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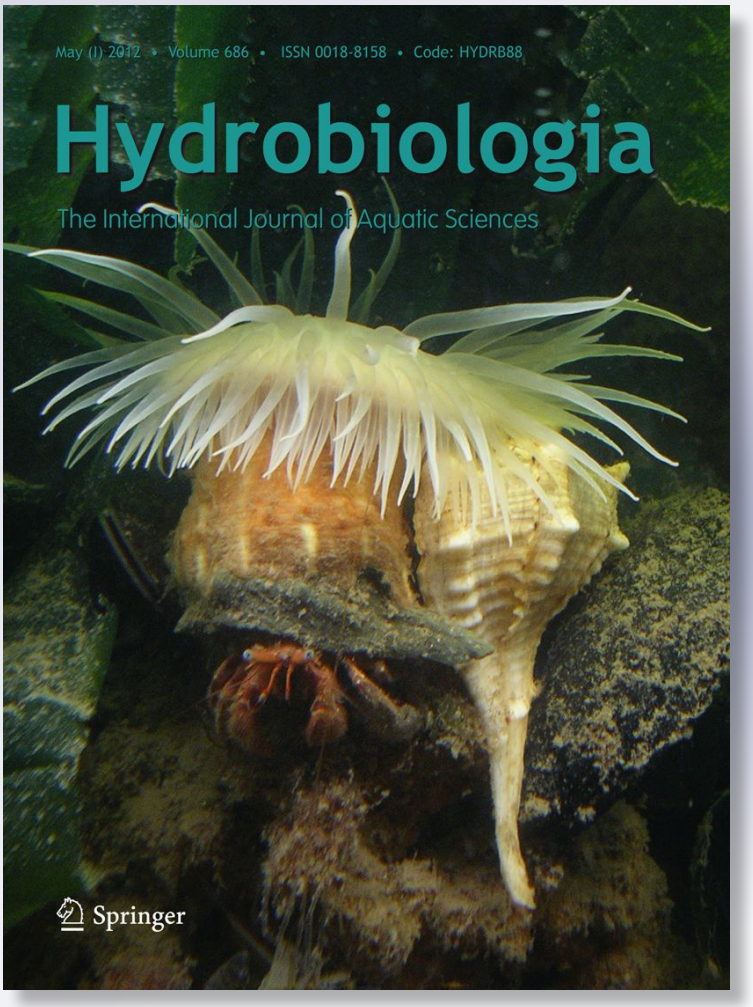

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PRIMARY RESEARCH PAPER

Genetic variation of European grayling (*Thymallus thymallus*) populations in the Western Balkans

Saša Marić · Belma Kalamujić · Aleš Snoj · Andrej Razpet · Lada Lukić-Bilela · Naris Pojskić · Simona Sušnik Bajec

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Abstract In order to elucidate genetic composition of European grayling (*Thymallus thymallus*) populations in the Western Balkans, the partial mitochondrial DNA (mtDNA) control region was sequenced and 12 microsatellite loci genotyped in 14 populations originating from tributaries of the Adriatic and Danube drainages. Eleven mtDNA haplotypes were found, one confined to the Adriatic clade, one to the Alpine group and the rest to the 'Balkan' grayling phylogenetic clade. Haplotypes from the Balkan clade were confined to the Danube drainage and constituted two groups: northern group with haplotypes found in the Slovenian part of the Danube drainage, and southern group, consisting from Bosnia–Herzegovina and Montenegro. Substantial genetic distance between northern and southern groups of haplotypes (0.75–1.8%) and well supported divisions within the northern group indicate very structured grayling population within the studied Danube basin that most probably did not evolve due to vicariance but rather as a consequence of multiple colonization waves that might have occurred during the Pleistocene. Furthermore, genetic distance of ~4% between Adriatic and Danube populations' haplotypes, suggest that their separation occurred in mid-Pliocene. These findings imply a complex colonization pattern of the Western Balkans drainages. Microsatellite data also confirm high genetic diversity in Western Balkans populations of grayling (on average 7.5 alleles per microsatellite locus and H_{exp} 0.58). Limited stocking activities were detected based on microsatellites and mtDNA data. Regarding current knowledge of grayling phylogeography appropriate management strategies were proposed to preserve unique, autochthonous grayling populations in Western Balkan.

Keywords European grayling · Western Balkans · Genetic diversity · mtDNA · Microsatellites · Evolutionary significant units

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Introduction

European grayling (grayling, *Thymallus thymallus*, Linnaeus 1758) population have been suffering from

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considerable size reduction throughout the species distribution range, apparently due to e.g. habitat deterioration, water pollution, bird depredation, excessive fishing, competition with non-native fish species and so on (Northcote, 1995; Persat, 1996; Baars et al., 2001; Koskinen & Primmer, 2001; Uiblein et al., 2001; Carlstein, 2004; Sušnik et al., 2004; Gum, 2007). In order to supplement waning wild populations, conservation programmes based on restocking with farmed fingerlings have been launched. Best intentions notwithstanding, if parental brood-stocks represent non-native lineages such management measures can cause further damage, and potentially lead to a significant change in the genetic structure of the native population or even its break-down (Koskinen et al., 2002a; Sušnik et al., 2004; Duftner et al., 2005). Taking these considerations into account, several studies into genetic structure of grayling have been initiated since the beginning of this millennium, mainly to resolve its genealogy and population genetic structure (for detailed review, see Gum et al., 2009). Gum et al. (2009) also reviewed phylogeographic studies performed on grayling and proposed five major mitochondrial DNA (mtDNA) phylogenetic lineages, i.e. the northern/northeastern (N/NE), the central-eastern (CE), the central-western (CW), the Danubian and, finally, the Adriatic clade.

