Folia Zool. – 57(1–2): 71–82 (2008)

Genetic diversity and phylogenetic relationships of spined loaches (genus *Cobitis*) in Croatia based on mtDNA and allozyme analyses

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Received 22 December 2006; Accepted 21 March 2008

A b s t r a c t . Using mitochondrial DNA (mtDNA) variations, the phylogenetic position of Croatian populations of spined loaches within the European genus *Cobitis* was assessed. Spined loaches from the Matica, Neretva, Cetina, Zrmanja, Jadova, Sava, Drava and Kupa Rivers are included in two previously described groups: a) *Cobitis sensu stricto* group, and b) Adriatic group. The Danubian populations of *C. elongatoides* and its hybrids from the Kupa and Drava Rivers are related to the species included in the *Cobitis s. str.* group, whereas all other analysed populations clustered within the Adriatic group, which is divided into the "Bilineata", "Elongata" and "Ohridana-zanandreai" clades. The Croatian spined loaches from the Adriatic watershed are included in the first two clades. Four mitochondrial lineages were revealed within the "Bilineata" clade: 1."*C. bilineata*" lineage containing loaches from the Zrmanja River and Italian and Spanish representatives of *C. bilineata*; 2. "Neretva-Cetina" lineage included loaches from the Cetina River (*C. dalmatina*) and the Neretva River (*C. narentana*); 3. "Jadova" lineage included specimens from the Jadova River; and 4. "Matica" lineage comprised spined loaches from the Matica River. *Cobitis elongata* from the Kupa and Drava Rivers belonged to the "Elongata" clade with two sublineages. Allozyme analyses of the *C. taenia* complex revealed the presence of both *C. elongatoides* and its all-female triploid hybrids in the Danube basin of Croatia.

Key words: Cobitidae loaches, Danube watershed, Adriatic watershed, DNA markers, hybrids

Introduction

Recent phylogenetic studies have significantly unravelled phylogenetic interrelationships within the genus *Cobitis* in European waters and demonstrated an unexpectedly high diversity within this fish group (e.g. P e r d i c e s & D o a d r i o 2001, J a n k o et al. 2005a,b, B o h l e n et al. 2006). The Balkan region was identified as a centre of endemism for spined loaches, while also playing a role during its Quaternary colonisations (B o h l e n & R á b 2001, B o h l e n et al. 2006). However, a detailed level of both phylogenetic and taxonomic diversity of the genus *Cobitis* is still not well understood (M r a k o v č i ć et al. 2000).

The territory of Croatia is divided into two watersheds, the Danube and Adriatic, which are separated by the Dinaric mountain range. At present, five species of *Cobitis* are cited for Croatian waters (M r a k o v č i ć et al. 2000, S c h n e i d e r et al. 2000). Rivers of the Danube watershed are inhabited by *C. elongata* and *C. elongatoides*, whereas *C. bilineata*,

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C. dalmatina and *C. narentana* occur in three Adriatic rivers (Zrmanja, Cetina and Neretva) (M r a k o v č i ć et al. 2000). The recognition of *C. narentana* (from the Neretva River) and *C. dalmatina* (from the Cetina River) was based only on their morphological characters (S c h n e i d e r et al. 2000). The taxonomic status of the third species occurring in the Zrmanja River is uncertain (M r a k o v č i ć et al. 1995), though its relation to *C. bilineata* was hypothesized (S c h n e i d e r et al. 2000). The loaches occurring in the Jadova River morphologically differ from all other populations and might represent another *Cobitis* species occurring in the Adriatic area. However, genetic analyses have not been conducted to date.

Furthermore, the populations of *C. elongatoides* from Croatia were generally considered as pure nonhybrid spined loaches (M r a k o v č i ć et al. 2000, S c h n e i d e r et al. 2000), though taxonomic identification was based exclusively on morphological traits. Recent studies have also shown syntopic occurrence of gynogenetic hybrid lineages of different ploidy levels with parental genomes of *C. elongatoides* and *C. tanaitica* in the Danube drainage (S o f r a d ž i j a & B e r b e r o v i ć 1978, B o h l e n & R á b 2001, J a n k o et al. 2003, 2005a).

The present study addresses the phylogenetic assignment of spined loach populations occurring in the territory of Croatia into the recognized phylogenetic structure of the European *Cobitis* loaches, in an effort to contribute to the revision of their taxonomic status, and the determination of the presence and genome composition of hybrids in the middle Danube River basin in Croatia.

