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Genetic diversity and phylogenetic origin of brown trout *Salmo trutta* populations in eastern Balkans

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Abstract: The study focuses on the phylogenetic origin and genetic diversity of brown trout in the eastern part of the Balkan Peninsula. It further aims to reveal the impact of human-mediated transfers and stocking with non-indigenous trout on the populations in this area. For these purposes, mtDNA control region and microsatellite variation of 204 individuals from 16 populations were analysed. The results indicate that mtDNA haplotypes from the lower Danube basin and southern Black Sea basins differ substantially from a subclade of the Danubian lineage consisting of haplotypes found so far in the most of the Danube basin and in the Caspian and Aral Sea basins. Considering also the results of demographic analyses, this study evidences a complex evolutionary history of brown trout in the southern and western parts of the Black Sea basin. In the Aegean Sea basin, a high frequency of the central haplotype of Adriatic mtDNA lineage has been found. The other Adriatic lineage haplotypes found in this basin differ from the central haplotype by one mutational step only, indicating a recent evolution of the Adriatic lineage in the Aegean Sea basin. Substantial genetic differentiation among populations and basins was revealed. The hybridization with Atlantic brown trout was indicated in both sea basins, but especially in the Danube basin. Compared to other European regions, it can be inferred that the introgression of exogenous brown trout in the eastern Balkan populations is rather low.

Key words: Danube; microsatellites; mitochondrial DNA; stocking; introgression

Introduction

The Balkan Peninsula is considered as a hotspot in the evolution of many European species (Hewitt 2004). Due to the complex geological history, it also represents one of the most important areas of European ichthyofauna (Bianco 1990; Economidis & Banarescu 1991). In the eastern part of the Balkan Peninsula, the Ponto-Caspian and the Ponto-Aegean ichthyofaunas came into contact at the end of Pleistocene as a consequence of the salinity dilution and penetration of freshwater fishes via Black Sea-Aegean Sea junction (Bianco 1990).

The large part of the brown trout genetic as well as phenotypic variation was found in the Balkan Peninsula. Many taxa of trout were described in this region based on morphological features. During the last decades, the phylogenetic position of these taxa has been revaluated using genetic analyses. In general, five major groups have been identified within the brown trout based on the mitochondrial DNA (mtDNA) data (Bernatchez et al. 1992; Bernatchez 2001). They were named Atlantic (AT), Danubian (DA), Mediterranean (ME), *marmoratus* (MA) and Adriatic (AD) lineages. Bernatchez (2001) assumed that these lineages have evolved in geographic isolation and remained allopatric during the Pleistocene. The most ancient separation appeared between the Atlantic, the Ponto-Caspian and the Mediterranean drainages, giving rise to the three major groups of brown trout, the Atlantic, Danubian and Mediterranean lineages (Bernatchez 2001; Cortey et al. 2004). Subsequent fragmentation led to the separation between the Mediterranean, *marmoratus* and Adriatic lineages within the Mediterranean basin. Nevertheless, Sušnik et al. (2005) and Bardakci et al. (2006) revealed deeply divergent mtDNA haplotypes in the Tigris River basin indicating an existence of another lineage. Geographically restricted mtDNA lineage has been recently suggested also in the Duero River basin of the Atlantic catchment (Vera et al. 2010). The large part of the brown trout genetic as well as phenotypic

variation was found in drainages of the Mediterranean Sea. The present distribution of the brown trout lineages within the Mediterranean area shows a complex mosaic pattern (Apostolidis et al. 2008a). Bernatchez (2001) hypothesized that Adriatic and *marmoratus* lineages evolved in the Adriatic and Balkan part of the Mediterranean area, whereas the Mediterranean lineage originated in the western part of the Mediterranean area. Cortey et al. (2004) suggested that, beside allopatric isolation, parapatry might also have played an important role in the brown trout evolutionary history. According to the authors, the western part of the Mediterranean basin could have served as a centre for an expansion of the Adriatic lineage, although the Mediterranean lineage reveals the largest diversity of mtDNA in this area (Cortey et al. 2004; Sušnik et al. 2007). On the other hand, Bardakci et al. (2006) supposed pre-Pleistocene isolation and diversification of the Adriatic, Danubian and Tigris lineages in Turkey. A deep divergence of brown trout within the Black Sea basin has also been indicated (Weiss et al. 2001; Duftner et al. 2003; Bardakci et al. 2006; Marić et al. 2006; Turan et al. 2009). These findings suggest that beyond the Mediterranean region, the Black Sea basin have had a very important role in the formation of the brown trout diversity.