Despite the extensive palette of molecular-based studies conducted on grayling, the southernmost populations of the Balkan Peninsula remained largely unexplored. A few local investigations (Kalamujić et al., 2007; Kalamujić, 2008; Marić et al., 2011) focused on grayling populations south of Slovenia. The preliminary results from these studies further affirm the assumption of Koskinen et al. (2002c) that the level of genetic distinctiveness increases with approaching the southernmost border of the grayling range and underline the need to elaborate genetic surveys in this region. Based on the data by Janković (1960) that describe large pool of morphological variation among grayling populations in former Yugoslavia, it was suggested that the rivers of the Balkans represented major refugia for grayling during the last glaciations and that a significant amount of genetic variation remained uncovered in these southernmost populations (Koskinen et al., 2000; Weiss et al., 2002; Gum et al., 2005). Here, we report the results of the first comprehensive study on the genetic composition of grayling populations in the Western Balkans based on mtDNA control region (CR mtDNA) and microsatellite DNA markers. The aims of this study were (1) to assess the genetic diversity of grayling in Bosnia–Herzegovina, Montenegro and Slovenia, (2) to determine phylogenetic relationships among these populations as well as their relation to other European populations, (3) to detect potential effects of stocking activities and propose appropriate conservation and management strategies in the region with emphasis on populations from the Danubian basin.

Materials and methods

Sampling, DNA isolation and reference material

A total of 227 grayling specimens (subadult and adult individuals) were collected by electrofishing and angling from 14 locations across the Danube and Adriatic drainages in Slovenia, Bosnia–Herzegovina and Montenegro from 1997 until 2008 (Table 1; Fig. 1). Fin clips were sampled and stored in 96% ethanol. Total DNA was isolated using Wizard Genomic DNA Purification Kit (Promega), following the supplier's instructions.

Newly described haplotypes were deposited in GenBank (accession numbers JF703125–JF703129; JQ611728–JQ611729). Additional grayling haplotypes from GenBank (accession numbers AF522395–AF522452; HM636922–HM636924) were used in analysis. To assign individual haplotypes and clades to the previously identified within the European grayling, data from this study were aligned against haplotypes from each clade (Weiss et al., 2002; Marić et al., 2011) and compared with them. *T. arcticus* (AF522453), *T. grubei* (AF522454) and *T. brevirostris* (AF522455) were used as outgroup taxa.

Molecular techniques

MtDNA sequencing

CR mtDNA was amplified in 87 individuals (Table 1) using polymerase chain reactions (PCR) and primers LRBT-25 and LRBT-1195 (Uiblein et al., 2001).

Each PCR reaction (total volume 30 µl) contained 21.6 µl H_2O, 3 µl 10× PCR buffer, 0.75 µl 10 mM of each primer, 1.2 µl 25 mM $MgCl_2$, 1.5 µl 0.2 mM

Table 1 Details of samples, sample locations, numerical code, region of origin (*MNT* Montenegro, *BIH* Bosna-Herzegovina, *SVN* Slovenia), the number of individuals analysed (*N*) and summary of haplotype frequencies

River basin	River system	Codes, populations and regions	Year of sampling	mtDNA CR (*N*)	Haplotype frequency											Microsatellite DNA N	Ar	H_e	H_o	F_{IS}
					Da23	Da25	Da26	Da27	Da28	Da29	Da30	Da31	Da32	Da33	Ad7					
Danubian	Sava	1. Ljuča (MNT)	2008	6	–	–	–	6	–	–	–	–	–	–	–	6	1.83	0.2292	0.2917	−0.186
Danubian	Sava	2. Drina (BIH)	2005	5	–	–	–	3	–	1	–	–	–	1	–	17	3.50	0.3978	0.4088	0.004
Adriatic	Neretva	3. Neretva (BIH)	2005	8	2	3	–	–	–	–	3	–	–	–	–	16	2.58	0.3315	0.2917	0.152
Danubian	Sava	4. Bosna (BIH)	2005	7	–	–	–	–	–	5	–	–	2	–	–	21	1.75	0.1902	0.1921	0.015
Danubian	Sava	5. Vrbas (BIH)	2005	1	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–
Danubian	Sava	6. Sanica (BIH)	2005	5	–	–	5	–	–	–	–	–	–	–	–	13	2.42	0.3311	0.2885	0.168
Danubian	Sava	7. Sana (BIH)	2005	6	–	–	6	–	–	–	–	–	–	–	–	5	1.83	0.2783	0.3333	−0.088
Danubian	Sava	8. UnaII (BIH)	2005	5	–	–	–	–	–	–	–	5	–	–	–	30	2.17	0.2207	0.2130	0.052
Danubian	Sava	9. UnaI (BIH)	2005	6	–	–	–	–	–	–	–	6	–	–	–	10	1.83	0.1908	0.2417	−0.217
Danubian	Sava	10. Krušnica (BIH)	2005	6	–	–	4	–	2	–	–	–	–	–	–	25	3.25	0.3893	0.3096	0.225
Danubian	Sava	11. Obrh (SVN)	1999	7	–	7	–	–	–	–	–	–	–	–	–	18	2.25	0.2953	0.3102	−0.022
Danubian	Sava	12. Unec (SVN)	1999	6	2	4	–	–	–	–	–	–	–	–	–	10	2.00	0.3258	0.3917	−0.151
Danubian	Sava	13. Sava (SVN)	1998	9	3	6	–	–	–	–	–	–	–	–	–	22	2.50	0.2744	0.2734	0.027
Adriatic	Soča	14. Tolminka (SVN)	1997	10	2	–	–	4	–	–	–	–	–	–	4	34	4.08	0.5846	0.5441	0.084
		∑		87	9	20	15	13	2	6	4	11	2	1	4	227				
		%			10	23	17	15	2	7	5	13	2	1	5					

Haplotypes first described in this study are underlined. Genetic diversity indices of microsatellite marker data: *Ar* allelic richness; H_E expected heterozygosity in population; H_O observed heterozygosity; deviations of F_{IS} values from HWE are not significant except in Krušnica ($P < 0.001$)

