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Jihočeská univerzita
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Population genetic structure of brown trout as groundwork for efficient management of fisheries in central European salmonid waters

Populačně genetická struktura pstruha obecného jako základ úspěšného obhospodařování lososových vod ve střední Evropě

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. DIVERSITY AND TAXONOMY OF BROWN TROUT

There are few European fishes, whose economic and ecological importance combined with scientific interest have resulted in such a volume of research like in case of brown trout. Many studies have revealed considerable variability of brown trout. In the past, great number of taxa has been described based on differences in morphology, ecology, behaviour and life-history. On the contrary, based on the ability to change its appearance in depends on environmental conditions, brown trout has been considered as one polytypic species *Salmo trutta*, Linneaus 1758. Recent investigations have shown that neither of the two views is unambiguously correct (Turan et al., 2009). Whereas several species and subspecies described on the basis of morphology have not been supported by genetic analyses, there are some evidences of the existence of sympatric populations that are completely or partly reproductively isolated (Allendorf et al., 1976; Ryman et al., 1979; Ferguson, 2004; Sušnik et al., 2005; Duguid et al., 2006; Turan et al., 2009). Assuming also differences in morphology, genetic variation and life-history, they could be considered as different species under most species concepts including the Evolutionary Species Concept (Mayden, 2002; Kottelat, 1997; Turan et al., 2009). Based on available data, Kottelat and Freyhof (2007) supposed at least 29 European species of brown trout. New species have been recently described in other areas, for example in North Africa and Anatolia (Delling and Doadrio, 2005; Turan et al., 2009). In general, regardless of the point of view on brown trout taxonomy, all available data evidence deep phenotypic, life-history and genetic diversity within brown trout.

1.2. EVOLUTIONARY HISTORY OF BROWN TROUT

Genetic diversity of brown trout has been studied extensively over the last three decades. First genetic studies on brown trout were conducted based on allozyme data and several evolutionary scenarios were proposed (e.g. Allendorf et al., 1976; Ferguson and Fleming, 1983; Ryman, 1983; Hamilton et al., 1989; Ferguson, 1989; Guyomard, 1989; Osinov, 1990; Ferguson and Taggart, 1991; García-Marín et al., 1999). In these studies, the *LDH-C1** (lactatdehydrogenase-C1) locus was found to be one of the most useful marker outlining the evolutionary pattern of brown trout, especially in the Atlantic basin. The two most common alleles of this locus are **100* and **90*. The allele **90* is specific for 'modern' race of brown trout from the Atlantic basin. The allele **100* has been found throughout the brown trout range, including some populations of the Atlantic basin. It is considered to be specific for an 'ancestral' race since it was found also in other salmonids. The two races probably reflect an allopatric evolution of brown trout in different refugia and subsequent recolonization of North Europe since glacial retreat at the end of last glaciation (10000–18000 years b.p.). The main distribution area of brown trout in the Atlantic basin during the last glaciation is supposed to be in Iberian Peninsula, France and North Africa. However, several glacial refugia existed at the margin of the ice sheets (Ferguson and Fleming, 1983; García-Marín et al., 1999; Laikre, 1999; Weiss et al., 2000; McKeown et al., 2010). Most detailed information on the brown trout evolutionary history has been obtained on the basis of mitochondrial DNA analyses. Five major evolutionary groupings have been proposed: Atlantic, Danubian, Adriatic, Mediterranean and *marmoratus*

(Bernatchez et al., 1992). These lineages were supported also in subsequent studies using sequencing and RFLP analyses of mitochondrial DNA fragments (Bernatchez and Osinov, 1995; Apostolidis et al., 1997; Machordom et al., 2000; Bernatchez, 2001; Suárez et al., 2001). Most of the subsequent phylogenetic studies were based on the mitochondrial DNA control region (D-loop).

The Atlantic lineage is distributed throughout the Atlantic river systems from the Atlas mountains in North Africa and the western part of Iberian Peninsula in the south to Scandinavia and White Sea basin in the north. The Black, Caspian and Aral Sea basins are inhabited by the Danubian lineage (Bernatchez, 2001; Weiss et al., 2001; Marić et al., 2006; Griffiths et al., 2009; Vera et al., 2011; Segherloo et al., 2012). Another three lineages have been found in peri-Mediterranean area. The Adriatic lineage is distributed across the northern Mediterranean and Turkey, including Iberian and Apenine Peninsulas, Adriatic, Ionian and Aegean Sea basins (Apostolidis et al., 1997; Bernatchez, 2001; Cortey et al., 2004; Sušnik et al., 2006, 2007; Marić et al., 2006; Snoj et al., 2009). The Mediterranean lineage inhabits the western and central part of the Mediterranean, whereas it has not been found in the eastern Mediterranean. The *marmoratus* lineage, named according to the species *Salmo marmoratus* Cuvier, 1829 is restricted to several rivers in the Adriatic-Ionian basin. It was revealed in several locations of the Balkan Peninsula that the *marmoratus* haplotypes and marbled phenotype may not be associated (Razpet et al., 2007; Snoj et al., 2009). Recently, new lineages of brown trout have been suggested in the Tigris River basin of the Persian Gulf catchment (Sušnik et al., 2005; Bardakci et al., 2006) and in the Duero River basin of the Atlantic catchment (Vera et al., 2010).

Although many studies on the distribution of the main evolutionary groups have been published, several phylogeographic questions have remained unresolved, especially in regions where the distributions of two or more lineages overlap. For example, a mosaic distribution of the three Mediterranean clades in the western and central Mediterranen has been found. Nevertheless, the evolutionary history of brown trout in this area is not clear, since hypotheses on the origin of the main lineages are inconsistent. Whereas some authors suggested centre of expansion of the Adriatic lineage in eastern Mediterranean (Bernatchez, 2001; Bardakci et al., 2006), Cortey et al. (2004) suggested parapatric evolution of the Adriatic and Mediterranean lineages in the western part of Mediterranean. Another question arose in the upper part of the Danube River basin, where a high frequency of the Atlantic lineage haplotypes has been reported (Weiss et al., 2001). It could be assumed that this finding is a consequence of multiple anthropogenic transfers and stocking. However, there are some indications of natural penetration of the Atlantic lineage into the Danube River basin already during the Pleistocene (Weiss et al., 2001; Duftner et al., 2003). A secondary contact between the Atlantic and Danubian lineages may occur also via interconnection between the Volga River basin and the Baltic Sea basin, but the human-mediated introduction cannot be ruled out in this case as well (Osinov and Bernatchez, 1996).