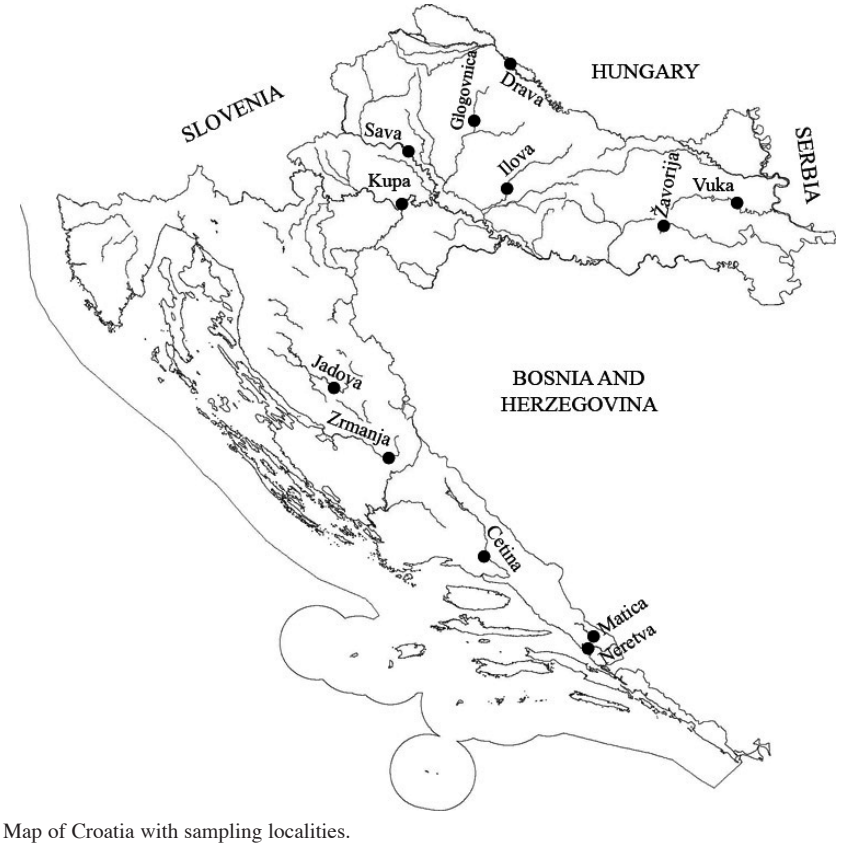

Fig. 1. Map of Croatia with sampling localities.

Materials and Methods

Materials

Mitochondrial DNA (mtDNA) analyses were conducted on 38 individuals and allozyme analysis on 125 specimens throughout Croatia (Fig. 1). Table 1 summarizes sampling details.

DNA extraction, polymerase chain reaction (PCR) amplifications and sequencing

To avoid amplification of nuclear pseudogenes (*numts*) that might be present, DNA was purified from fresh or deep-frozen muscle tissues following the procedure of Beckman et al. (1993). PCR amplifications were performed using the MasterTaq Kit (Eppendorf) according to manufacturer instructions. Primer combinations **L14725** (Hrbek et al. 2004) + **H16460**, and **L8331** + **H9236** (see Perdices & Doadrio 2001 for primer details) were used for the cytochrome *b* (cyt*b*) and the ATP synthase 8/6 genes (ATPase 8/6), respectively. PCR products were purified with the PCR purification kit (Roche). Sequencing was carried out by Macrogen Service Centre (Seoul, South Korea) with the newly designed internal primers **H-COB_cyt638** (5' TGA TAC TTT ATC TGC GTC NG 3') and **L-Cyp_425** (5' GGA CAA ATA TCC TTT TGA GG 3') for the cyt*b* gene, and amplification primer **L8331** for the ATPase 8/6 genes.

The complete cyt*b* gene (1140 bp) was sequenced in 38 specimens from 8 localities. Sequences of ATPase 8/6 genes (a total of 828 bp comprising the complete ATPase 8 and partial ATPase 6 gene) were obtained only from individuals exhibiting substantial differences between their cyt*b* haplotypes (Table 1).

The sequences have been deposited in the GenBank under the following accession numbers: EF605290-EF605301 (ATPase 8/6) and EF605302-EF605329 (cyt*b*).

Sequence data and phylogenetic analyses

Pairwise comparisons of uncorrected sequence divergence (p-distances) in the cyt*b* gene were obtained using MEGA software, version 3.1 (Kumar et al. 2004).

Phylogenetic analyses were conducted on three data sets. Data set I contained concatenated cyt*b* and ATPase 8/6 sequences from this study, as well as homologous sequences of all other European *Cobitis* species available from GenBank (Perdices & Doadrio 2001, Bohlen et al. 2006). This was analysed in order to estimate the phylogenetic position of the Croatian *Cobitis* populations within the European *Cobitis* species.