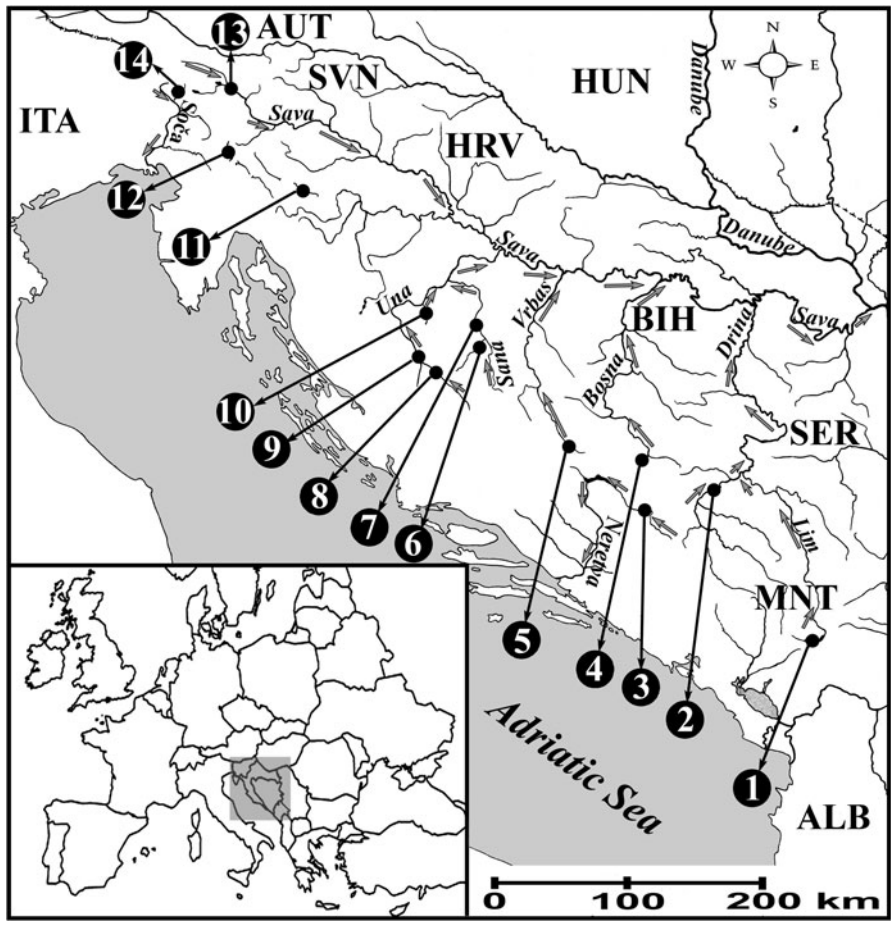

Fig. 1 Sampling locations with geographic coordinates. *1* Ljuča (42°34′30.00″N; 19°53′27.20″E), *2* Drina (43°37′46.04″N; 18°56′17.99″E), *3* Neretva (43°29′50.12″N; 18°08′14.18″E), *4* Bosna (43°41′22.24″N; 18°26′51.69″E), *5* Vrbas (44°08′33.74″N; 17°23′51.57″E), *6* Sanica (44°22′03.45″N; 16°22′16.26″E), *7* Sana (44°45′52.22″N; 16°39′24.19″E), *8* Una II (44°30′03.88″N; 16°08′59.48″E), *9* UnaI (44°33′10.48″N; 16°06′12.28″E), *10* Krušnica (44°51′0.09″N; 16°09′22.38″E), *11* Obrh (45°41′47.35″N; 14°28′10.14″E), *12* Unec (45°51′05.34″N; 14°15′35.45″E), *13* Sava (46°20′38.64″N; 14°09′19.60″E), *14* Tolminka (46°11′59.62″N; 13°44′32.60″E)

dNTP's, 0.2 µl Fermentas *Taq* polymerase (5 U/µl) and 1 µl of template (∼100 ng DNA). The cycle parameters were: initial denaturation (95 °C, 3 min) followed by 32 cycles of strand denaturation (95 °C, 45 s), primer annealing (55 °C, 45 s) and DNA extension (72 °C, 2 min). All PCR amplifications were performed in a programmable thermocycler GeneAmp® PCR System 9700 (Applied Biosystems). Amplified DNA fragments were run on a 1.5% agarose gel and were purified from the gel using the QIAEX II gel Extraction Kit (QIAGEN) prior sequencing.

All sequencing reactions were prepared using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's recommendations. The 3′-end of 595 bp fragment of control region and 74 bp of flanking tRNA (Phe) were sequenced using primer LRBT-1195 (Uiblein et al., 2001). The sequencing products were salt-precipitated and analyzed on an ABI Prism 3130xl Genetic Analyser.

Also, we have sequenced 45 individuals for the ATP6 gene (data not shown). However, this marker turned out to be completely non-informative in phylogeographic aspect at the same catchment area, so we dropped these results from further analysis, and proceeded with the results of CR analysis.

Microsatellite genotyping

Twelve microsatellite loci, i.e. BFRO004 (Snoj et al., 1999), BFRO005, BFRO006, BFRO007, BFRO008, BFRO009 (Sušnik et al., 1999a), BFRO010, BFRO011 (Sušnik et al., 2000) BFRO014, BFRO015, BFRO016 and BFRO017 (Sušnik et al., 1999b) were amplified in 227 individuals using fluorescently labelled forward primers. PCR amplification and genotyping of microsatellites were conducted according to previously published protocols (Snoj et al., 1999; Sušnik et al., 1999a, b, 2000). Each PCR reaction (total volume 10 µl) contained 6.3 µl H_2O,

1 µl 10× PCR buffer, 0.25 µl 10 mM of each primer, 0.6 µl 25 mM $MgCl_2$, 0.5 µl 0.2 mM dNTP's, 0.1 µl Fermentas *Taq* polymerase (5 U/µl) and 1 µl of template (~100 ng DNA). The cycle parameters were: initial denaturation (94 °C, 3 min) followed by 30 cycles of strand denaturation (94 °C, 45 s), primer annealing (55 or 60 °C, 30 s) and DNA extension (72 °C, 5 s). Aliquots of amplified DNA, mixed with formamide and GeneScan-500 ROX Size Standard (Applied Biosystems) were run on an ABI Prism 310, 377 and 3130xl Genetic Analyzers. A subset of samples was genotyped on all genetic analyzers to ensure consistent allele reading.