1.3. DISTRIBUTION OF GENETIC VARIABILITY IN BROWN TROUT

Except for the main evolutionary groupings, there is further genetic structuring of brown trout resulting from more recent events. Substantial part of the brown trout genetic variability is distributed among populations of particular areas representing different water systems. However, high genetic differentiation has been observed among populations within basins and even within particular rivers and lakes (Allendorf et al., 1976; Ryman et al., 1979; Ferguson and Fleming, 1983; Ferguson, 1989; Bernatchez et al., 1992). These population subdivisions are maintained due to restricted gene flow, which is a result of strong homing behaviour, existence of impassable geographic barriers and/or differences in spawning place and time. In general, populations in northern part of the Atlantic area including the North, Baltic and White Sea drainages are less genetically differentiated compared to populations from southern drainages. It is a consequence of shorter evolutionary history of these populations since this area was repeatedly ice-covered during the Pleistocene. Nevertheless, the post-glacial colonization and subsequent isolation resulted in substantial genetic differentiation among populations (Hansen and Mensberg, 1998; Sønstebø et al., 2007; McKeown et al., 2010). On the other hand, lack of differentiation between genetically monomorphic populations was found in Scotland (Prodöhl et al., 1997). The highest genetic diversity of brown trout has been observed in the Mediterranean-Adriatic region. It corresponds with its highest phenotypic diversity in this region (Behnke, 1968; Banarescu et al., 1971; Kottelat, 1997; Kottelat and Freyhof, 2007) and the fact that three of the five main evolutionary lineages recognized by Bernatchez et al. (1992) are restricted to this area. There is also very strong genetic differentiation among populations, especially in the eastern part of the area, i.e. Balkan Peninsula and Anatolia (Apostolidis et al., 2008, 2011; Bardakci et al., 2006). Based on microsatellite analyses, extremely deep genetic differentiation was observed between the brown trout populations in Greece (Apostolidis et al., 2008) and between the pure populations of marble trout, *Salmo marmoratus* in Slovenia (Fumagalli et al., 2002). Compared to other regions, there is a lack of information on genetic variability of brown trout in the Black, Caspian and Aral Sea basins, especially at the population level (Togan et al., 1995; Bardakci et al., 2006; Weiss et al., 2001; Duftner et al., 2003). Phylogeographic studies did not provide sufficient support for taxonomic distinction of several species previously described in this area, as all analysed populations belonged to one cluster based on allozyme and mitochondrial DNA (Danubian lineage) markers (Bernatchez and Osinov, 1995; Griffiths et al., 2009; Vera et al., 2011) or to a sublineage within the Danubian lineage (Segherloo et al., 2012). Although two differentiated species were recently recognized and supported by genetic analyses in tributaries of southern Black Sea basin (Turan et al., 2009), no information has been provided on genetic population structure of these species.

1.4. STOCKING AND HATCHERY REARING OF BROWN TROUT

Brown trout is one of the most popular and valuable fish in terms of recreational and production fisheries in fresh waters. As a consequence, it has been transferred and artificially propagated for centuries. Brown trout is bred in hatcheries and farms. Release of fish from hatcheries and farms to the wild is called stocking. Transfers and stocking are performed by fisheries managers, owners and scientists for many purposes. Most of the stocking activities have arisen from a presumption that it is beneficial and lead to increase in catches or enrichment of local fauna (Cowx, 1994). However, many stocking programmes lack well-defined objectives and definitions of success criteria and an efficiency of the programmes is not monitored (Cowx, 1994; Laikre, 1999). In the last decades, stocking strategies have been extensively discussed and re-evaluated (Cowx, 1994; Cowx, 1998; Laikre, 1999). Ferguson (2007) divided stocking according to its objectives and origin of stocked fish into four main categories:

1. Put-and-take stocking: Release of brown trout to water with little or no natural spawning with the aim to produce fish available for angling.
2. Stocking to restore: Stocking with purpose of saving populations from extinction and for re-establish populations, which had become effectively extinct.
3. Supplemental stocking: Release of farm-reared (for more than one generation) or wild non-native brown trout. The aim of this stocking is to improve fisheries or to increase number of fish for conservation purposes.
4. Supportive breeding: Stocking with first generation hatchery-reared offspring of wild native brown trout.

1.5. EFFECTS OF STOCKING ON GENETIC VARIABILITY OF BROWN TROUT POPULATIONS

Genetic changes of wild brown trout populations due to introgression with non-indigenous trout were reported in many European countries. First such studies used differential frequencies of the two *LDH-C1** alleles in farm-reared and wild populations to estimate the level of introgression in Northern Ireland (Taggard and Ferguson, 1986), Spain and Mediterranean drainages in France (García-Marín et al., 1991; Almodóvar et al., 2006). The hatchery strains used for stocking in Spain are mostly of Northern or Central European origin and are fixed or close to fixation for the *LDH-C1*90* allele. On the other hand, this allele is absent in native Iberian populations (García-Marín et al., 1991, Morán et al., 1991). Also other nuclear (allozymes and microsatellites) markers have been used to analyse the extent of introgression (e.g. Poteaux et al., 1998; Heggenes et al., 2002, 2006; Sanz et al., 2002, 2006; Jug et al., 2005; Sønstebø et al., 2007). In some cases, non-native origin of wild populations was detected based on mitochondrial DNA (Weiss et al., 2001). However, mitochondrial DNA alone cannot be used as direct indicator of introgression especially in populations where repeated stocking has been practised, since it reflects only maternal lineage origin and says nothing about intermixing between different gene pools. Moreover, the studies using both mitochondrial and nuclear markers showed discrepancies in introgression rate between markers probably due to assortative mating and/or sex-specific viability (Avise, 1994; Sanz et al., 2006). This lack of congruence between the two types of markers has been widely reported in brown trout and is probably caused by different modes of transmission and evolution (Bernatchez and Osinov, 1995; Apostolidis et al., 1997; Lu et al., 2001). Sanz et al. (2006) suggested a using of large number of genetic markers for increasing an accuracy of introgression rate evaluation. To reliably assess the genetic contribution of released fish to wild populations, the genetic structure of native population before stocking should be known.