Data set II comprised all available cyt*b* sequences belonging to the Adriatic group in addition to representatives of all other main European *Cobitis* lineages (Perdices & Doadrio 2001, Doadrio & Perdices 2005, Bohlen et al. 2006). Data set II was analysed in order to provide a detailed phylogenetic structure of the Adriatic group. For both of the above data sets, *Sabanejewia romanica*, *S. balcanica* and *Misgurnus fossilis* (Perdices & Doadrio 2001) were used as outgroups.

Finally, data set III contained *C. elongatoides* cyt*b* sequences obtained in this study as well as those retrieved from GenBank (Janko et al. 2005a, Perdices & Doadrio 2001, Bohlen et al. 2006). The intraspecific analysis of *C. elongatoides* (data set III) was

performed to clarify the position of Croatian populations within the global phylogeographic pattern of this species.

Data sets I and II were analysed using three different methods of phylogenetic inference: maximum parsimony (MP) and maximum likelihood (ML) as implemented in PAUP (version

Table 1. Geographical origin, taxonomy, cytochrome *b* (cyt*b*) haplotype and sample vouchers of the *Cobitis* samples analysed in the study. Underlined samples were sequenced also for ATP8/6 genes. (A) Adriatic watershed, (D) Danube watershed *Samples identified by allozymes.

LOCALITY	TAXA	NUMBER OF SAMPLES	CYT*b* HAPLOTYPE	SAMPLE VOUCHER
Cetina (A)	*C. dalmatina*	5	CET1 CET2 CET3 CET4 CET5	CBCE1 CBCE2 CBCE3 CBCE4 CBCE5
Matica (A)	*C. narentana*	5	MAT1 MAT2 MAT3	CBMA1, CBMA4-5 CBMA2 CBMA3
Neretva (A)	*C. narentana*	3	NER1 NER2 NER3	CBNE1 CBNE2 CBNE3
Zrmanja (A)	*Cobitis* sp.	4	ZRM	CBZR1-4
Jadova (A)	*Cobitis* sp.	3	JAD	CBJA1-3
Sava (D)	*C. elongata*	4	SAV1 SAV2 SAV3 SAV4	CBSA1 CBSA2 CBSA3 CBSA4
Kupa (D)	*C. elongata*	5	KUP1 KUP2 KUP3 KUP4	CBKU1 CBKU2-3 CBKU4 CBKU5
	C. elongatoides *C. elongatoides* Hybrid*	4 21 3	 KUP5 KUP6 KUP7 KUP8	 CBKU6 CBKU7 CBKU9 CBKU10
Drava (D)	*C. elongatoides* Hybrid*	2 3	DRA2 DRA3 DRA4 DRA1	CBDR3 CBDR4 CBDR5 CBDR1-2
Glogovnica (D)	*C. elongatoides* Hybrid*	11 16		
Ilova (D)	*C. elongatoides* Hybrid*	7 2		
Žavorija (D)	*C. elongatoides*	29		
Vuka (D)	*C. elongatoides* Hybrid*	26 1		

4.0b10, Swofford 2002), and Bayesian inference as implemented in MrBayes (version 3.1.2, Ronquist & Huelsenbeck 2003). For ML and Bayesian analyses, the best-fitting model of molecular evolution (HKY+I+Γ model for both data sets) was selected by hierarchical likelihood ratio tests using the ModelTest software (version 3.06, Posada & Crandall 1998). Unweighted MP analyses were conducted using the heuristic search mode with 100 replicates, randomized input orders of taxa, and tree bisection-reconnection (TBR) branch swapping. Branch support was assessed by bootstrap (BS) analysis (1000 BS replications, 10 addition-sequence replicates). ML analyses were performed using heuristic search mode and TBR branch swapping algorithm. Bayesian analyses consisted of two simultaneous runs. For each, four Markov Chain Monte Carlo chains were run for three million generations with trees sampled every 100 generations. The first 20% of the sampled trees were discarded and Bayesian posterior probabilities (BPP) were estimated from the 50% majority-rule consensus tree of the retained trees. Data set III was analysed by statistical parsimony method (Templeton et al. 1992) as implemented in the TCS program (v1.13:2; Clement et al. 2000) under 95% parsimony connection limit.

Allozyme and ploidy level analysis

For allozyme analysis, a piece of muscle tissue was frozen in an extraction buffer (0.1 mol.l^{-1} Tris-HCl pH 8.5, Valenta et al. 1971). Variable allozyme loci previously known to be species specific (Janko et al. 2007) were analyzed following the methods of Šlechtová et al. (2000). The ploidy level was estimated by the number of observed alleles at each locus and/or by the estimation of gene dosage effect (Vrijenhoek 1975). Here, in the case of triploids, the products of species genome present in a single copy should be two times less intensive than products of genome present in a double copy.