Data analysis

Sequence analysis

DNA sequences were aligned using the computer programme ClustalX (Thompson et al., 1994). Sequence polymorphism was assessed using DNAsp ver. 4.10 (Rozas et al., 2003) and sequence divergence was calculated with net nucleotide divergence (Da) in MEGA version 2.1 (Kumar et al., 2001).

Aligned haplotypes were imported into the programme PAUP Version 4.0b10 (Swofford, 2000) for phylogenetic analysis. Neighbour-joining (NJ), maximum likelihood (ML) and maximum parsimony (MP) analyses were used for phylogenetic reconstruction. For NJ and ML the best-fit model of nucleotide substitution evolution TPM3uf+I+G was selected according to corrected Akaike Information Criterion (AICc) using JModelTest 0.1.1 (Posada, 2008). For ML, after choosing a model, a heuristic search (10 replicates) was used to estimate the most likely topology. For MP, insertions or deletions (indels) were included as a fifth character. A heuristic search (10 replicates) with tree bisection reconnection (TBR) branch-swapping was employed to find the most parsimonious trees. Support values for the nodes were obtained with 1,000 bootstrap replicates for NJ, ML or MP analysis, whereby the fast stepwise addition method was used for ML.

In addition, relationships among haplotypes were depicted by a haplotype network using TCS 1.2 programme (Clement et al., 2000) with fixed connection limit at 16 steps to include all different haplotypes. Gaps were treated as the fifth character.

Microsatellite analysis

Expected (H_e), and observed (H_o) heterozygosities were calculated with GENETIX 4.04 (Belkhir et al. 1996–2004). FSTAT 2.9.3.2 (Goudet, 2002) was used to calculate deviations from Hardy–Weinberg equilibrium (HWE), allelic richness and pair-wise F_{ST} values, all based on 1,000 permutations. Genetic relationships between individuals were estimated as the proportion of shared alleles at each locus, i.e. allele sharing distances (D_{AS}) (Bowcock et al., 1994). A matrix of D_{AS} was used to construct NJ trees of individuals and populations with POPULATIONS software (Langella, 2002).

Genetic population structure was inferred using the hierarchical STRUCTURE analysis approach (Vähä et al., 2007). STRUCTURE 2.3.2.1 programme runs Markov chain Monte Carlo (MCMC) simulations to partition individuals into *K* clusters. Basic assignment criteria are the minimization of Hardy–Weinberg and linkage disequilibria (Pritchard et al., 2000). For runs estimating ln Pr($X|K$) under a certain *K*, different run lengths were used (from 20,000 to 100,000 burn-in and 100,000 to 2,000,000 total length, repeated seven times for each *K*) depending on convergence. We applied the ΔK method (Evanno et al., 2005) to estimate the most probable K (see Appendix 1 in electronic supplementary material).

Results

Mitochondrial DNA sequence analysis

Aligned sequences of 595 bp 3′-end CR mtDNA and 74 bp tRNA (Phe) gene obtained from 87 individuals grouped into eleven haplotypes, seven of which had not been previously described (i.e. Da26, Da28, Da30, Da31, Da32, Da33 and Ad7). All the new haplotypes were detected in the Danube drainage (Sava River) in Bosnia–Herzegovina, except for haplotype Ad7, which was found in the Adriatic drainage (Tolminka River, Soča Basin) in Slovenia and clusters within to the Adriatic clade. The other four haplotypes (i.e. Da23, Da25, Da27 and Da29) had been previously identified in the Danube drainage of Slovenia and Serbia (Weiss et al., 2002; Marić et al., 2011). In this study, the haplotypes Da23, Da25 and Ad7 were found to predominantly occur in Slovenia (frequency of