The supplemental stocking may have very variable and hardly predictable effects on genetic structure of wild brown trout populations. Whereas stocking with farm-reared trout has often resulted in no or low genetic changes in native population, extensive introgression leading to a total replace of original population was reported in some cases (Ferguson, 2007). For example, approximately half of Iberian populations have shown introgression from hatchery-reared fish (Almodóvar et al., 2006). Whereas low level of introgression due to stocking was observed in some populations (Morán et al., 1991, 1995; Arias et al., 1995; Martínez et al., 1993), substantial introgression was revealed in another ones (Martínez et al., 1993; Aparicio et al., 2005). Very variable levels of introgression were found also in Mediterranean and Atlantic drainages in France (Guyomard, 1989; Poteaux et al., 1998; Berrebi et al., 2000). No or very low genetic contribution of hatchery-reared trout to wild populations was reported in Danish lake Hald and river Karup (Hansen et al., 1993, 1995; Hansen, 2002). On the other hand, stocking fish from the same domesticated strains into other rivers caused strong introgression (Hansen, 2002; Hansen et al., 2009). Such contrasted results were obtained also from Norway; despite of intensive stocking, very low introgression was observed in two lakes of Skiensvassdraget River system (Heggenes et al., 2002; Heggenes et al., 2006), whereas high introgression was found in three lakes of Hardangervidda mountain plateau (Sønstebø et al., 2008). The stocking had resulted in extensive introgression in Italy (Marzano et al., 2003; Caputo et al., 2004; Splendiani et al., 2006). High introgression level from hatchery-reared trout was found also in *Salmo marmoratus* populations in Slovenia (Delling et al., 2000; Jug et al., 2005). Despite the long-term and extensive mixing and stocking hatchery-reared trout in Central Europe, substantially variable frequencies of *LDH-C1*90* allele and Atlantic lineage haplotypes were found in German and Austrian parts of the Danube River basin (Riffel et al., 1995; Weiss et al., 2001).

1.6. FACTORS INFLUENCING THE LEVEL OF INTROGRESSION FROM HATCHERY-REARED TROUT

There are several reasons for inconsistent genetic consequences of stocking. Reduced survival and lower performance of stocked hatchery-reared compared to wild trout was reported in many studies (e.g. García-Marín et al., 1991, 1998; Martínez et al., 1993; Weiss and Schmutz, 1999; Fjellheim et al., 2003; Baer, 2004). It can be a result of differential environmental conditions, unsuitable techniques of stocking and hatchery rearing, behavioural, morphological and physiological differences and other factors. For example, greater survival of hatchery-reared stocked trout was reported in lakes than in rivers (Martínez et al., 1993; White et al., 1995). It corresponds with findings that a level of introgression depends on physical and hydrological conditions such as discharges, water level and temperature (Madeira et al., 2005; Almodóvar et al., 2006). Non-random mating between hatchery-reared and wild fish can also influence the introgression level (Largiadèr and Scholl, 1996; Almodóvar et al., 2006). It can be a result of mate choice or spatial and temporal differences in spawning (Shields et al., 2005). Hansen and Mensberg (2009) hypothesised that later spawning of indigenous trout compared to farm one rescues a part of wild population from introgresion. This suggestion was supported by the statement that broodstocks are often selected for early spawning (Hansen et al., 2002). Origin of stocked fish is another important factor. Each population is characterised by its unique adaptability to the local environment as a result of the interaction between many genes, so-called "co-adapted gene complexes" (Templeton et al., 1986; Laikre, 1999). Since stocked non-indigenous trout are often not adapted to the new environment, the effectiveness of stocking program can be hampered (Templeton et al., 1986). Low success of stocking and subsequent introgression is correlated with genetic changes in stocked farm trout, which have been reared in captivity for more generations. Founder effect, genetic drift and inbreeding may result in loss of genetic variability. Relaxed natural selection due to survival of fish, which would be eradicated in the wild, brings disadvantageous alleles to next generations (Elliott, 1989; Einum and Fleming, 2001). Artificial selection for growth, reproduction rate and other traits contributes to increasing differentiation between stocked and native trout (Ryman and Ståhl, 1980; Guyomard, 1989; Poteaux et al., 1998). Effectiveness of stocking such domesticated trout is usually very low. Poteaux et al. (1998) suggested that natural selection against domesticated genes of Atlantic origin trout in Mediterranean populations is responsible for limited hybridization between the two groups. It seems that higher densities of wild trout lead to stronger selection against stocked hatchery fish. However, if immigration rate by stocked trout override the selection, strong introgression may occur (Hansen, 2002; Hansen et al., 2009).

1.7. CONSEQUENCES OF INTERBREEDING BETWEEN NATIVE AND NON-INDIGENOUS BROWN TROUT

Stocking of hatchery-reared trout usually causes reducing of genetic variability among populations. Such genetic homogenisation was found in Great Britain and in Iberian Peninsula (Ferguson, 1989; Machordom et al., 1999; Almodóvar et al., 2006). It seems that not only direct introgression is responsible for the decrease of genetic differentiation among populations. Svärdson and Faderström (1982) suggested heritable differences in migratory pattern among brown trout populations. Hatchery fish usually have weaker homing than wild fish (Jonsson and Jonsson, 2006). It was hypothesised that stocked trout may increase rate of gene flow resulting in disruption of reproductive isolation between wild populations and subsequent homogenisation (Fergusson and Taggart, 1991). If stocked fish are of multiple origins, an increase of genetic variability among populations due to stocking may occur. Thaulow et al. (2013) compared historical and contemporary samples of brown trout from two river systems in Norway. Whereas historical samples from this area showed low genetic variability and represented one microsatellite cluster, contemporary samples were distinct from each other in proportions of another four clusters, presumably as a result of different origins of stocked fish. Stocking has also various effects on within-population genetic variability. It results in increase of genetic variability if the native population is sufficiently abundant and in all cases where hatchery-reared trout has higher genetic variability than the native population. If the stocked trout has reduced genetic variability and represents substantial proportion of a population into which they are stocked, genetic variability of the wild population decreases.

The interbreeding between non-native and native trout may have deleterious effect on population viability. Change in effective population size can cause inbreeding depression characterised by reduced survival, decrease in feed conversion efficiency, growth rate and increase in developmental abnormalities (Ferguson, 2007). Since released fish are genetically distinct from the wild populations into which they are stocked, the interbreeding may result in breakdown of co-adapted gene complexes and subsequent erosion of local adaptations, decrease in fertility, viability and other changes, which are more significant in later hybrid generations, i.e. outbreeding depression (Templeton et al., 1986).

1.8. HISTORY OF STOCKING AND HATCHERY-REARING IN CENTRAL EUROPE

Artificial reproduction and hatchery rearing of brown trout started in the middle of 19th Century. It was associated with transport of brown trout eggs, even between substantially distant locations, and their distribution in particular areas. First farm stocks originated from Central and Northern Europe. Hatchery-reared fish were stocked across this region. Subsequent farm strains were often derived from the first farms, but new farms with native trout were also established. There are some records from 19th century on transport of brown trout eyed eggs between substantially distant locations. For example, brown trout from German farms were imported into England (Ferguson, 2007). Until recently, all farm strains in Spain, Italy and France were established with exogenous brown trout of north or central European origin (Poteaux et al., 1998; Almodóvar et al., 2001). Farm strains in Slovenia are also of non-indigenous Atlantic or hybrid (Atlantic x *marmoratus*) origin (Jug et al., 2005). Based on the facts described above and the political history of Central Europe, it is not surprising that stocking in the present Czech Republic has a very long tradition. Since the middle of the 19th Century, eyed eggs of brown trout were distributed across the area of Bohemia and Moravia (Frič, 1875). In 1904, 45 salmonid hatcheries were in Bohemia, nine in Moravia and seven in Silesia (Lusk, 1989). After the World War II, a production of hatcheries and farms rapidly increased and stocking and transfers of fingerlings, eggs and brood fish across the whole Czechoslovakia became much more extensive. Moreover, imports of brown trout from other countries have been performed in order to "enrich" local ichthyofauna or increase catches (Kálal, 1989). Documented imports are listed below:

1862 – transport of eggs from Salzburg (Austria) to Nedošín (eastern Bohemia)
1946 – import of eggs from Denmark
1977 – import of eggs and fish from an Austrian farm (population Kolowrat)
1954, 1956, 1968, 1969, 1970, 1972 – eggs of 'sea trout' and 'lake trout' imported from Poland, fingerlings released in several lakes and reservoirs especially in Slovakia
1994, 1995 – imports of eggs from an Italian farm

Between the 1950s and 1990s, brown trout catches increased more than threefold. However, in the last two decades the catches and abundances of many brown trout populations have been decreasing, although the quality of water and other environmental conditions were significantly improved. The decrease may be caused by many factors and it is very difficult to estimate proportions of each of them. However, changes in genetic structure may be one of most important.

The aims of this thesis were:

1) To reveal the genetic structure of brown trout populations in the Czech Republic and Slovakia based on mitochondrial and nuclear DNA markers. The patterns of genetic diversity were analysed to determine extent of genetic changes due to human-mediated transfers and stocking.

2) To compare the genetic diversity of Central European populations and populations from eastern Balkans, where stocking activities have probably been limited.

3) To answer the phylogeographic questions regarding the colonization of the Danube River basin.

4) To outline management strategies for brown trout in the Czech Republic on the basis of results of the genetic analyses. An identification of populations or groups of populations, which show some unique traits, will serve for future management and conservation purposes.

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CHAPTER 2

EFFECTS OF STOCKING ON THE GENETIC STRUCTURE OF BROWN TROUT, *SALMO TRUTTA*, IN CENTRAL EUROPE INFERRED FROM MITOCHONDRIAL AND NUCLEAR DNA MARKERS

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Effects of stocking on the genetic structure of brown trout, *Salmo trutta,* in Central Europe inferred from mitochondrial and nuclear DNA markers

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Abstract Stocking has had a considerable effect on wild brown trout, *Salmo trutta* L., populations throughout Europe. To elucidate this impact and to outline further management strategies, the genetic structure of 25 wild populations and five hatchery stocks from Czech Republic and Slovakia were analysed using mitochondrial (control region) and nuclear DNA (microsatellites, *LDH-C1**) markers. Stocking practices have caused massive hybridisation between the Atlantic and Danube brown trout strains in the central Danube basin and have lead to a loss of among-population divergence in Slovakia and the eastern part of Czech Republic. Comparison with studies from neighbouring countries revealed substantial differences in haplotype, allele frequencies and genetic diversity across Central Europe. Differences in stocking management and origin of breeding stocks appear to be crucial factors for the spatial variability of the genetic structure of brown trout.

KEYWORDS: control region, Danube, introgression, *LDH-C1**, microsatellites, population genetics.

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Introduction

Efforts to increase the production of brown trout, *Salmo trutta* L., in Central European waters have been made since at least the Middle Ages (Andreska 1987; Largiadèr & Scholl 1995). During the last two centuries, the management of brown trout has been aimed at supporting populations, which have been weakened by environmental changes and overfishing. Extensive stocking was originally initiated as a putatively beneficial practice. However, together with degradation of the environment and harvesting practices, stocking has had unfavourable effects on wild populations (Laikre 1999). Farm-reared brown trout released into the wild differ from wild brown trout in many aspects including genetic, behavioural and other phenotypic characteristics (Ferguson 2007). Genetic changes caused by founding effects and subsequent domestication lead to reduced survival and ability to breed in the wild as a result of a few generations of farm rearing. Nevertheless, some farm-reared individuals usually survive and breed successfully in the wild. Supplemental stocking has an unpredictable effect on wild populations. While little or no effect was reported in some cases (Arias *et al.* 1995; Poteaux *et al.* 1998; Weiss & Schmutz 1999), extensive interbreeding between wild and farm-reared trout has been documented in many wild populations (Largiadèr & Scholl 1996; Weiss *et al.* 2001; Cagigas *et al.* 2006; Hansen & Mensberg 2009). Such interbreeding often causes a reduction in fitness and loss of local adaptation to various environmental conditions. The extent of such unfavourable changes is very hard to predict and may not be visible until it leads to strong population decline. Genetic variability among and within populations and the current introgression rates, therefore, need to be examined in specific populations to develop further strategies for stocking and conservation of the brown trout.

According to mtDNA analysis, there are five main lineages of brown trout, three of which are restricted to the peri-Mediterranean area (Bernatchez *et al.* 1992). The remaining two lineages have wide distribution areas. The Atlantic lineage is naturally distributed in the Atlantic basin northwards from North Africa, and it shows its greatest genetic variability in the Iberian Peninsula (Cortey *et al.* 2009). The majority of domesticated strains of brown trout were derived from this lineage. Human-mediated spread of the Atlantic lineage also occurred outside its natural distribution area, where it introgressed into many local populations of brown trout (e.g. Largiadèr & Scholl 1996; Poteaux *et al.* 1998; Weiss *et al.* 2001; Sanz *et al.* 2006; Meraner *et al.* 2007; Razpet *et al.* 2007). A lineage that is widely distributed throughout the Black, Caspian and Aral Sea basins has been described as the Danubian lineage. The upper part of the Danube River basin, in addition to the Danubian lineage, is also the habitat of the Atlantic lineage. In Austria, 44% of all individuals belong to the Atlantic lineage (Weiss *et al.* 2001). However, the Atlantic lineage is quite rare in the Danube River basin in Serbia (Marić *et al.* 2006).

Based on allozyme analyses, the *LDH-C1** locus was found to be a useful marker to distinguish the modern race of the Atlantic brown trout from brown trout of different origins (Ferguson & Fleming 1983). In northern Europe and in most of the farm stocks, the allele **90* predominates, whereas the allele **100* has been fixed in many wild populations found within the brown trout distribution area (Bernatchez & Osinov 1995). McMeel *et al.* (2001) described a protocol for routine typing of the *LDH-C1** by polymerase chain reaction (PCR).