Results

Phylogenetic relationships

By analysing a complete cyt*b* gene from 38 specimens included in the study, a total of 29 different haplotypes were found (Table 1).

All three methods of phylogenetic reconstruction based on data set I yielded to similar overall trees (Fig. 2). MP analysis resulted in 12 equally parsimonious trees (length = 2536, consistency index (CI) = 0.431, retention index (RI) = 0.720 and rescaled consistency index (RC) = 0.310) and ML in single tree (-ln *L* = 13630.60024). All Croatian specimens clustered within two main European *Cobitis* groups: *C. elongatoides* and its asexual hybrids clustered within the *Cobitis s. str.* group, while all other *Cobitis* taxa were included in the Adriatic group. These clusterings are very well supported by both BS values and BPP.

MP analysis of data set II yielded the 12 most parsimonious trees (length = 1434, CI = 0.423, RI = 0.700 and RC = 0.296). ML analysis recovered three ML trees (-ln *L* = 7670.30110) with congruent overall topologies. The existence of three main clades within the Adriatic group as shown in several previous studies based on a smaller sample size (Perdices & Doadrio 2001, Bohlen et al. 2006) was confirmed by all three phylogenetic methods (Fig. 3). All three main clades, designated hereafter as “Bilineata”, “Elongata” and “Ohridana-zanandreai”, are very well supported by both BS values and BPP. The newly analysed Croatian loaches clustered within the first two clades.