In Europe, stocking by man has altered the genetic diversity of brown trout populations. The hybridisation of local populations with non-indigenous brown trout, mostly of Atlantic origin has been widely observed (e.g., García-Marín et al. 1998; Hansen 2002; Sanz et al. 2006; Thaulow et al. 2013). Extensive hybridization between the Atlantic and Danubian mtDNA lineages due to the repeated transfers and long-term stocking have been reported in the upper/middle Danube River basin (Weiss et al. 2001; Duftner et al. 2003; Kohout et al. 2012). Stocking activities and their impacts on genetic structure has been reported also in eastern Balkans. The Nestos (Mesta) River was stocked in its Greek part about 30 years ago by fish from Acheloos River (Apostolidis et al. 1997), which belongs to the Adriatic-Ionian Ichthyogeographical zone (Economidis & Banarescu 1991), and mtDNA haplotypes that most likely originated from the released individuals have been frequent in the population 20 years after stocking (Apostolidis et al. 1997). Fishes from the Acheloos River have also been released into the Venetikos River, a tributary of the Aliakmon River, and a high number of individuals with the Acheloos haplotype was detected in the Venetikos River by Apostolidis et al. (2008a). Impact of stocking with exogenous fish was indicated using microsatellite markers in the Axios River basin (Apostolidis et al. 2008b). Marić et al. (2006) reported occasional stocking with non-indigenous fish in some rivers of Serbia. Brown trout had also been transported from former Czechoslovakia to Bulgaria during the second half of the 20th century. Nevertheless, it is very difficult to come across any written evidence regarding the transfers from those times and the only information source is a weak one, restricted to local fishermen.

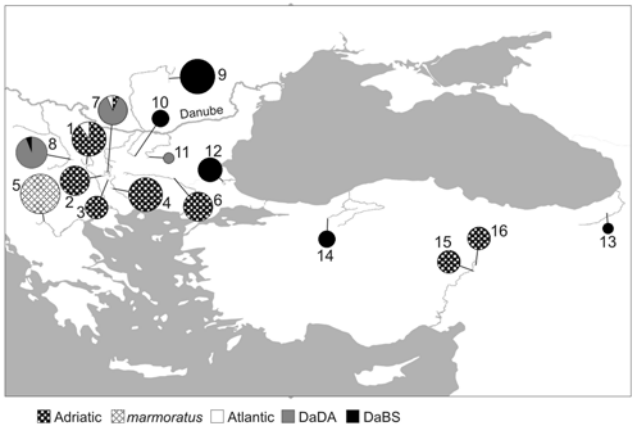

Fig. 1. Map showing the geographic origin of the samples. Pie charts display the frequencies of haplotype groups for each sample. The size of the circle corresponds to the number of analysed individuals from each sampling site. The numbers of sampling sites correspond to the numbers of populations in Table 1.

The extensive impact of stocking has been reported in central European parts of the North, Black and Baltic Sea basins, where the genetic variability among populations is almost lost (Kohout et al. 2012).

Although the brown trout *Salmo trutta* L., 1758 belongs to the most intensively studied freshwater fishes in Europe, our knowledge of its genetic variation is decreasing from the west to the east, and there is only little information on the eastern Balkan populations. Therefore the aim of this study was to uncover the brown trout genetic diversity in the eastern Balkan Peninsula. The phylogenetic relationships of brown trout from the eastern Balkans to the brown trout from other parts of its distribution were evaluated on the basis of mtDNA control region sequence data. In the second step, tracing of the origin of populations from the Black Sea and Aegean Sea drainages were based on mtDNA and microsatellite analyses. The data were searched for indications of hybridization between the eastern Balkan basins and introgression from the Atlantic drainage.

Material and methods

Sampling and laboratory analyses

A total of 204 individuals of brown trout were collected from 2006 to 2008. Samples originated from four river drainages of the Aegean Sea basin and from two river drainages of the Black Sea basin in the eastern Balkans. For comparison, samples from four Turkish locations, including the flathead trout *Salmo platycephalus* Behnke, 1968 from a tributary of Zamanti River (Seyhan River basin), were included. If possible, individuals of various age classes were sampled from a longer section of rivers (at least 100 m) to reduce a probability of family sampling. Details about the samples origin and numbers of specimens are given in Table 1 and Fig. 1.

Fin clips were preserved in 96% ethanol and stored at 4 ℃. The genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, CA, USA). MtDNA control region of 994 bp was amplified using primers L19 (Bernatchez et al. 1992) and HN20 (Bernatchez & Danzmann 1993) with PCR conditions: 94 ℃ for 5 min, 35 cycles at 94 ℃ for

Table 1. Sample sizes and frequencies of mtDNA haplotypes in brown trout populations analysed in the present study. Newly described haplotypes are written in bold type. Populations 1 and 2 belong to the Struma (Strymon) River basin and populations 3 and 4 belong to the Mesta (Nestos) River basin.