The watercourses of Czech Republic and Slovakia represent the upper parts of four large European river systems – Danube, Elbe, Oder and Vistula. Many streams and rivers offer suitable habitats for brown trout. However, as in other parts of Europe, anthropogenic activities have introduced serious threats for local populations, which have led to decreases in trout densities. The stocking of non-indigenous brown trout has been very extensive in Central Europe. It was initiated during the Austrian-Hungarian Empire in the 19th Century. For example, in 1862, eggs of brown trout were transported from Salzburg (Danube basin) to Nedošín (North Sea basin), where the stock was set up (http://www.vackuvchovpstruhu.estranky.cz). For several years, fish from the stock were released into many rivers in the region as well as in neighbouring countries. During the 1940s, millions of fingerlings were transported from Denmark (Atlantic basin) to Czechoslovakia (Vejrychová-Solarová 1950). Since 1994, domesticated brown trout of Atlantic origin have been imported from Italy to the Czech Republic several times (Pokorný *et al.* 2000). Moreover, transport between populations, including those occurring within different sea basins, is often not documented. Currently, many more or less domesticated strains of brown trout are bred in farms. Extensive stocking with both hatchery-reared and domesticated farm-reared brown trout of diverse origins has occurred, but in recent years, anglers' unions have reported a substantial decrease in catches in the Czech Republic (http://www.rybsvaz.cz; http://www.mrsbrno.cz).

This study aims to determine the genetic structure of brown trout in the Czech Republic and Slovakia using mitochondrial (control region) and nuclear (*LDH-C1**, microsatellites) DNA markers. Because of the extensive transfer and stocking of brown trout, the routes and extent of interbreeding are traced. Several hatchery populations were analysed to evaluate genetic changes and possible effects of these stocks on wild populations. In addition, the present findings are compared with the results of previous studies carried out in neighbouring countries, and the possible threats posed by the current brown trout management in Central Europe are discussed. Recommendations for improving management and conservation are also included.

Materials and methods

Sampling and laboratory analyses

A total of 658 individuals from 30 populations of brown trout were collected from 2005 to 2009. Samples from all four river basins in the territory of Czech Republic and Slovakia and five hatchery populations were included. The wild samples represent populations with various stocking histories and angling pressure. Individuals of various age classes were sampled to eliminate sampling of siblings and individuals released in one stocking event. Details about the origin of the samples and numbers of specimens are given in Table 1. Wild individuals were caught by electric

Table 1. Sampling locations, status of populations and number of analysed individuals for each population and marker type

			No. of analysed individuals		
Abbreviations	Population	Status	mtDNA	*LDH-C1**	Microsatellites
Elbe R. basin					
KA	Kamenice	Wild	15	15	15
KW	Turnov – 'Kolowrat'	Hatchery	21	22	27
IT	Turnov – 'Italian'	Hatchery	22	22	38
LI	Liběchovka	Wild	11	11	11
UP	Úpa	Wild	20	19	20
LO	Nedošín	Hatchery	6	6	17
ZP	Zlatý stream	Wild	24	24	24
LA	Jezerní stream	Wild	21	21	21
ZE	Zelenský stream	Wild	19	19	19
PP	Pramenský stream	Wild	18	18	18
JP	Jiřetínský stream	Wild	27	30	24
BI	Bílý stream	Wild	19	19	19
Oder R. basin					
OD	Odra	Wild	28	25	24
VP	Vrchovištní stream	Wild	20	21	20
Vistula R. basin					
PO	Poprad	Wild	16	15	15
KV	Kežmarská Biela voda	Wild	15	15	15
Danube R. basin					
CP	Celní stream	Wild	32	34	24
ME	Medvědí stream	Wild	16	20	20
LP	Liščí stream	Wild	25	25	25
MP	Mlýnský stream	Wild	41	48	48
MD	Moravská Dyje	Wild	18	21	17
DY	Dyje	Wild	30	31	34
JI	Jihlava	Wild	26	21	16
DE	Desná	Wild	21	22	21
VB	Vsetínská Bečva	Wild	20	23	15
LT	Východná	Hatchery	28	28	28
BP	Biely stream	Hatchery	27	29	27
VA	Váh	Wild	22	23	23
TO	Topl'a	Wild	16	16	16
MU	Muráň	Wild	14	15	14
Total			638	658	655

fishing, while hatchery samples were provided by angling associations and fish farmers. Fin clips were preserved in 96% ethanol and stored at 4 °C. Genomic DNA was extracted using NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). A mtDNA control region of 992 bp was amplified using primers L19 (Bernatchez *et al*. 1992) and HN20 (Bernatchez & Danzmann 1993) under the following PCR conditions: 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Amplified fragments were sequenced on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequences were revised and aligned using BIOEDIT version 7.0.9 (Hall 1999). The nuclear *LDH-C1** locus was amplified according to the protocol described in McMeel *et al*. (2001). The PCR product of 440 bp was digested using *BseL*I (Fermentas Inc., Burlington, Canada), and fragments were separated by horizontal agarose gel electrophoresis. Homozygotes **90/90* possessed two bands, heterozygotes **90/100* three bands and homozygotes **100/100* had one band (see McMeel *et al*. 2001). For microsatellite analyses, the conditions and primers used were as described in Lerceteau-Köhler and Weiss (2006). Two multiplex PCR sets with eight and four primer pairs were used. However, the locus OMM1064 showed non-unambiguous allele sizes and it was, therefore, removed from further analyses. Amplified fragments were separated on an ABI Prism 3130 Genetic Analyzer and analysed in relation to ROX-labelled size standards using GENEMAPPER 3.7 software (Applied Biosystems).

Data analyses

The haplotype (h) and nucleotide (π) diversities (Nei 1987) were estimated with DNAsp v5 (Librado & Rozas 2009). The *LDH-C1** locus was tested for deviations from Hardy–Weinberg equilibrium using the Fisher's exact test in GENEPOP v.4.0. (Raymond & Rousset 1995). For microsatellites, deviations from Hardy–Weinberg equilibrium were evaluated based on F_{IS} values obtained using GENETIX software (Belkhir *et al*. 2000). Allelic richness (AR), the measure of the number of alleles independent of the sample size, was calculated using FSTAT (Goudet 2001). The GENALEX 6 software (Peakall & Smouse 2006) was used to calculate the expected and observed heterozygosity for each population. Genetic assignment tests were performed using the program GENECLASS 2 version 2.0 (Piry *et al*. 2004). The Bayesian model-based method of Rannala and Mountain (1997) was used. The probability that the individual belongs to particular population was calculated by simulating of 10 000 genotypes. Each individual was assigned to the population with the highest likelihood of its genotype. The Bayesian-based clustering method in STRUCTURE software (Pritchard *et al*. 2000) was applied to infer population structure and to reveal level of hybridisation between clusters. The most probable number of genetic clusters (K) was estimated based on the posterior probability of the data for a given K ($Pr(X/K)$) and clarified using a ΔK (Evanno *et al*. 2005). Ten Markov Chain runs were performed for each value of K, each consisting of 600 000 iterations with the first 100 000 iterations discarded as a burn-in, and the number of groups ranged between $K = 1$ and $K = 30$. F_{ST} between pairs of populations were calculated for mitochondrial and microsatellite data using Arlequin ver. 3.11 (Excoffier *et al*. 2005). The significance levels for multiple comparisons were adjusted using the sequential Bonferroni correction (Rice 1989). The levels of genetic diversity within and among populations were assessed by a hierarchical analysis of molecular variance (AMOVA) using the same software. Non-random *a priori* grouping models based on hydrological (sea basins and river basins) and political regions (countries) were compared. The significance of differences in population diversity indices (h, π) and in haplotype and allele frequencies was tested for predefined dichotomous categories using Student's t-tests. A paired sample Student's t-test was used to compare H_O and AR in all nuclear loci. Alleles frequencies from Germany (Riffel *et al*. 1995) and haplotypes frequencies from Austria (Weiss *et al*. 2001) were included in the tests.

