al., 2001), promoted the isolation of the *Bicanestrinia* I–IV from each other and from *Cobitis* s. str. V. Local co-occurrence of *Bicanestrinia* loaches and other

Cobitis can be only found along the Bulgarian Black Sea drainage and in some Bulgarian tributaries of the Danube, where the distribution areas of the bicanestrine *C. strumicae* overlaps with that of Danubian *Cobitis* species. Exclusive distribution ranges and lack of co-occurrence has been described as rather typical for spined loaches across Europe by Bohlen and Ráb (2001) and was ascribed both to vicariance effects of river history and a large ecological niche overlap of similar species. However, subsequent and reciprocal faunal exchanges with the Danubian basin happened at least three times, while local speciation gave rise to the subgenus *Beysehiria* in Beysehir Lake. The first Danubian faunal exchange probably was a river capture of a tributary of the Morava River (Danubian) by the upper Vardar River (Bănărescu, 1960; Economidis and Bănărescu, 1991; Karakousis et al., 1995). A second exchange was illustrated by the Danubian influence of the coastal rivers of the Black Sea until the Turkish Aegean basin (Bănărescu, 1960, 1990; Economidis and Nalbant, 1996). Most likely, the *Bicanestrinia* loaches in this area were replaced by *Cobitis* s. str., otherwise it is difficult to understand the absence of *Bicanestrinia* in the Eastern Aegean basin. A *Cobitis* exchange from the Vardar system to the Eastern Aegean basin appears to be more unlikely due to the closer phylogenetic relationships of the *Cobitis* from western Turkey with lower Danubian *Cobitis* species than to *Cobitis* species from Eastern Greece. Moreover, *Cobitis* s. str. has no circum-Aegean distribution as it is found in some other freshwater fishes of Danubian origin (e.g., *Rhodeus*, *Rutilus*, and *Scardinius*) due to the repeated sea level lowering during Pleistocene

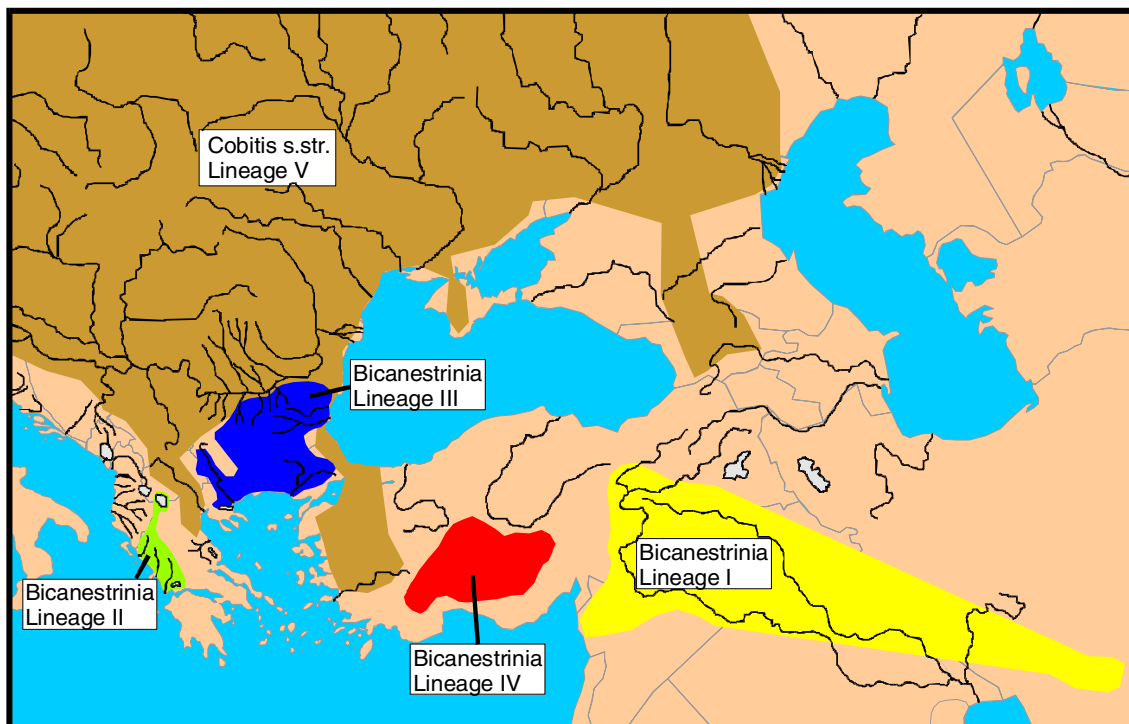


Fig. 3. Geographic distribution of the five identified ingroup lineages. Current knowledge about the distribution of spined loaches indicates no contact between the lineages *Bicanestrinia* I–IV.