Population	No. of individuals assessed		mtDNA haplotype																								
	mtDNA	msat	AdAE3	ADcs1	AdAE2	AdZA1	ADcs20	AdAE1	H13	H1	Da1A	Da26	DaDA2	Da2	DaDA1	DaBS9	DaBS3	DaBS4	DaBS2	DaBS5	DaBS6	DaBS7	DaBS8	DaBS1	MAcs1
Aegean Sea basin																									
1. Treklianska R.	22	20		14				4		2															
2. Rilska R.	16	16						16																	
3. Dobarska R.	9	9	3	6																					
4. Tufca R.	20	20		16	4																				
5. Aliakmon R.	27	27																							27
6. Maritza R.	15	15		12			3																		
Black Sea basin – Danube																									
7. Cerny Iskar R.	15	15						1	1		6	5	1	1											
8. Džepska R.	17	17									2			2	12	1									
9. Timiş R. (Olt)	21	21															2	5	11	3					
10. Beli Vit R.	5	5															3							2	
11. Vidima R.	2	2										2													
Black Sea basin – non-Danube																									
12. Rezovska R.	10	10																						10	
13. Çoruh R.	2	2																					2		
14. Lake Abant	5	5																			3	2			
Seyhan R. basin																									
15. Örenşehir	9	11				9																			
16. Karagöz	9	9				9																			

30 s, 50 °C for 30 s and 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Amplified fragments were sequenced on ABI Prism 3130 Genetic Analyzer (Applied Biosystems, CA, USA). Sequences were revised and aligned using BioEdit version 7.0.9 (Hall 1999). For microsatellite analyses, the same conditions and the same primers as described in Lerceteau-Köhler & Weiss (2006) were applied. Two multiplex PCR sets with eight and four primer pairs were used. However, one locus (OMM1064) had shown non-unambiguous allele sizes and it was therefore removed from further analyses. Amplified fragments were separated on an ABI Prism 3130 Genetic Analyzer and determined relative to ROX size standard using GeneMapper 3.7 software (Applied Biosystems, CA, USA).

Data analyses

For mtDNA, the number of haplotypes was computed using DnaSP v5 (Rozas et al. 2005). The phylogenetic relationships among haplotypes were evaluated using median-joining network in Network 4.6. software (Bandelt et al. 1999). In order to identify new haplotypes and to reveal their phylogenetic relationships, all sequences of the brown trout control region of appropriate length available from GenBank were included in the first analysis. For better clarity of the network the subsequent analysis was conducted with representative haplotypes of the five lineages described by Bernatchez (2001), with an exception of the Danubian lineage, for which all available haplotypes were used (Fig. 2). Average Tamura-Nei nucleotide distances were computed for the pairs of haplotype groups. The phylogenetic reconstruction using maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) analyses were implemented in order to specify the relationships outlined by haplotype network. Prior to the analyses, the best fitting model of nucleotide substitution was assigned using Modeltest 3.7 (Posada & Crandal 1998). Under Akaike information criterion (AIC), the TRN+I+G model was selected. ML analysis was performed in GARLI v. 0.95 (Zwickl 2006) with the parameter setting as estimated by Modeltest. MP and NJ analyses were performed in PAUP 4.0b10 (Swofford 2002). For MP, insertions and deletions were included as a fifth character. Statistical support for branching patterns was estimated by 1000 bootstrap replications.

To trace the demographic changes in the populations, the DnaSP v5 software (Rozas et al. 2005) was employed. First, the distribution of the number of pairwise mutation differences between sequences (the mismatch distribution), which is expected to be unimodal in recently expanded populations, but irregular in shape in stationary populations (Rogers & Harpending 1992), was assessed. The raggedness index, which quantifies the smoothness of the observed mismatch distribution, was tested against the null distribution based on 1000 coalescent simulations for neutral populations of the same genetic diversities. Next, the Fu's test of neutrality, which is expected to take large negative values in expanded populations (Ramos-Onsins & Rozas 2002), was applied. Last, the Tajima's D test of neutrality, where the presence of significant departures from the null hypotheses may suggest either selective pressures on the locus under study, or changes in the population size, was performed.

For microsatellites, allele frequencies, F_{ST} values between pairs of populations and values of F_{IS} were computed in Genetix v.4.05 (Belkhir et al. 2000). Allelic richness, the measure of the number of alleles independent of the sample size, was calculated using FSTAT (Goudet 2001). All 11 loci were tested for deviations from Hardy-Weinberg equilibrium by the Fisher's exact test in GENEPOP v.4.1 (Raymond & Rousset 1995). Using the same software, each pair of loci was tested for genotypic (linkage) disequilibrium. The significance levels for multiple comparisons were adjusted using the sequential Bonferroni correction (Rice 1989). The GenAlEx 6.5 software (Peakall & Smouse 2006) was used to determine private alleles in each population and in the three basins (Black Sea, Aegean Sea, the Zamanti River). The analysis of molecular variance (AMOVA) performed by