Results

Based on the sequence variation of mtDNA, 27 haplotypes were identified, 19 of which were assigned to the Atlantic lineage and eight to the Danubian lineage. The Atlantic lineage was distributed in all localities, whereas the Danubian lineage was found only in populations of the Danube River and Vistula River basins (Fig. 1). The Atlantic lineage predominated in each of the populations. The frequencies of the Danubian haplotypes did not exceed 44%. There were three populations in the Danube River basin that did not contain individuals carrying the Danubian haplotype. The four most frequent Atlantic haplotypes, namely A1, A2, A3 and A4, were found in all four river basins. These correspond to the GenBank sequences of haplotypes AF273088, AF273087, AF274574 and AF273086 (Cortey & García-Marín 2002). These haplotypes are widely distributed in the

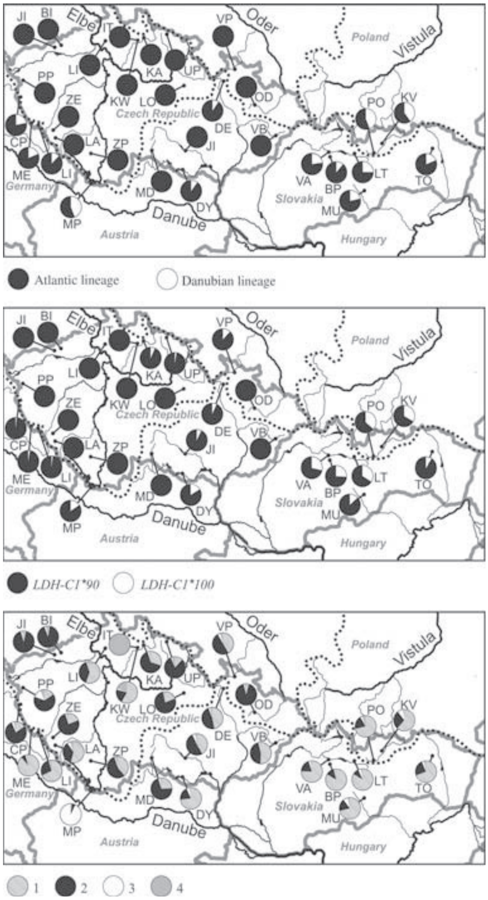

Figure 1. Maps of sampling localities with pie charts based on frequencies of the mtDNA haplotype groups, the *LDH-C1** alleles and the microsatellite clusters.

Atlantic basin and commonly found in hatchery stocks (Cortey & García-Marín 2002; Duftner *et al.* 2003; Cortey *et al.* 2004, 2009). Another four haplotypes, namely A6, D1, D2, D6, were previously found in Austria (GenBank accession numbers AY185577, AY185568, AY185573, AY185571; Duftner *et al.* 2003). One or two of the most frequent Atlantic lineage haplotypes (A1 and A2) were detected in five Atlantic basin populations and in one Danube basin population. A2 was the most abundant haplotype occurring in the three hatchery strains of the Elbe River basin, as well as in the two hatchery strains of the Danube River basin (Table 2). The haplotype A3, which was not found in any wild population from the Elbe River basin, was the second most abundant haplotype in the hatcheries. A1 was the most abundant haplotype in the Elbe, Oder and Vistula River basins. In the Danube River basin, haplotypes A1 and A2 were found with similar frequencies. Not considering singletons, private haplotypes were found in populations of Mlýnský potok (Danube River basin), Liběchovka (Elbe River basin), Váh (Danube River basin), Celní potok (Danube River basin) and Kolowrat hatchery population (Table 2). Newly found haplotypes were deposited in GenBank under the accession numbers HQ848357–HQ848375. The overall genetic differentiation for the mitochondrial DNA data was 0.209. Most of the values for pairwise comparisons were not significant after applying sequential Bonferroni correction (Table 3).

Both the **90* and the **100* alleles of nuclear fragment *LDH-C1** were found. The allele **90* predominated in all analysed populations. The allele **100* was found in all basins, with the highest frequencies in the Vistula River and Danube River basins. In the Elbe River basin, the allele **100* was identified in only two of 12 populations at very low frequencies (Fig. 1). Deviations from Hardy–Weinberg expectations were not detected in any of the analysed populations.

Pairwise F_{ST} values for microsatellite data revealed middle to low levels of genetic differentiation between populations (0.005 – 0.209). The overall F_{ST} was 0.071. The hatchery population of Italian origin and the Mlýnský potok population were the most divergent groups. The F_{IS} values indicated deviation from Hardy–Weinberg equilibrium because of heterozygote excess in the hatchery population of Italian origin and heterozygote deficiency in three wild populations of the Danube River basin. Mean values of *AR* for particular populations ranged from 3.298 to 6.883. Four main clusters were revealed using the Bayesian clustering method in STRUCTURE (Fig. 2). Two of the clusters were distributed throughout the sampled area (Fig. 1). Cluster one dominated in most of the wild populations of the Elbe and Oder river basins, as well as in two populations of the Danube River basin. Cluster two dominated in most populations of the Danube and Vistula river basins. The remaining two clusters corresponded to the Mlýnský potok population and the hatchery population of Italian origin.