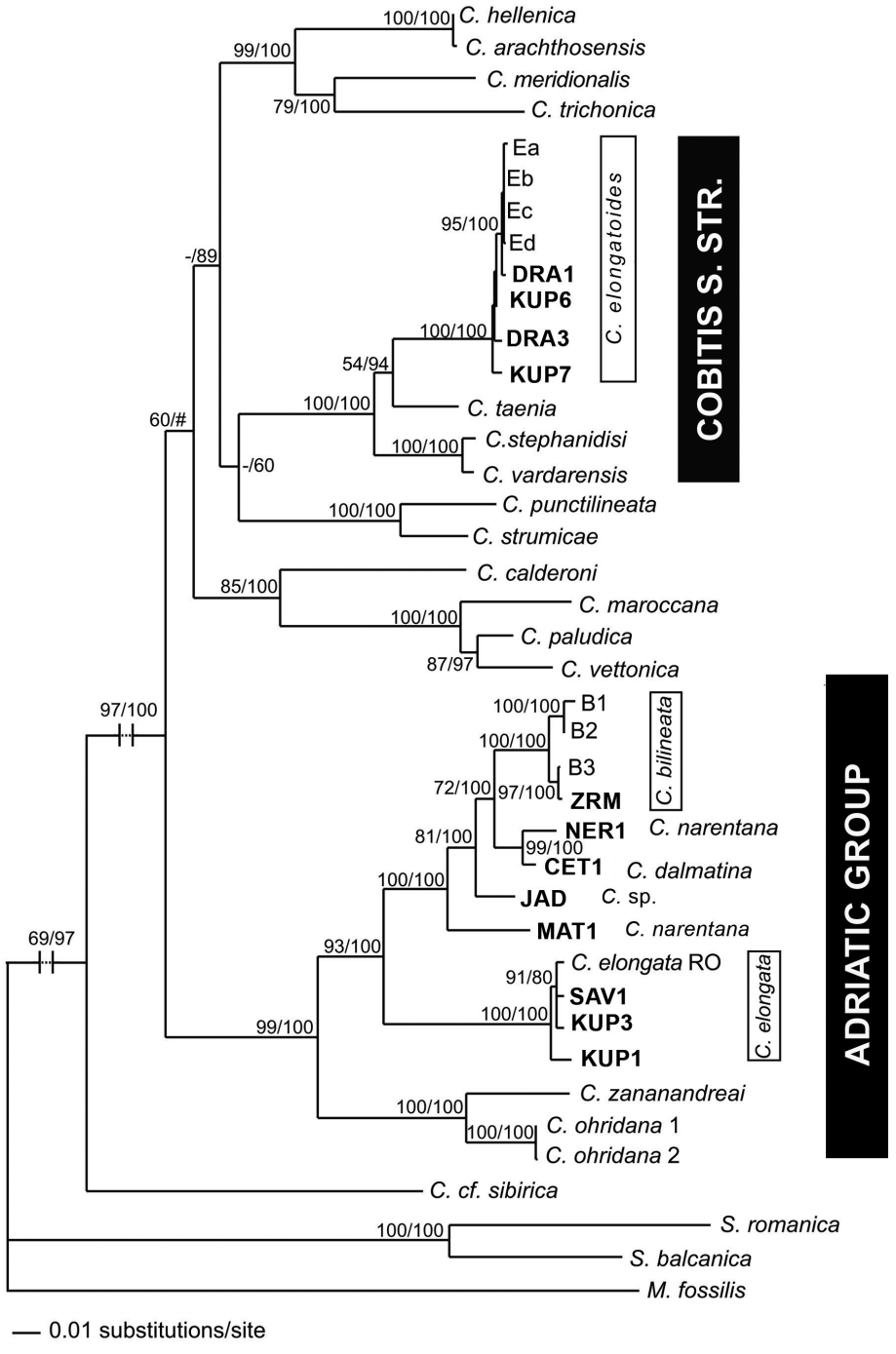

Fig. 2. Phylogenetic relationships of the Croatian *Cobitis* populations within the European *Cobitis* species based on the ML analysis of data set I (combined cyt*b* and ATPase 8/6 data set). Numbers at nodes represent MP bootstrap (BS) values and Bayesian posterior probabilities (BPP), respectively. BS values ≤ 50 are indicated with a dash. Samples from this study are in bold.

The "Bilineata" clade comprised four main mitochondrial lineages designated as: 1) "C. bilineata" lineage which included the Italian and Spanish (specimen from Lake Banolas) haplotypes of *C. bilineata* as well as the single haplotype of the specimens from the