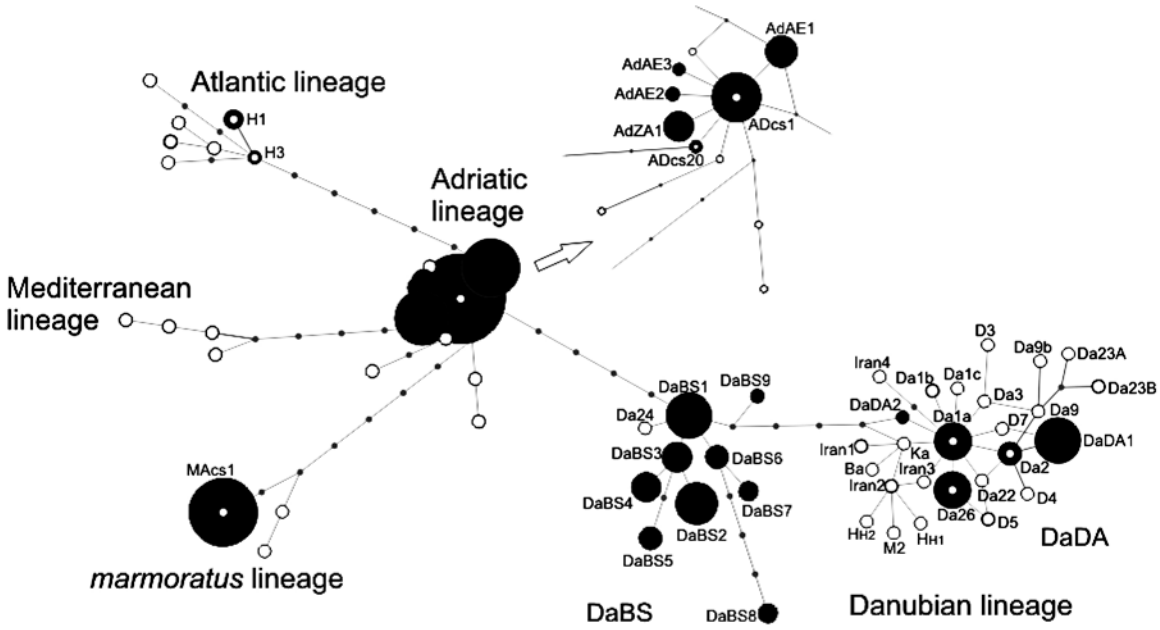

Fig. 2. Median-joining network of brown trout control region haplotypes. Original data (Tables 1 and 2), as well as previously published haplotypes are included: H1, H3 (Cortey & García-Marín 2002); Da1a, Da1b, Da2, Da22, Da23a, Da23b, Da3, Da9, Da24 (Duftner et al. 2003); ADcs1, ADcs20, MAcs1 (Cortey et al. 2004); Da26 (Meraner et al. 2007); Da1c, Da9b (Griffiths et al. 2009); Iran1-4 (Vera et al. 2011); D3-5, D7 (Kohout et al. 2012); Ka, Ba, H_{H1}, H_{H2}, M_2 (Segherlo et al. 2012). Size of the circles corresponds to the haplotype abundance in the sample analysed in the present study. The white circles determine previously found haplotypes, the white dot in the centre of bigger circles designate previously found haplotypes revealed also in the present study.

Arlequin v.3.5 (Excoffier et al. 2005) was applied to estimate partitioning of diversity among the sea basins, among populations within the sea basins and within the populations, using 10,000 permutations. This analysis was employed for mtDNA as well as microsatellite data. For the population-based analyses only samples with at least 15 individuals were included.

The Bayesian-based clustering method in STRUCTURE software (Pritchard et al. 2000) was applied to infer the population structure and to reveal potential hybridisation between populations, without a priori assigned individuals to populations. The most probable number of genetic clusters (K) was estimated based on posterior probability of the data for a given K (Pr (X/K) and clarified using a ΔK (Evanno et al. 2005). For the estimation, genotypes were assigned into one to 15 groups and ten runs with 100 000 burn-in and 500 000 MCMC (Monte Carlo Markov Chain) iterations were applied for each K. For the graphic visualization of the results the bar plot implemented in STRUCTURE has been displayed. First, the Bayesian analysis was performed for all sampled individuals. In the second analysis, two hatchery populations of different Atlantic origin (IT, LO) and three wild populations (ZP, JP, OD) from the North and Baltic Sea basins in the Czech Rep. was included. These populations were found to be of pure Atlantic origin in the previous study (Kohout et al. 2012). Populations with extremely low sample size (? 9 individuals), the Seyhan River basin populations and the Aliakmon River population were excluded from this analysis.