The GENECLASS test assigned 36% of the individuals to the population they were collected from (Table 4). The frequency of self-assignment varied from 0% (Jihlava) to 67% (Mlýnský potok). Most of the individuals from the whole area were assigned to the Danube River basin. The number of self-assigned

Table 2. Frequencies of mtDNA haplotypes and the values of genetic diversity (h, π) for the 30 analysed populations

	Haplotype																													
Population	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	A17	A18	A19	D1	D2	D3	D4	D5	D6	D7	D8	Total	h	π
Elbe R. basin																														
KA	13	2																										15	0.248	0.000
KW	2	2	13	1								2			1													21	0.614	0.001
IT	1	21																										22	0.091	0.000
LI	6	1							3								1											11	0.673	0.001
UP	4	16																										20	0.337	0.000
LO		1	2			3																						6	0.733	0.001
ZP	14	9					1																					24	0.540	0.001
LA	21																											21	0.000	0.000
ZE	11	1		7																								19	0.556	0.001
PP	7			3	7													1										18	0.706	0.002
JP	24	2														1												27	0.211	0.000
BI	11	8																										19	0.515	0.001
Oder R. basin																														
OD	15	6			4	1	2																					28	0.704	0.002
VP	10	3	5	1		1																						20	0.695	0.001
Vistula R. basin																														
PO	4	3	2																	5	1		1					16	0.833	0.006
KV	4	2	1	1			1													3	2			1				15	0.895	0.006
Danube R. basin																														
CP	5	16				1				1				2						7								32	0.683	0.004
ME	7	4								2										2					1			16	0.642	0.004
LP	10	12																		2					1			25	0.627	0.003
MP	11	5						5			2									14		4						41	0.789	0.006
MD	11	7																										18	0.503	0.001
DY	13	6	7			1														1				2				30	0.736	0.001
JI	4	13	3	3		1	1												1									26	0.723	0.001
DE	10	5	1	1		2														1							1	21	0.733	0.003
VB	6	7		1		1	5																					20	0.758	0.001
LT	8	9	3	1																6	1							28	0.783	0.005
BP	7	11	4	2																1			2					27	0.761	0.003
VA	5	5	3	2									2							1	3		1					22	0.879	0.005
TO	1	6	3	3																1	1			1				16	0.825	0.005
MU	4	4	2	1																1	1					1		14	0.890	0.005
Total	249	187	49	27	11	11	10	5	3	3	2	2	2	2	1	1	1	1	1	45	9	4	4	4	2	1	1	638		

Table 3. Pairwise divergences between the brown trout populations using F_{ST} for microsatellites (below diagonal) and mitochondrial DNA data (above diagonal)