glaciations that connected river systems and homogenised the ichthyofauna around the Aegean Sea (Economidis and Nalbant, 1996; Erkakan et al., 1999). The third and probably most recent faunal exchange is evidenced by the *Bicanestrinia* species *C. strumicae* inhabiting the Balkans Aegean basin and currently distributed across the coastal rivers along the Black Sea and some southern tributaries of the Danube. The small genetic distinctiveness observed between *C. strumicae* from Aegean and Danubian localities as well as no occurrence of this species in northern tributaries of the Danube (e.g., in Romania) suggest a postglacial exchange. In conclusion, vicariance seems to have played a major role in the diversification of *Bicanestrinia* loaches in Asia Minor and the Balkans area, whereas colonisation events and local speciation were geographically restricted to some species.

Acknowledgments

We thank P.P. Bănărescu, M. Breil, J.A. Carmona, B. Coad, J. Cubo, I.S. Dobrovolov, H.R. Esmacili, B. Güler, P. Ivanova, L. Kalous, A. Marchordom, D. Memiş, M. Mrakovcic, T. Nalbant, M. Povz, and V. Šlechtová jr for their help with obtaining samples as well as F. J. Garitagoita, F. Alda, L. Alcaraz, and V. Šlechtová jr, for help in the laboratory work. The study was supported by Grants 206/05/2556 and 206/00/0668 of the Grant Agency of the Czech Republic, Grants S5045111 and IAA600450508 of the Grant Agency of the Academy of Sciences of the Czech Republic and by the European Commission HUMAN POTENTIAL PROGRAMME under BIODIBERIA at the Museo Nacional de Ciencias Naturales in Madrid. A.P. was supported by a FCT grant (SFRH/BPD/14367/2003). The study was partially supported by POCI/BIA-BDE/56073/2004 and by the IRP IAPG No. AV0Z50450515.