Results

Mitochondrial DNA

Sequencing of mtDNA control region provided readable sequences of 994 bp corresponding to the segment analysed in the previous studies from Central Europe (Duftner et al. 2003; Kohout et al. 2012). Among 204 individuals originating from 16 sampling sites, a total of 23 haplotypes was revealed, 15 of which were found for the first time (Tables 1 and 2). The haplotype diversity within the whole sample set was 0.851 $\pm$ 0.011 and the nucleotide diversity 0.0063 $\pm$ 0.0002, defined by 23 polymorphic sites, four of which were insertions/deletions. The two-base deletion in positions 928-929 was unique for the haplotype DaBS8 found in both individuals from Olgunlar. The insertion in position 111 was characteristic for the haplotype MAcs1 fixed in the Aliakmon River sample. The insertion in position 938 distinguished the haplotypes of the Adriatic lineage and DaBS group (see below) from all other haplotypes, with the exception of DaDA2 haplotype. Within the Black Sea basin, the number of haplotypes was 14, the haplotype diversity 0.904 $\pm$ 0.013, the nucleotide diversity 0.0045 $\pm$ 0.0001 and the number of polymorphic sites 18. Within the Aegean Sea basin, the number of haplotypes was six, the haplotype diversity 0.692 $\pm$ 0.029, the nucleotide diversity 0.0028 $\pm$ 0.0001 and the number of polymorphic sites ten.

The median-joining network (Fig. 2) indicated six haplotype groups: Atlantic, Adriatic, Mediterranean, *marmoratus* and two groups from the Ponto-Caspian and Aral Sea area. The first of the Ponto-Caspian groups, further referred to as 'Danubian group' (DaDA), included all haplotypes from the Cerni Iskar River, the Džepska River (except for one haplotype), the Vidima River, as well as almost all published Danubian lineage haplotypes from Austria (Weiss et al. 2001; Duftner et al. 2003), Serbia (Marić et al. 2006), Switzer-

Table 2. Variable base positions among newly found *Salmo trutta* haplotypes based upon 994 bp of the control region. Nucleotide positions are numbered according to the Da1a haplotype (Duftner et al. 2003).

		Variable sites																					
Haplotype	GenBank No.	24	59	225	231	232	233	259	387	400	527	539	540	545	660	786	814	910	928	929	938	967	991
Da1a	AY185568	A	C	T	G	A	G	G	C	T	C	A	C	T	T	T	T	T	A	T	–	T	C
DaDA1	GQ357906	.	.	.	.	G	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DaDA2	GQ357907	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
DaBS1	GQ357897	.	.	.	.	.	.	.	.	.	T	G	G	C	C	.	.	.	.	.	T	.	.
DaBS2	GQ357898	.	.	G	.	.	.	.	.	.	T	G	G	C	C	A	.	.	.	.	T	.	.
DaBS3	GQ357899	.	.	G	.	.	.	.	.	.	T	G	G	C	C	.	.	.	.	.	T	.	.
DaBS4	GQ357900	.	.	G	.	.	.	.	T	.	T	G	G	C	C	.	.	.	.	.	T	.	.
DaBS5	GQ357901	.	.	G	.	.	.	.	.	.	T	G	G	C	.	.	C	.	.	.	T	.	.
DaBS6	GQ357902	.	.	.	.	G	.	.	.	.	T	G	G	C	C	.	.	.	.	.	T	.	.
DaBS7	GQ357903	.	.	.	.	G	.	.	T	.	T	G	G	C	C	.	.	.	.	.	T	.	.
DaBS8	GQ357904	.	.	.	A	G	.	.	.	.	.	G	G	C	C	.	.	.	–	–	T	.	.
DaBS9	GQ357905	.	T	.	.	.	.	.	.	.	.	G	.	C	C	.	.	.	.	.	T	.	.
AdAE1	GQ357908	T	.	.	.	.	T	C	.	C	T	G	G	C	.	.	.	.	.	.	T	C	.
AdAE2	GQ357909	C	.	.	.	.	T	C	.	C	T	G	G	C	.	.	.	C	.	.	T	C	.
AdAE3	GQ357910	C	.	.	.	.	T	C	.	C	T	G	.	C	.	.	.	.	.	.	T	C	.
AdSE1	GQ357911	C	.	.	.	.	T	C	.	C	T	G	G	C	.	.	.	.	.	.	T	C	T

land (Meraner et al. 2007), Czech Republic and Slovakia (Kohout et al. 2012). All haplotypes found in the Caspian Sea basin and two inland lake basins in Iran (Vera et al. 2011; Segherloo et al. 2012) and the Aral Sea basin (Griffiths et al. 2009) belonged also to this group. The second group, in this study named as the 'Black Sea group' (DaBS), included the haplotypes from the Timiş River, Beli Vit River, one haplotype from the Džepska River (carried by one individual) and all found haplotypes from the non-Danube locations of the Black Sea catchment. One haplotype from the DaBS group, previously published in GenBank, was reported from the Waldaist River in Austria (Weiss et al. 2001; Duftner et al. 2003). Another two haplotypes of the DaBS group (561 bp of length) were found in two tributaries of the Južna Morava River in Serbia (Marić et al. 2006). The average Tamura-Nei distance between the DaDA and DaBS haplotypes was 0.007.