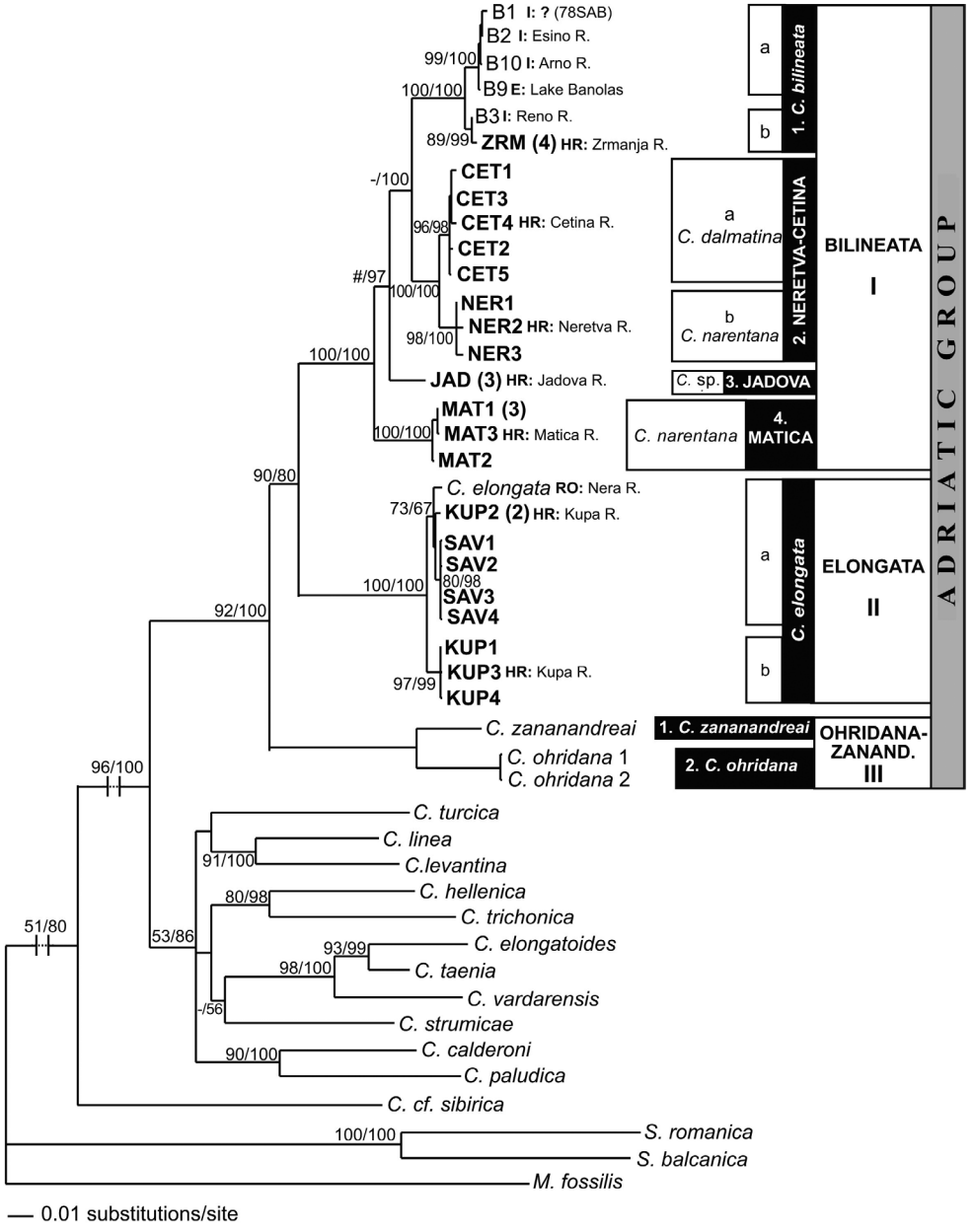

Fig. 3. Phylogram derived from ML analysis of the data set II (cyt*b*) showing phylogenetic relationships within the Adriatic group. Numbers at nodes represent MP bootstrap (BS) values and Bayesian posterior probabilities (BPP), respectively. BS values ≤ 50 are indicated with a dash. Cyt*b* haplotypes from this study are in bold. Numbers in parenthesis refer to the total number of samples in which the respecting haplotype was found. "#" refers to the node that was not revealed by MP analysis (see text). Abbreviations: I = Italy, HR = Croatia, RO = Romania.

Zrmanja River, 2) "Neretva-Cetina" lineage containing haplotypes belonging to the species *C. dalmatina* and *C. narentana* from the Cetina and Neretva Rivers, respectively, and 3) "Jadova" and 4) "Matica" lineages that represent mitochondrial lineages not previously observed.

ML and Bayesian analyses were congruent with very high BPP values: the "Matica" lineage appeared as the most basal branch, the "Jadova" lineage was recovered as the sister lineage of the cluster formed by "C. bilineata" and "Neretva-Cetina" lineages. However, the MP phylogeny revealed a trichotomy between "C. bilineata" lineage, "Neretva-Cetina" lineages and fairly supported clade comprising the "Matica" and "Jadova" lineages. Since MP and ML/Bayesian analyses yielded incongruent topologies (Fig.3), the evolutionary relationships of major "Bilineata" lineages are treated as an unresolved tetratomy. P-distances between the "Bilineata" lineages ranged from 3.3–5.7%. The "Bilineata" lineage is further subdivided into two sublineages (p-distances up to 1.3%). Within the "Neretva-Cetina" lineage, *C. dalmatina* and *C. narentana* appeared as monophyletic sublineages. However, p-distances observed between them (1.1–1.6%) are rather low and comparable with p-distances found within *C. bilineata*. P-distances found between the Jadova and Matica River populations were 3.5–3.6%, and ranged from 3.3 to 5.7% between them and the remaining three species from the Adriatic drainage.

All specimens belonging to *C. elongata* were contained within the "Elongata" clade. This clade could be further separated into two lineages ("a" and "b") with p-distances between them in the range of 1.0–1.1%. All specimens from the Sava River as well as a Romanian specimen belong to sublineage "a", whereas the cyt*b* haplotypes found in the Kupa River occur in both lineages. P-distances between "Elongata" and "Bilineata" clades were in the range of 7.0–8.1%.

Finally, the intraspecific analysis of 9 specimens of *C. elongatoides* revealed 8 haplotypes: 4 in the Kupa River and 4 in the Drava River. Intra-population cyt*b* p-distances found in the Drava and Kupa River populations of *C. elongatoides* were in the range of 0.1–0.5 and 0.1–0.7%, respectively, and therefore were similar to the inter-population p-distances of 0.1–0.7%. The highest observed intraspecific p-distance was 1.1%.

In the 95% parsimony network (Fig. 4), several Croatian haplotypes appeared as interior and therefore probably ancestral haplotypes within the clades defined by J a n k o et al. (2005a). The haplotypes KUP7 and KUP8 found in the Kupa River appeared as ancestral haplotypes within the northernmost clade (2–1). On the other hand, the two remaining haplotypes from the Kupa River belong to the clade 2–3 as well as haplotype DRA3 from the Drava River. Both, DRA3 and KUP6 represent ancestral haplotypes within this clade. Haplotype DRA2 is encompassed within the northern clade (2–2). The two remaining Drava haplotypes (DRA1 and 4) were found in hybrid specimens and are included within the "Hybrid clade" I of J a n k o et al. (2005a).