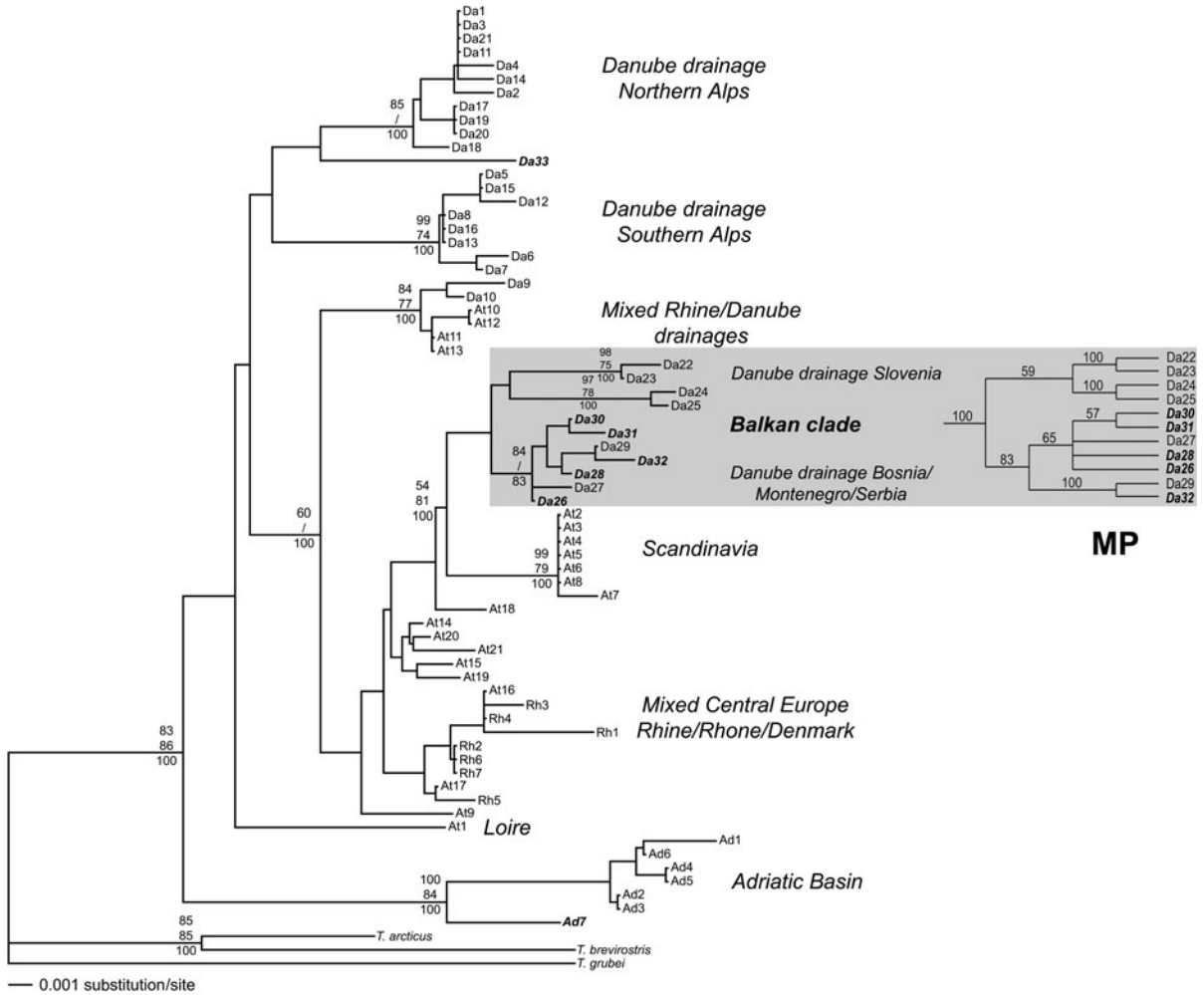

Fig. 2 NJ phylogram of mtDNA control region haplotypes. Node support is shown by per cent bootstraps (1,000 replicates) for NJ and ML above, and MP 50% consensus below; italicized and bold taxa represent newly sampled haplotypes. *MP* maximum parsimony topology for Balkan clade. *Slash* represents bootstrap <50%. GenBank accession numbers JF703125–JF703129, JQ611728–JQ611729, AF522395–AF522455, HM636922–HM636924

80–100%), while the others (i.e. Da26, Da27, Da28, Da29, Da30, Da31, Da32 and Da33) prevailed in Bosnia–Herzegovina and Montenegro (frequency of 70–100%).

Tree topologies of NJ, ML and MP approaches revealed a group of haplotypes found in the Danube drainage in Slovenia, splitting into two well supported subgroups (i.e. Da25–Da24 bootstrap 98/75/100; Da22–Da23 bootstrap 97/78/100; Fig. 2). Haplotypes Da22 and Da24 had been previously reported in the Sava drainage in Slovenia (Weiss et al., 2002). This group forms a sister group with the one consisting of the haplotypes from the Danube drainage in Bosnia–Herzegovina, Montenegro and Serbia. Unlike the Slovenian haplotypes, haplotypes found in Bosnia–Herzegovina and Montenegro (Da26, Da28, Da30, Da31, Da27, Da29 and Da32) form a rather homogenous group. Both sister groups belong to the so-called "Balkan clade" (Marić et al., 2011). Haplotype Da33 is the only haplotype found in the Danube drainage in the western Balkan that does not confine to the Balkan clade (Table 1). This haplotype belongs to the Alpine group and is most closely related to the northern Alpine clade (Fig. 2).

Genealogical relationships among the haplotypes, seen in the minimum spanning haplotype network

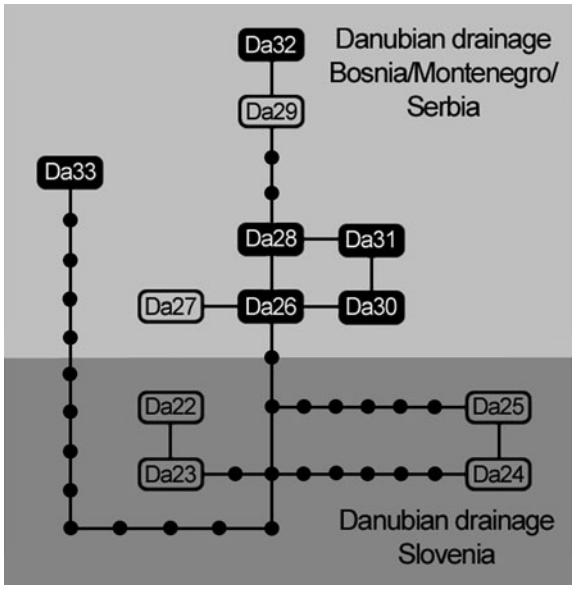

Fig. 3 mtDNA control region haplotype network relating grayling with previously published data (Weiss et al., 2002; Marić et al., 2011). *Lines*, regardless of length, represent single mutational events and link the haplotypes; *small black circles* represent missing or theoretical haplotypes; six new haplotypes are *shaded in black*

(Fig. 3), also revealed differentiation of haplotypes into two groups from the Danube drainage in Slovenia (northern groups) and group of haplotypes from the Danube drainage in Bosnia–Herzegovina, Montenegro and Serbia (southern group). Two Slovenian haplotype subgroups, (Da25 & Da24) and (Da22 & Da23), differ in 8–10 mutations. Within the southern group, haplotypes vary up to three mutations, except haplotypes Da29 and Da32 which are three to six mutations away from other southern haplotypes. Number of variable sites between northern and southern groups' haplotypes ranges from five to twelve, with corresponding genetic distance of 0.75–1.8%. Haplotype Da33 is most closely associated with the haplotypes of the northern Alpine clade, from which it differs in 10–12 mutation, with corresponding genetic distance of 1.5–1.8%. Haplotype Da33 is separated from the most related haplotype of the Adriatic clade by 26 mutations, corresponding to the genetic distance of 3.9%, and it is 15–17 mutations away from all other clades in Fig. 2, with the equivalent genetic distance of 2.25–2.55%. The most divergent haplotype within Adriatic clade is Ad7, six to nine mutations distant from other Ad haplotypes. Haplotype Ad7 is separated from the most closely related haplotype of the Balkan clade by 23 mutations, corresponding to the genetic distance of 3.45%.