More than half of the individuals from the Aegean Sea basin (excluding the Aliakmon River) carried the most common haplotype of the Adriatic lineage, reported by Cortey et al. (2004) from the western part of the Mediterranean area. Each of the five remaining Adriatic haplotypes, four of which were reported here for the first time, diverged from the most common haplotype by one mutational step only. One of them was fixed and the only found haplotype within 18 individuals of *S. platycephalus* from the Seyhan River basin. This haplotype differed by one base substitution from the 740 bp sequence of *S. platycephalus* published by Sušnik et al. (2004). All 27 individuals from the Aliakmon RIVER carried the most widely distributed haplotype of the *marmoratus* lineage (Berrebi et al. 2000; Cortey et al. 2004; Meraner et al. 2007).

The tree topologies of all three phylogenetic analyses (ML, MP, NJ) revealed four (*marmoratus*, Mediterranean, Atlantic and Danubian) of the five described clusters (Bernatchez, 2001), whereas the Adriatic haplotypes did not form sufficiently supported cluster in any case. Within the Danubian cluster the DaDA group, indicated by haplotype network, created well-supported subclade. The relationships of the remaining haplotypes of the Danubian cluster, corresponding to the DaBS group of the haplotype network, were not statistically supported.

AMOVA showed that the highest portions of mtDNA variance were distributed among the basins (47.53%) and among the populations within the basins (43.55%), whereas the percentage of variance within populations was 8.93%. The unimodal trend of the mismatch distribution and the raggedness index (0.169, $P < 0.01$) suggested a recent expansion of the Adriatic lineage in the Aegean Sea basin. The results of Fu's test and Tajima's D test were negative, however not significant. The expansion was indicated also for the Adriatic lineage in its whole distribution area (samples of Cortey et al. 2004; Sušnik et al. 2007; Marić et al. 2006; Snoj et al. 2009 and this study were included). Significant values of the Fu's Fs (–25.749, $P < 0.001$) and Tajima's D test (–1.621, $P < 0.01$) were also observed. Although a value of the raggedness index was not significant, the unimodal shape of the curve of mismatch distribution indicates a recent expansion. In contrast, no expansion was revealed for the whole data set from the Ponto-Caspian basin. However, concerning only the frequencies of the haplotypes within the DaDA group, an expansion was indicated by the unimodal trend of the mismatch distribution (raggedness index not significant), by the Tajima's D test (–1.436, $P < 0.05$) and by the Fu's Fs (–14.227, $P < 0.001$). Frequencies of haplotypes revealed in this as well as previous (Duftner et al. 2003; Meraner et al. 2007; Griffiths et al. 2009; Vera et al. 2011; Segherloo et al. 2012) studies were included in this analysis.

Microsatellites

All 11 microsatellite loci were polymorphic, with five to 37 alleles per locus. The average allelic richness per

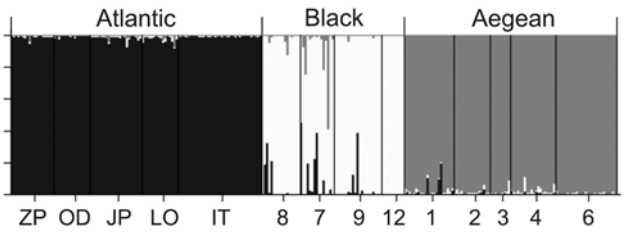

Fig. 3. Individual membership of the samples from the Atlantic (including North and Baltic Seas), Black Sea and Aegean Sea basins in each cluster ($K = 3$) estimated using STRUCTURE. Each individual is represented by a vertical line. The letter codes correspond to the Atlantic origin populations analysed in the previous study (Kohout et al. 2012), the numbers correspond to the populations analysed in this study (Table 1).

locality was 4.31. None of the 55 pairs of loci differed significantly from linkage equilibrium. Significant departures from Hardy-Weinberg equilibrium were found after Bonferroni correction in the Džepska River and the Treklianska River ($\alpha = 0.05$). A significantly positive value of F_{IS} in the Džepska River (0.142, $P < 0.01$) indicated that this departure was due to heterozygote deficiency. In total, 35 (19% of all observed alleles) population-specific alleles and 53 (29% of all observed alleles) group-specific alleles were found. The Black Sea basin included 22, the Aegean Sea basin 18 and the Seyhan River basin 13 private alleles. The F_{ST} value across all eastern Balkan samples with more than 15 individuals excluding exogenous Aliakmon River sample was 0.23. A highly significant ($P < 0.001$) genetic differentiation was found for all pairs of populations, with F_{ST} values ranging from 0.11 (the Timiş River vs. the Cerni Iskar River) to 0.32 (the Treklianska River vs. the Džepska River). Since no genetic differentiation was revealed between the two samples from Seyhan River basin, these samples were further considered as one (F_{IS} for the grouped sample was not significant). Values of F_{ST} for the Seyhan River sample and the eastern Balkan samples then ranged between 0.39 and 0.47. AMOVA showed that 21.24% of variation was distributed among the sea basins, 14.50% among the populations within basins and 63.55% within the populations. Excluding the samples from the Seyhan River basin, 17.38% was distributed among the Sea basins, 14.76% among the populations within basins and 67.89% within the populations.