H y b r i d r i c h n e s s

Allozyme analyses of the *C. taenia* complex (*sensu* J a n k o et al. 2005b) revealed the presence of both *C. elongatoides* and its asexual hybrids in the Danube drainage of Croatia. From 125 analyzed samples, 25 were hybrid biotypes (Table 1). Hybrids were all-female triploid forms with two haploid sets of *C. elongatoides* and one haploid set of a second species. In 12 hybrids, the *C. elongatoides*-specific allozyme products showed a heterozygous pattern, whereas other hybrids showed a homozygous pattern, but twice as contrated as the

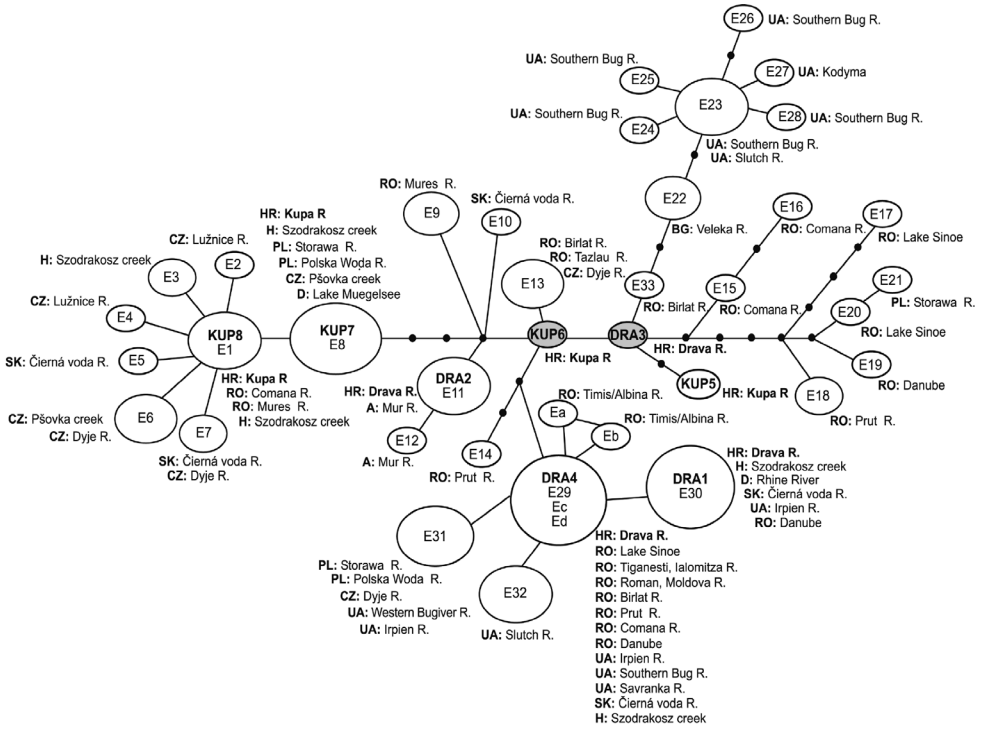

Fig. 4. 95% parsimony network of the cyt*b* haplotypes in *C. elongatoides* and hybrids (data set III). Size of ovals corresponds to haplotype frequency. Black dots are missing (unobserved) haplotypes. Missing haplotypes from the study of J a n k o et al. (2005a) found in this investigation are presented as grey circles. Abbreviations: HR = Croatia, RO = Romania, A = Austria, SK = Slovakia, PL = Poland, CZ = Czech Republic, H = Hungary, D = Germany, UA = Ukraine, BG = Bulgaria.

genome of a second species. Allozyme studies did not distinguish whether the second genome present in hybrids was that of *C. taenia*, *C. tanaitica* or *C. taurica*.

Discussion

P h y l o g e n e t i c r e l a t i o n s h i p s

Phylogenetic analyses placed Croatian spined loaches within two main European *Cobitis* groups (Figs. 2–4): the Adriatic group (all populations besides the populations of *C. elongatoides*) and the *Cobitis s. str.* group (*C. elongatoides* and its hybrids).

The Adriatic group has previously been only initially investigated using molecular markers (P e r d i c e s & D o a d r i o 2001, D o a d r i o & P e r d i c e s 2005, B o h l e n et al. 2006). The recognition of three main clades ("Bilineata", "Elongata" and "Ohridana-zanandreai") within this group is in accordance with lineages recognized already by P e r d i c e s & D o a d r i o (2001) and B o h l e n et al. (2006). However, the present study provides new data concerning the number of distinct mtDNA lineages and sublineages as well as phylogenetic relationships within the first two clades.

According to the molecular clock calibration for the cyt*b* (0.68% divergence per Myr) for the genus *Cobitis* (D o a d r i o & P e r d i c e s 2005), the separation of the "Bilineata" and

"Elongata" clades likely occurred 10.7–12.4 Myr ago. P-distances between the four main lineages within the "Bilineata" clade suggest that they could have diverged from a common ancestor around 4.9–8.4 Myr ago. We have shown that the "Elongata" clade contains at least two well-separated lineages that likely split from a common ancestor some 1.5–1.6 Myr ago.