References

- Albertson, R.C., Streebman, J.T., Kocher, T.D., 2003. Directional selection has shaped the oral jaws of Lake Malawi cichlid fishes. *Proc. Natl. Acad. Sci. USA* 100, 5252–5257.
- Bănărescu, P.M., 1960. Einige Fragen zur Herkunft und Verbreitung der Süßwasserfischfauna europäischmediterranean Unterregion. *Archiv. Hydrobiol.* 57, 16–134.
- Bănărescu, P., 1990. Zoogeography of Fresh Waters. Vol. 1, AULA-Verlag, Wiesbaden.
- Bohlen, J., Ráb, P., 2001. Species and hybrid richness in spined loaches of the genus *Cobitis* L. (Teleostei: Cobitidae), with a checklist of European forms and suggestions for their conservation. *J. Fish Biol.* 59a, 75–89.
- Doadrio, I., Perdices, A., 2005. Phylogenetic relationships among the Ibero-African cobitids (*Cobitis*, Cobitidae) based on cytochrome *b* sequence data. *Mol. Phylogenet. Evol.* 37, 484–493.
- Economidis, P.S., Bănărescu, P., 1991. The distribution and origins of freshwater fishes in the Balkan Peninsula, especially in Greece. *Int. Rev. Ges. Hydrobiol.* 76, 257–283.
- Economidis, P.S., Nalbant, T.T., 1996. A study of the loaches of the genera *Cobitis* and *Sabanejewia* (Pisces, Cobitidae) of Greece, with description of six new taxa. *Trav. Mus. Natl. Hist. Nat. Grigore Antipa* 26, 295–347.
- Erkakan, F., Atalay-Ekmekci, G., Nalbant, T.T., 1999. A review of the genus *Cobitis* in Turkey (Pisces: Ostariophysii: Cobitidae). *Hydrobiologia* 403, 13–26.
- Frickhinger, K.A., 1991. Fossilien Atlas Fische. Mergus Verlag, Melle.
- Hewitt, G.M., 1999. Post-glacial re-colonization of European biota. *Biol. J. Linnean Soc.* 68, 87–112.
- Hillis, D.M., Huelsenbeck, J.P., 1992. Signal, noise, and reliability in molecular phylogenetic analyses. *J. Hered.* 83, 189–195.
- Hrbek, T., Kücün, F., Frickey, A., Stölting, K.N., Wildekamp, R.H., Meyer, A., 2002. Molecular phylogeny and historical biogeography of the *Aphanius* (Pisces, Cyprinodontiformes) species complex of Central Anatolia, Turkey. *Mol. Phylogenet. Evol.* 25, 125–137.
- Hrbek, T., Meyer, A., 2003. Closing of the Tethys Sea and the phylogeny of Eurasian killifishes (Cyprinodontiformes: Cyprinodontidae). *J. Evol. Biol.* 16, 17–36.
- Huelsenbeck, J.P., Ronquist, F.R., 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17, 754–755.
- Humphries, J.M., Miller, R.R., 1981. A remarkable species flock of pupfishes, genus *Cyprinodon*, from Yucatán, Mexico. *Copeia* 1, 52–64.
- Humphries, J.M., 1984. Genetics of speciation in pupfishes from Laguna Chichancanab, Mexico. In: Echelle, A.A., Kornfield, I. (Eds.), *Evolution of fish species flocks*. Univ. Maine, Orono, pp. 129–139.
- Karakousis, Y., Marchordom, A., Doadrio, I., Economidis, P.S., 1995. Phylogenetic relationships of *Barbus peloponnesius* Valenciennes, 1842 (Osteichthyes: Cyprinidae) from Greece and other species of *Barbus* as revealed by allozyme electrophoresis. *Biochem. Syst. Ecol.* 23, 365–375.
- Kosswig, C., 1964. Bemerkungen zur Geschichte und zur Ökologie der Ichthyofauna Kleinasiens, besonders seines abflußlosen Zentralbeckens. *Zool. Anz.* 172, 1–15.
- Ludwig, A., Bohlen, J., Wolter, C., Pitra, C., 2001. Phylogenetic relationships and historical biogeography of spined loaches (Cobitidae, *Cobitis* and *Sabanejewia*) as indicated by variability of mitochondrial DNA. *Zool. J. Linnean Soc.* 131, 381–392.
- Macey, J.R., Schulte, J.R., Ananjeva, N.B., Larson, A., Rastegar-Pouyani, N., Shammakov, S.M., Papenfuss, T.J., 1998. Phylogenetic relationships among lizards of the *Laudakia caucasica* species group: testing hypotheses of biogeographic fragmentation and an area cladogram for the Iranian plateau. *Mol. Phylogenet. Evol.* 10, 118–131.
- Nagelkerke, L.A.J., Sibbing, F.A., 1997. A revision of the large barbs (*Barbus* spp., Cyprinidae, Teleostei) of Lake Tana, Ethiopia, with a description of seven new species. In: Nagelkerke, L.A.J. (Ed.), *The barbs of Lake Tana, Ethiopia: morphological diversity and its implications for taxonomy, trophic resource partitioning and fisheries*. Wageningen University, The Netherlands, pp. 105–170.
- Neveščakaja, L.A., Goncharova, I.A., Iljina, L.B., Paramonova, N.P., Popov, S.V., Voronina, A.A., Chepalyga, A.L., Babak, E.V., 1987. History of Paratethys. *Ann. Inst. Geol. Publ. Hung.* 70, 337–342.
- Page, R.D.M., Holmes, E.C., 1998. *Molecular Evolution: A Phylogenetic Approach*. Blackwell Science, Oxford.
- Palumbi, S., Martin, A.P., Romano, S., McMillan, W.O., Stice, L., Grabowski, G., 1991. The simple fool's guide to PCR, version 2.0. Univ. Hawaii Press, Honolulu.
- Palumbi, S.R., 1996. Nucleic acids II: The polymerase chain reaction. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), *Molecular Systematics*. Sinauer Associates, Sunderland, pp. 205–247.
- Perdices, A., Doadrio, I., 2001. The molecular systematics and biogeography of the European Cobitids based on mitochondrial DNA sequences. *Mol. Phylogenet. Evol.* 19, 468–478.
- Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Rögl, F., 1998. Palaeogeographic considerations for Mediterranean and Paratethys Seaways (Oligocene to Miocene). *Ann. Naturhist. Mus. Wien.* 99, 279–310.
- Rögl, F., 1999. Circum-Mediterranean Miocene Palaeogeography. In: Rössner, G., Heissig, K. (Eds.), *The Miocene Land Mammals of Europe*. Pfeil Verlag, München, pp. 39–48.
- Rokas, A., Atkinson, R.J., Webster, L.M.I., Csoka, G., Stone, G.N., 2003. Out of Anatolia: longitudinal gradients in genetic diversity support an eastern origin for a circum-Mediterranean oak gallwasp *Andricus quercustozae*. *Mol. Ecol.* 12, 2153–2174.

- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
- Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of Log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.* 16, 1114–1116.
- Schlieven, U.K., Tautz, D., Pääbo, S., 1994. Sympatric speciation suggested by monophyly of crater lake cichlids. *Nature* 368, 629–632.
- Swofford, D.L., 2002. PAUP*: Phylogenetic analysis using parsimony (* and other methods). v. 4.0, Sinauer Associates, Sunderland.
- Weisrock, D.W., Macey, J.R., Ugurtas, I.H., Larson, A., Papenfuss, T.J., 2001. Molecular phylogenetics and historical biogeography among salamandrids of the “true” salamander clade: rapid branching of numerous highly divergent lineages in *Mertensiella luschani* associated with the rise of Anatolia. *Mol. Phylogenet. Evol.* 18, 434–448.