The Bayesian analysis in STRUCTURE revealed four groups that correlate geographically with the Black Sea basin, the Aegean Sea basin, the Seyhan River basin and the Aliakmon River. After excluding the Aliakmon and Seyhan River populations and including the three Atlantic basin populations from the Czech Republic three clusters were revealed (Fig. 3). The clusters corresponded to: 1. North and Baltic Sea basins samples including hatcheries, 2. samples from the Danube River basin in Balkans, 3. samples from the Aegean Sea basin. Nevertheless, certain level of admixture between the clusters one and two and clusters two and three was revealed in the Danube River basin. The Cerni Iskar River population had the highest membership of both alien clusters (0.099 and 0.084, respectively).

Discussion

Black Sea basin

Analyses of mtDNA revealed the new haplotypes in the Danube River basin and the southern Black Sea basin, which differ substantially from the most of published Danubian lineage haplotypes and are closely related to the three haplotypes previously reported from the upper and middle Danube River basin. In the haplotype network, the DaBS haplotypes formed an interior group located between the remaining haplotypes of the Danubian lineage and the Adriatic lineage. Whereas the ML, MP and NJ analyses resulted in paraphyly of all DaBS haplotypes, the remaining haplotypes of the Danubian lineage formed a statistically well supported cluster that includes also previously published haplotypes from Caspian and Aral Sea basins. In the haplotype network, all mutation paths from the Danubian lineage haplotypes to other lineages passed through the DaBS1 haplotype, which was fixed in the Rezovska River and found also in the Beli Vit River population (Danube River basin). According to mtDNA-RFLP analyses of Turkish brown trout, a most common haplotype BM12 was found in seven populations across the Turkish Black/Marmara Sea basins (Bardakci et al. 2006). Since it was fixed in three western populations including the Rezovska (Rezve) River population, it can be inferred that this haplotype may correspond to haplotype DaBS1. Assuming the position of DaBS1 in the haplotype network and the extensive distribution of BM12/DaBS1 across the Black Sea basin, this haplotype may be the ancestral haplotype of the other DaBS haplotypes. The most distinct haplotype DaBS8 was found in the easternmost sample from Çoruh River. This finding confirms the distinctiveness of the populations in eastern Anatolia, where two sympatric species of brown trout, differing in life-history, morphological and genetic characteristics, were recently described (Turan et al. 2009). The migratory species *S. coruhensis* occurs in lower parts of streams and rivers, whereas *S. rizeensis* is resident and inhabits upper parts of streams and rivers. Assuming all available data, the findings of the DaBS haplotypes are very rare in the upper/middle Danube River basin (Fig. 4). Moreover, the DaBS and DaDA haplotypes have been found together in only one population (Džepska River). It can be speculated that the occurrence of the distinct haplotypes in the Danube River basin may reflect an existence of different groups of populations. Nevertheless, this hypothesis cannot be tested based on our limited data. A more complex study analysing morphology, ecology and genetic variation must be performed to resolve this problem. Bernatchez (2001) suggested the Pleistocene expansion of the Danubian lineage, which was probably enabled by the cyclic glacial events that caused the water level and salinity changes of the Black Sea and the repeated interconnections of the Black,

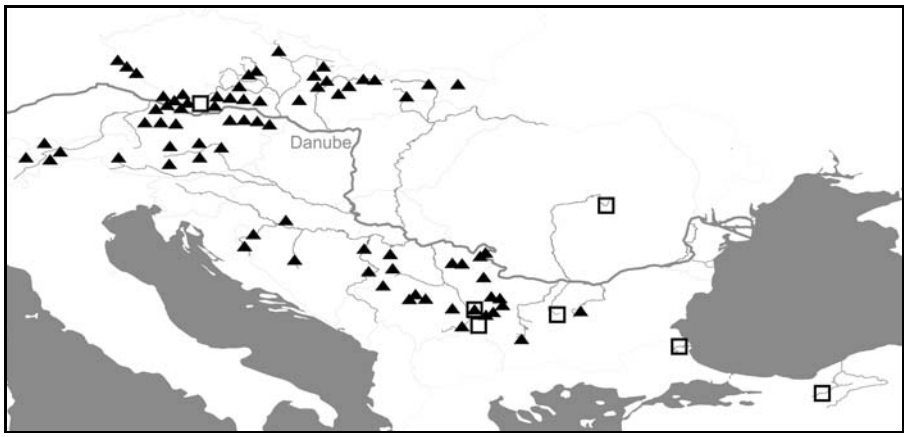

Fig. 4. Distribution of the DaDA (triangles) and DaBS (squares) haplotypes in the western part of the Black Sea basin using the data of Weiss et al. (2001), Duftner et al. (2003), Marić et al. (2006), Kohout et al. (2012), this study and our unpublished data. Some sample locations in Austria were not precisely resolved; therefore they were placed to the corresponding region according to Weiss et al. (2001).