Microsatellite DNA analysis

Allelic richness ranged from 1.75 to 4.08 and the observed heterozygosity ranged from 0.19 to 0.59 within the studied populations (Table 1). The highest levels of allelic richness (4.08), the observed (0.59) and the expected (0.58) heterozygosities were detected in the Tolminka River samples, while those of the Bosna River had very low levels of heterozygosity. Without Tolminka, overall allelic richness was 6.33 and observed heterozygosity 0.28. Deviation from HWE was detected only in the population from the Krušnica River (Table 1).

Population differentiation, clustering and introgression

Pair-wise F_{ST} comparison shown non-significant differences between a small number of locations, e.g. Sana/Sanica (converging rivers), Krušnica/Sanica (tributaries to Una and Sana, converging rivers), Sava/Obrh and Obrh/Unec [Obrh and Unec grayling populations were introduced from the Sava River (Fisheries Research Institute of Slovenia, personal communication)). Non-significant difference between geographically remote Ljuča and Sana has no obvious explanation (Table 2); however, a very small sample size was available for these locations. Close relationship among certain populations was also evident from D_{AS} tree (Fig. 4), where Sava/Unec/Obrh, and Ljuča/Drina created subtrees while Krušnica/Sana/Sanica/UnaI/UnaII created closely associated subtrees. From D_{AS} tree, close relationship between Tolminka (Adriatic drainage) and Slovenian populations from the Danube drainage was evident. Also, Neretva population was the closest to three Slovenian populations of the Danube drainage (Table 2).

Hierarchical clustering using STRUCTURE separated grayling of Slovenian origin from the other populations in the first step (Fig. 5). Samples from the Neretva population represented a mixture of both groups. In the second step, more profound separation within grayling populations from Slovenia was evident; genetically distinct grayling introgressed with Sava genes in Tolminka was separated from grayling

Table 2 Paired values of F_{ST} above and D_{AS} below the diagonal for microsatellite marker data

	Ljuča	Drina	Neretva	Bosna	Sanica	Sana	UnaII	UnaI	Krušnica	Obrh	Unec	Sava	Tolminka
Ljuča		0.224**	0.465**	0.658**	0.465**	0.529^{NS}	0.578**	0.534**	0.386**	0.476**	0.491*	0.500**	0.346**
Drina	0.188		0.398**	0.560**	0.359**	0.378**	0.433**	0.315**	0.296**	0.473**	0.459**	0.494**	0.346**
Neretva	0.427	0.425		0.577**	0.358**	0.392**	0.492**	0.472**	0.309**	0.345**	0.328**	0.336**	0.258**
Bosna	0.537	0.553	0.499		0.577**	0.599**	0.680**	0.684**	0.511**	0.634**	0.636**	0.638**	0.453**
Sanica	0.415	0.359	0.317	0.474		−0.016^{NS}	0.503**	0.405**	0.002^{NS}	0.532**	0.513**	0.538**	0.366**
Sana	0.439	0.400	0.369	0.431	0.014		0.562**	0.512*	0.006^{NS}	0.558**	0.540**	0.568**	0.358**
UnaII	0.423	0.347	0.372	0.578	0.369	0.427		0.309**	0.409**	0.572**	0.581**	0.583**	0.435**
UnaI	0.341	0.265	0.380	0.551	0.292	0.372	0.119		0.285**	0.571**	0.576**	0.591**	0.398**
Krušnica	0.371	0.301	0.286	0.463	0.001	0.033	0.308	0.222		0.474**	0.455**	0.478**	0.349**
Obrh	0.395	0.514	0.258	0.579	0.556	0.589	0.461	0.479	0.517		0.026^{NS}	0.043^{NS}	0.200**
Unec	0.439	0.534	0.281	0.603	0.567	0.602	0.491	0.507	0.532	0.022		0.067*	0.177**
Sava	0.404	0.521	0.230	0.566	0.525	0.557	0.469	0.499	0.488	0.024	0.047		0.212**
Tolminka	0.555	0.587	0.362	0.637	0.601	0.637	0.569	0.589	0.573	0.257	0.260	0.259	

NS non-significant after Bonferroni-type correction

** $P < 0.01$; * $P < 0.05$

populations from the Sava drainage. In the southern Sava cluster, genetically homogeneous populations appeared in the rivers Bosna, Drina, Sana/Sanica, and UnaI/II, and a mixed population in Krušnica (the only one deviating from HWE), probably derived from closely related Una and Sana/Sanica. Neretva population formed a separate cluster in the second step regardless whether included among the samples of Slovenian origin (Fig. 5) or among the other southern populations (data not shown). In the third step, the populations containing hybrid individuals (Tolminka, UnaII and Krušnica) were even more evident than in the second step. Separate analysis of UnaII in the fourth step (not shown) did not reveal any intrapopulation structure.

Discussion

Phylogenetic relationship among the studied populations and their relation to other European populations

Based either on mtDNA or on nuclear microsatellite DNA, we demonstrated high genetic diversity of grayling populating the western Balkan area [14 mtDNA haplotypes altogether (Weiss et al., 2002 and this study)]. Comparing this genetic diversity of west Balkan's populations to that found in other studied European grayling populations (Koskinen et al., 2002b; Weiss et al., 2002; Gum et al., 2003, 2005), it is evident that the objects of this study represent a substantial part of the entire European grayling genetic pool.

Distribution pattern of genetic variation of the studied grayling implies two clearly separated evolutionary groups, represented by grayling native to the Adriatic drainage in Slovenia, and grayling from the Sava River system (Danube drainage). This is an expected observation, as the Adriatic and Danube populations have been split for ~4 MY, which also lines up with previous studies (Sušnik et al., 2001; Weiss et al., 2002). Given geographical, genetic and morphological distinction (Janković, 1960), we believe that these two evolutionary groups should be considered subspecies. Here we support the nomenclature using designations the Adriatic grayling, as previously proposed by Sušnik et al. (2001), and the tentative name Balkan grayling.