Finally, the phylogenetic relationships of *C. elongatoides* from two Croatian populations with other *C. elongatoides* populations from Europe were analysed. The overall high haplotype diversity, but only moderate nucleotide diversity found in Croatian samples is consistent with results reported by J a n k o et al. (2005a). Incorporated into the 95% parsimony network with all published cyt*b* haplotypes of this species (including those found within hybrid lineages), most of the Croatian haplotypes obtained from the Kupa and Drava River populations appeared as ancestral (central) haplotypes within several clades discovered by J a n k o et al. (2005a). Such a phylogeographic pattern suggests the important role of the Drava and Kupa Rivers in the postglacial northward and northwestward spread of *C. elongatoides*. The great haplotype diversity observed within the Kupa and Drava River populations, especially their ancestral positions within the *C. elongatoides* haplotype network, would corroborate the hypotheses of J a n k o et al. (2005a), assuming that the Pannonian basin could have served as one of the refugia during glacial periods, and a source for the postglacial expansion of this species.

H y b r i d r i c h n e s s

The co-occurrence of *Cobitis elongatoides* with all female triploid hybrid biotypes was revealed, similarly to that found in the upper and lower Danube basin (B o h l e n & R á b 2001, J a n k o et al. 2003, 2005a). Surprisingly, hybrids were absent or rare in some localities (Vuka and Kupa Rivers, Žavorija stream), a pattern that is quite exceptional (see B o h l e n & R á b 2001). However, only one locality per river was sampled and thus sampling deficiency cannot be excluded. Since the genome in double dose was that of *C. elongatoides*, our nuclear markers did not distinguish the third set in triploids. Subsequent analysis of PCR-restriction fragment length polymorphism of ribosomal S7 nuclear gene in one hybrid per locality excluded the presence of the *C. taenia* genome (for method see J a n k o et al. 2007), and the chromosome study of two another hybrids showed a number of 75 chromosomes with several acrocentrics (C h o l e v a et al., unpublished). The haploid set in *C. taurica* has only one acrocentric chromosome (J a n k o et al. 2005b). Moreover, the *C. taurica* and *C. taenia* genomes have not yet been observed in hybrid genomic composition in the upper and lower Danube River system (B o h l e n & R á b 2001, J a n k o et al. 2003, 2005a, 2007). Thus, it is concluded that the third genome in hybrids originates from *C. tanaitica*. However, more variable nuclear markers need to be found in order to clearly identify *C. tanaitica* from *C. taurica*. The resolution is well supported by S o f r a d ž i j a & B e r b e r o v i ć (1978) who described 75 chromosome females near the Ukrinski Lug River (middle Danube system in Bosnia and Herzegovina) and its karyotype dissections suggest the presence of the *C. tanaitica* genome.

Allozyme analyses confirmed that the DRA1 and DRA4 cyt*b* haplotypes belong to hybrid biotypes, while DRA2, DRA3 and KUP5-8 are from the pure *C. elongatoides*. The clustering of DRA1 and DRA4 haplotypes within "Hybrid clade" I of J a n k o et al. (2005a) would corroborate the suggestion of J a n k o et al. (2005a) that hybrid lineage I survived in refuge areas together with their maternal species, *C. elongatoides*.

Taxonomic implications

Genetic distances between the Jadova and Matica River lineages (3.5–3.6%) as well as between these and three other species comprised within the "Bilineata" clade (3.3–5.7%) are in the same range or even greater than distances found between well defined *Cobitis* species. In comparison, divergence among species within main Asia Minor and Balkan lineages was 4.5±2.9% (Bohlen et al. 2006). Similarly, p-distances found between some closely related Ibero-African *Cobitis* species were in the range of 2.1–6.9% (Doadrio & Perdices 2005). The genetic distinctiveness of the Matica and Jadova River populations suggest that these populations most likely represent *Cobitis* species that may deserve formal description. The morphological analysis of specimens from the Jadova River corresponds with the results of genetic analyses and the Jadova River population should be considered a separate species (Mustafić 2008).

On the other hand, the genetic distances found between C. *dalmatina* and *C. narentana* are similar to those found within the *C. bilineata* species. Such low genetic divergences as found between those two isolated and morphologically well described species might be a consequence of rapid local speciation in karst streams. However, more detailed taxonomic revision necessitates the use of other genetic markers.

Acknowledgements

We are grateful to P. Ráb for useful comments and J. Kopecká for help in the laboratory. The study was a part of the project No. 119102 of Croatian Ministy of Science, Education and Sports and has also been supported by the grant GAUK No. 187/2005 B BIO from the Grant Agency of the Charles University in Prague and partially by grant GAČR 206/05/P586 and GAČR 206/06/1763 and Biodiversity Research centre LC0607 (L.Ch.). The Laboratory of Fish Genetics at Liběchov receives continuous support from the Academy of Sciences of the Czech Republic (IRP IAPG No. AV0Z50450515).

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