Caspian and Aral Sea basins (Arkhipov et al. 1995; Kotlík et al. 2008). The past expansion of the DaDA haplotype group was indicated by the results of our demographic analyses. If the expansion was not revealed for the whole Danubian lineage including the DaBS haplotypes, our results may indicate separate evolution of the brown trout populations in the Black Sea basin. Unfortunately, despite our effort, only limited number of populations and individuals was available for this study. Further investigations analysing more individuals from more localities are needed to assess evolutionary history of brown trout in the Ponto-Caspian basin.

Mediterranean basin

The central haplotype of Adriatic lineage ADcs1 has an extensive occurrence in the Western Mediterranean (Cortey et al. 2004). Shorter sequences of the control region corresponding to ADcs1 were found in some drainages of Ionian Sea basin, Aegean Sea basin and Lake Prespa (Apostolidis et al. 1997, 2011; Marić et al. 2006; Snoj et al. 2009). The frequency of this haplotype was very high also in the Aegean Sea basin populations analysed in this study (61% considering the Adriatic lineage only). Except for this haplotype, no common haplotype have been found in the western and eastern Mediterranean. The distribution of the Adriatic lineage haplotypes and their relationships could be explained by a Pleistocene expansion of the lineage throughout the whole northern Mediterranean (Bernatchez 2001). This assumption is supported by the results of our demographic analyses, by the star-like shape of the haplotype network and by the high frequency of the ancestral haplotype ADcs1 in the western and the eastern Mediterranean. Subsequent isolation caused a genetic diversification of Adriatic lineage brown trout among regions and local populations. This process is particularly apparent in western Greece, where extremely strong differentiation among populations and low variability within populations have been reported based on microsatellite and mtDNA analyses (Apostolidis et al. 2008a, b, 2011). Our analyses on populations of eastern Balkans generated considerably different results. The F_{ST} values were significant but substantially lower compared to the values published for the Greek populations (Apostolidis et al. 2008b, 2011). Whereas the Greek populations were often fixed for one allele at several microsatellite loci, at least two alleles were found at each locus in all populations from the eastern Balkans. AMOVA revealed that the proportion of within-population variability is higher and the among-population variability is much lower in the eastern Balkan samples compared to the Greek populations. Such contrasted results are probably caused by a larger effective population size and/or incomplete isolation of the eastern Balkan populations resulting in a lower impact of genetic drift compared to the small isolated populations in Greece. Substantial differences in the partitioning of genetic variability between mtDNA and microsatellites were found in both regions. They may result from a different influence of genetic drift and different modes of transmission (Avise 2000; Haavie et al. 2000). The population of the Aliakmon River in central Greece was the only population with an mtDNA haplotype of the *marmoratus* lineage. Also microsatellite analysis showed substantial differentiation of this population and the other populations analysed in this study. In the Venetikos River (tributary of the Aliakmon River), Apostolidis et al. (2008a) found the *marmoratus* haplotype in high frequency (62%) and suggested that this river was repeatedly stocked by brown trout from hatcheries in the Ionian Sea catchment. Based on our data, the *marmoratus* haplotype seems to be rather fixed, since it was the only haplotype hold by 27 individuals of variable sizes (total length 80 to 400 mm). This finding could indicate that trout of non-indigenous, probably hatchery origin have established the population at least in the sampled part of the Aliakmon River. Analysis of the two Seyhan River basin populations confirmed the results of Sušnik et al. (2004), showing that the flathead trout possess reduced genetic variability and recent evolution within the Adriatic mtDNA lineage (ADcs1). Fixation for the

unique haplotype and the results of microsatellite analyses, however, revealed the substantial differentiation between the flathead trout and all other analysed populations.

Introgression and conservation implications

The hybridization between indigenous and non-indigenous brown trout was indicated in the lower Danube River basin and the Aegean Sea basin by mitochondrial DNA and microsatellite analyses. One individual in the Black Sea basin and two individuals in the Aegean Sea basin possessed Atlantic lineage haplotypes. Moreover, one individual in the Black Sea basin had the Adriatic lineage haplotype. Based on the admixture analysis in STRUCTURE, a contribution of the Central European and Aegean clusters to the populations in the lower Danube River basin was indicated. The introgression was most pronounced in the Cerni Iskar River, where Atlantic and Adriatic mtDNA haplotypes were found. The samples from the Aegean Sea basin consisted of clearly defined cluster, with only a minor level of admixture. In general, assuming also the substantial differentiation among populations and basins, it can be inferred that the introgression of exogenous brown trout to the eastern Balkan populations is rather low for the present. The natural spread of fish from upper/middle Danube River basin to its lower part is not possible since 1970s, when the Iron Gate dams were built. Transfers and stocking are thus the only mechanisms enabling further spreading of strongly introgressed Central European trout to the lower Danube River basin. Future management and conservation strategies should avoid such activities to prevent disruption of unique genetic structure of local populations.

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