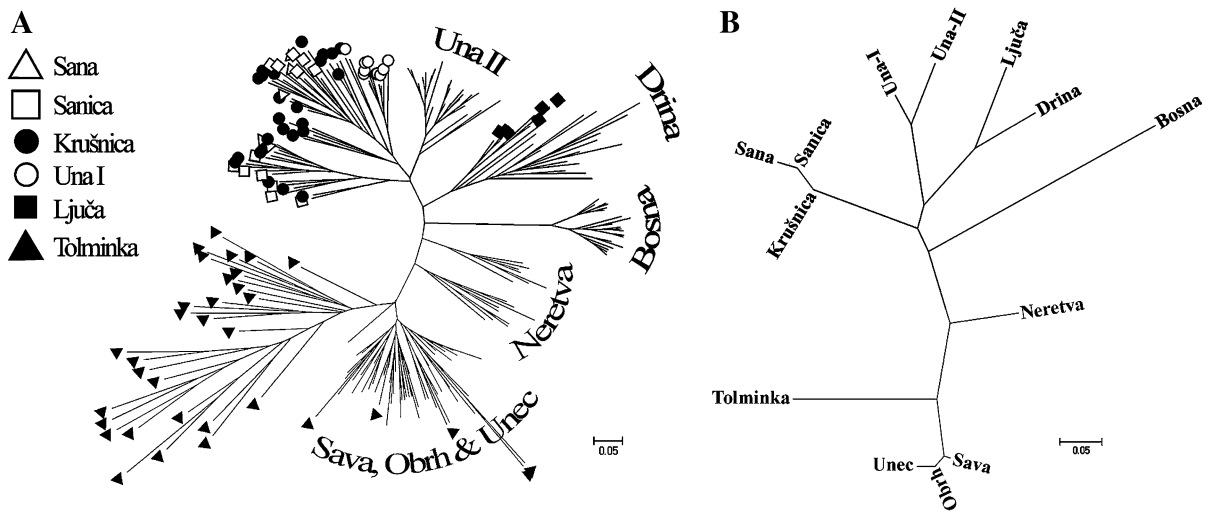

Fig. 4 NJ individuals (**a**) and population (**b**) trees based on D_{AS} estimated from 12 microsatellite DNA loci

Contrasting the distant relationship of geographically remote Adriatic and Balkan grayling, populations of the Balkan grayling, ranging from Slovenia to Montenegro, are much more related, which appears to be a direct consequence of the shared both, ancestry and the river system. The clade they form occupies comparably distinct position in the tree in relation to other previously described clades (Weiss et al., 2002) or lineages (Gum et al., 2009) and may thus be considered an evolutionary distinct lineage. The Balkan grayling turned out to be a well supported sister clade to the clade of Scandinavian haplotypes, which implies their common ancestry, most likely in the Caspian basin refuge as previously proposed (Koskinen et al., 2000; Weiss et al., 2002; Marić et al., 2011).

However, haplotype Da33, found in the Drina River basin, substantially differs from all the other haplotypes of the Balkan grayling and thus cannot be considered a member of this clade. Rather, it exhibits a considerable similarity with the Alpine group of haplotypes, particularly with the northern Alpine clade, as suggested by genetic distance data. Two possible scenarios may thus explain this finding, (1) this haplotype was introduced from unknown location of the upper part of the Danube River into the Drina River, which is known to be stocked with foreign grayling in the past (Marić et al., 2011), and (2) this is an ancestral haplotype, native also to the western Balkans suggesting that this area served as a colonization corridor of the upper Danube.

The genetic variation of the studied grayling was proven to be geographically localized to specific river systems or even separate rivers (Table 1; Fig. 5). This observation is in accordance with the proposed stationary life of grayling (Ovidio et al., 2004; Heggenes et al., 2006) and confirms a notion that present-day grayling is a poor invader in waters already inhabited by a local population (Gardiner, 2000). High level of overall genetic variation and genetic distinctiveness of local populations are also concordant with an extensive morphological variation of grayling observed in former Yugoslavia as well as with highly distinct morphological characters not only between grayling populations from different river systems but also within neighbouring populations of the same river system (Janković, 1960).

Despite populating the same river system, the Balkan grayling appeared to have evolved into two genetically differentiated sub-lineages represented by northern Sava population in Slovenia (northern group) and mid-Sava population observed in Bosnia–Herzegovina, Serbia (Marić et al., 2011) and Montenegro (southern group). However, a deep and long-branched split, giving rise to two sister subgroups within the northern group (Fig. 2) along with well differentiated southern group may also suggest that these three groups did not evolve due to vicariance but rather as a consequence of multiple colonization waves that might have occurred in the studied Danube area. Taking into account the molecular clock of 1% per million years (Koskinen et al., 2002c; Weiss et al., 2002; Froufe et al., 2003a), the

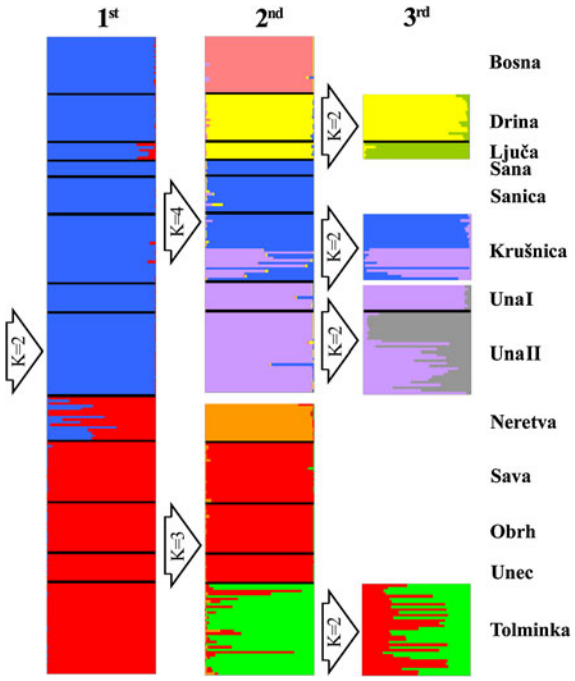

Fig. 5 Estimated population structure as inferred by hierarchical STRUCTURE analysis of microsatellite marker DNA data. *Black lines* separate sampling sites, after three steps nine different clusters were identified. The most probable K for analyzed samples given in *arrows* is based on ΔK method; no further structures were detected in subsequent rounds and within sampling locations ($K = 1$)

hypothesized colonization events occurred in the period from 750,000 to 1.65 million years ago or during the Pleistocene, well before the glacial events (Penck & Brückner, 1909; Gibbard & van Kolfschoten, 2004). This notion is in accordance with Weiss et al. (2002) and implies that grayling has survived considerable temperature oscillations during glacial events in distinct refugia, also within the Danube drainage of the Balkan Peninsula. The hypothesis gains significance if the scenario of autochthonous haplotype Da33 is accepted. Interestingly, four highly divergent grayling clades [Danube Northern Alps, Danube Southern Alps, Mixed Rhine/Danube (Weiss et al., 2002) and the Balkan one (Marić et al., 2011 and this study)] along with some well supported divisions within certain clades characterize the Danube drainage, indicating profoundly structured Danube grayling population as suggested by Gum et al. (2009). This points out to a very complicated colonization pattern of the drainage and/or complex paleo-hydrological events shaping the drainage prior to the glaciation events.

A pronounced split of the haplotype groups, pointing to the coexistence of divergent grayling groups, was found also in the Adriatic grayling (Fig. 2). This observation suggests a similar scenario of multiple colonization and/or complex paleo-hydrological events within the Adriatic drainage, too. However, genetic distances between the Balkan and Adriatic clades range from 3.45 to 4.05%, suggesting that their split occurred in mid-Pliocene, which is consistent with the previous reports (Sušnik et al., 2001; Koskinen et al., 2002c; Froufe et al., 2003a, b; 2005).

Observed stocking activities

Although there was a substantial part of phylogeographic structure found in patterns of haplotype distribution in this study, some unexpected haplotypes as well as introgression of non-native microsatellite alleles were detected in certain sampling locations.

Aquaculture of grayling in the western Balkans first began to develop in Slovenia in the 1960s (Fisheries Research Institute of Slovenia, personal communication); as a consequence Northern Sava haplotypes were distributed across several locations in the Danube and Adriatic drainages in the western Balkans.

One clear example of translocating grayling is the distribution of haplotype Da25 which is, based on phylogenetic analysis, clearly of the northern Sava origin, but was detected also in the Rzav River in Serbia (Marić et al., 2011) and the Neretva River in Bosnia–Herzegovina. Furthermore, the haplotype Da23 being of northern Sava origin, was also detected in the Neretva River and the Adriatic basin in the Tolminka River in Slovenia. Grayling is not native to the Rzav and Neretva rivers nor is the Danube one native to the Adriatic part of the river system in Slovenia (Sušnik et al., 1999a, 2004; Weiss et al., 2002). However, it is reported (Fisheries Research Institute of Slovenia, personal communication) that Tolminka and Neretva rivers have been stocked with grayling from the northern Sava River system, which thus resulted in introgressed Adriatic population in Tolminka River, and in a formation of novel population in the Neretva River, which became apparent after microsatellite analysis (Fig. 5). Interestingly, another haplotype, Da30, appearing in the nearby Vrbas River (Table 1), was also found in the Neretva River, where the admixture with microsatellite alleles of the

southern Balkan grayling was also evident (Fig. 5, 1st hierarchical level). This suggests that the grayling population in the Neretva River was formed not only of the Northern Sava population as generally presumed, but also of geographically much closer populations from Bosnia–Herzegovina.

Aside from having been stocked and introgressed with nearby northern Sava population from Slovenia, grayling population from the Tolminka River has been introgressed even with geographically very distant populations as evidenced by the presence of haplotype Da27 in this river, which is clearly of southern Balkan origin (Figs. 2, 3). Our results (STRUCTURE and D_{AS}) support previous observation of widespread introgression of grayling in the Soča River basin (Sušnik et al., 2004) and are also congruent with the results of wild male genotyping being annually performed in a frame of Adriatic grayling action plan (Jesenšek & Šumer, 2004), which have revealed only hybrid individuals with varying proportion of parental alleles (D. Jesenšek, personal communication). The Adriatic grayling conservation programme in Slovenia thus relies upon hybrid conservation, because this appears to be the only available option to avoid the complete loss of the hybridized native population.

Lower part of the Drina River also suffered from significant stocking with the material from the Sava River in Slovenia (Marić et al., 2011). However, as inferred from microsatellite DNA analysis of the present study, the upper part has not been affected and that native population appears to remain pure (Figs. 4, 5); however, the presence of haplotype Da33 should be born in mind, as its origin is not clear.

Implications for conservation and management strategies in the region

Based on the results, introduction of non-native grayling into the rivers of the Western Balkans has been confirmed only on a small number of locations. The remaining unaffected populations certainly deserve a protection from possible stocking with non-native material in the future.

For the effective conservation and management of biological resources, assigning evolutionary significant units (ESUs) and management units (MUs) is very important (Waples, 1991; Moritz, 1994). However, already assigned ESUs in grayling should be considered with caution; namely, only one ESU has been recently proposed for the entire Danube grayling population (see Gum et al., 2009). Taking into account at least three rather distinct clades within the basin (i.e., from the Danube Northern Alps, Danube Southern Alps and the Balkan, Fig. 2) and having in mind conservation and management strategies within the basin, introducing a single ESU does not seem to be an acceptable approach for effective conservation of the Danube grayling diversity and its phylogeographic structure in the area. Therefore, on the basis of current phylogeographic information, available for the Danube grayling, we propose to define at least three grayling ESUs: Danube Northern Alps, Danube Southern Alps and Balkan. Balkan ESU could further be divided into MUs associated with populations from the northern Sava and at least three MUs associated with populations from the Una River basin, Bosna River basin and Drina River basin, representing the middle and the southern Sava River system (Figs. 4, 5). In addition, there is no doubt that the Adriatic grayling should be considered a separate ESU, as first described in Sušnik et al. (2004). The assignment of the proposed categories would certainly help to sustain diversification of grayling populations in the region and preserve unique, autochthonous genetic resources.

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