	KA	KW	IT	LI	UP	LO	ZP	LA	ZE	PP	JP	BI	OD	VP	PO	KV	CP	ME	LP	MP	MD	DY	JI	DE	VB	LT	BP	VA	TO	MU
KA		0.588**	0.813**	0.107	0.588**	0.687**	0.065	0.105	0.207	0.244	−0.031	0.129	0.029	0.142	0.342	0.325	0.154	0.100	0.082	0.283	0.097	0.072	0.249	0.044	0.155	0.155	0.101	0.133	0.175	0.154
KW	0.090**		0.483**	0.398**	0.419**	0.235	0.466**	0.705**	0.599**	0.486**	0.662**	0.464*	0.401**	0.246	0.419*	0.431**	0.240	0.328**	0.265**	0.397**	0.473**	0.157	0.236	0.271**	0.396**	0.253	0.174	0.239*	0.210	0.257
IT	0.154**	0.077**		0.431**	0.063	0.533*	0.455*	0.951**	0.742**	0.553**	0.828**	0.486**	0.377**	0.338*	0.452*	0.463**	0.166	0.310**	0.166	0.391**	0.526*	0.154**	0.143	0.215**	0.368**	0.235	0.126	0.243**	0.220*	0.271**
LI	0.107**	0.081**	0.154**		0.245	0.334	0.037	0.284	0.178	0.203	0.206	0.045	0.049	0.058	0.271	0.256	0.095	0.068	0.031	0.257	0.040	0.033	0.090	0.012	0.063	0.108	0.037	0.089	0.087	0.082
UP	0.071**	0.099**	0.133**	0.125**		0.408	0.239	0.794**	0.591**	0.452**	0.659**	0.224	0.246**	0.175	0.404**	0.409**	0.130	0.224**	0.091	0.354**	0.261	0.088	0.043	0.120	0.233	0.188	0.070	0.189	0.165	0.199
LO	0.103**	0.147**	0.209**	0.114**	0.142**		0.478*	0.855**	0.649**	0.436	0.764**	0.491*	0.361	0.260	0.297	0.306	0.135	0.218	0.171	0.323	0.506*	0.109	0.189	0.163	0.329	0.171	0.107	0.157	0.119	0.155
ZP	0.054**	0.080**	0.134**	0.092**	0.041**	0.094**		0.268	0.225	0.287**	0.157	−0.039	0.032	0.037	0.380	0.368	0.133	0.121	0.043	0.317**	−0.044	0.044	0.079	0.021	0.062	0.157	0.056	0.145	0.153	0.149
LA	0.072**	0.108**	0.166**	0.124**	0.135**	0.145**	0.089**		0.306	0.309*	0.014	0.404	0.104	0.309*	0.412**	0.394**	0.220	0.165*	0.168*	0.317*	0.376	0.145	0.410**	0.124	0.285**	0.212	0.184**	0.191*	0.266**	0.242**
ZE	0.059**	0.115**	0.153**	0.119**	0.061**	0.140**	0.067**	0.112**		0.241	0.245	0.304	0.113	0.245	0.340	0.307	0.177	0.117	0.135	0.274	0.284	0.126	0.297*	0.088	0.118	0.161	0.135	0.133	0.168	0.158
PP	0.062**	0.082**	0.116**	0.123**	0.041**	0.167**	0.070**	0.103**	0.085**		0.246*	0.297**	0.083	0.274**	0.340*	0.318*	0.227	0.179	0.205**	0.306*	0.285*	0.196**	0.330**	0.175	0.241*	0.215	0.206*	0.179	0.221**	0.206
JP	0.091**	0.124**	0.175**	0.123**	0.085**	0.132**	0.090**	0.148**	0.094**	0.113**		0.240	0.078	0.236	0.435**	0.418**	0.217	0.168	0.149*	0.335**	0.205	0.131*	0.347**	0.105	0.239**	0.217	0.168**	0.199**	0.265**	0.243**
BI	0.091**	0.132**	0.177**	0.133**	0.064**	0.140**	0.072**	0.169**	0.099**	0.099**	0.034**		0.046	0.031	0.361	0.353	0.120	0.117	0.034	0.307*	−0.055	0.035	0.067	0.019	0.095	0.147	0.046	0.136	0.142	0.140
OD	0.064**	0.098**	0.159**	0.119**	0.104**	0.149**	0.083**	0.100**	0.101**	0.077**	0.114**	0.111**		0.077	0.361	0.340	0.154	0.111	0.076	0.310**	0.036	0.075*	0.134	0.044	0.066	0.164	0.090	0.141	0.160	0.146
VP	0.045**	0.044**	0.107**	0.071**	0.049**	0.117**	0.032**	0.084**	0.077**	0.039**	0.084**	0.070**	0.065**		0.361	0.331**	0.121	0.120	0.051	0.306	0.032	0.003	0.011	0.022	0.090	0.135	0.026	0.120	0.104	0.110
PO	0.051**	0.038**	0.109**	0.052**	0.071**	0.075**	0.037**	0.082**	0.089**	0.077**	0.089**	0.072**	0.083**	0.020		−0.057	0.069	0.065	0.171	−0.027	0.353	0.191	0.366*	0.181	0.314	0.019	0.167	0.022	0.052	0.029
KV	0.063**	0.064**	0.127**	0.049**	0.084**	0.104**	0.044**	0.100**	0.077**	0.087**	0.105**	0.081**	0.091**	0.043**	0.010		0.070	0.051	0.163	−0.027	0.344	0.182	0.361*	0.166	0.289	0.016	0.162	0.011	0.042	0.020
CP	0.046**	0.082**	0.125**	0.087**	0.066**	0.122**	0.054**	0.100**	0.058**	0.053**	0.064**	0.059**	0.091**	0.049**	0.046**	0.055**		−0.012	−0.003	0.104	0.120	0.028	0.113	0.015	0.114	−0.021	0.004	−0.010	−0.026	−0.037
ME	0.103**	0.109**	0.152**	0.089**	0.096**	0.150**	0.061**	0.126**	0.076**	0.130**	0.128**	0.116**	0.146**	0.075**	0.065**	0.085**	0.085**		−0.016	0.078	0.109	0.013	0.157	−0.016	0.102	−0.025	0.007	−0.028	−0.015	−0.047
LP	0.063**	0.105**	0.135**	0.080**	0.072**	0.132**	0.047**	0.104**	0.054**	0.099**	0.091**	0.085**	0.111**	0.070**	0.068**	0.051**	0.059**	0.041**		0.177	0.034	−0.013	0.054	−0.034	0.058	0.014	−0.024	0.013	−0.003	−0.018
MP	0.111**	0.109**	0.180**	0.095**	0.134**	0.150**	0.100**	0.113**	0.122**	0.113**	0.136**	0.139**	0.120**	0.080**	0.078**	0.071**	0.099**	0.124**	0.123**		0.301	0.206*	0.331**	0.183	0.282**	0.056	0.190	0.062	0.099	0.067
MD	0.040**	0.082**	0.150**	0.080**	0.033*	0.068**	0.031*	0.104**	0.048**	0.080**	0.059**	0.044**	0.081**	0.038**	0.027	0.039**	0.042**	0.075**	0.052**	0.091**		0.035	0.080	0.015	0.093	0.144	0.047	0.131	0.140	0.136
DY	0.051**	0.043**	0.109**	0.064**	0.068**	0.076**	0.045**	0.080**	0.090**	0.066**	0.072**	0.075**	0.077**	0.028**	0.002	0.026**	0.051**	0.080**	0.075**	0.079**	0.035**		0.029	−0.023	0.062	0.032	−0.023	0.022	−0.003	−0.008
JI	0.016	0.047**	0.118**	0.059**	0.040**	0.084**	0.027	0.072**	0.047**	0.039**	0.065**	0.057**	0.052**	0.013	0.016	0.022	0.026*	0.070**	0.052**	0.047**	0.008	0.017		0.048	0.071	0.149	0.023	0.141	0.100	0.127
DE	0.032*	0.047**	0.114**	0.057**	0.055**	0.072**	0.018	0.065**	0.064**	0.049**	0.085**	0.073**	0.057**	0.006	0.011	0.036**	0.037**	0.062**	0.059**	0.075**	0.027	0.019*	0.007		0.033	0.023	−0.021	0.016	0.003	−0.012
VB	0.060**	0.059**	0.136**	0.050*	0.080**	0.082**	0.041**	0.066**	0.066**	0.095**	0.089**	0.092**	0.091**	0.037**	0.022	0.013	0.059**	0.055**	0.051**	0.070**	0.027	0.030**	0.023	0.033*		0.129*	0.053	0.111	0.097	0.105
LT	0.095**	0.055**	0.119**	0.072**	0.097**	0.113**	0.068**	0.089**	0.114**	0.092**	0.099**	0.118**	0.111**	0.056**	0.030*	0.041**	0.071**	0.091**	0.091**	0.069**	0.075**	0.018**	0.038**	0.047**	0.040**		0.016	−0.032	−0.033	−0.051
BP	0.050**	0.058**	0.111**	0.054**	0.072**	0.076**	0.037**	0.082**	0.089**	0.080**	0.084**	0.077**	0.083**	0.036**	−0.003	0.017	0.046**	0.069**	0.067**	0.089**	0.040**	0.004	0.024	0.021	0.032**	0.033**		0.009	−0.020	−0.021
VA	0.058**	0.041**	0.111**	0.064**	0.057**	0.084**	0.037**	0.086**	0.082**	0.063**	0.078**	0.065**	0.080**	0.024**	−0.001	0.014	0.043**	0.073**	0.066**	0.072**	0.026	0.003	0.012	0.019	0.024	0.027**	0.010		−0.038	−0.053
TO	0.058**	0.046**	0.111**	0.054**	0.089**	0.099**	0.043**	0.065**	0.096**	0.067**	0.097**	0.105**	0.082**	0.033**	0.003	0.024	0.058**	0.079**	0.071**	0.067**	0.058**	0.012	0.023	0.024	0.034*	0.021	0.012	0.010		−0.061
MU	0.073**	0.048**	0.112**	0.049**	0.067**	0.087**	0.045**	0.099**	0.078**	0.089**	0.090**	0.079**	0.093**	0.037**	0.012	0.013	0.059**	0.056**	0.056**	0.078**	0.026	0.007	0.027	0.027	0.018	0.028	0.010	0.005	0.027	

The significance levels were adjusted using the sequential Bonferroni correction (Rice 1989; initial $k = 435$). $*P < 0.05$; $**P < 0.01$.

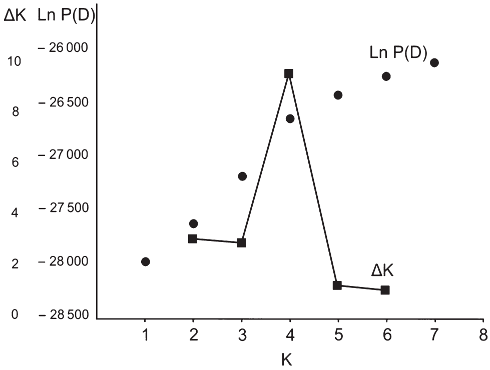

Figure 2. Probability of the data *P(D)* for a tested number of clusters (K = 1 to 8), and the rate of change in the log probability of data between successive K values, ΔK (Evanno *et al.* 2005). Based on ΔK, the most probable number of clusters was four.

individuals varied among particular river basins from 28% (Elbe River basin) to 96% (Danube River basin). Slight differences in assignment of individuals possessing the Atlantic and Danubian lineages were detected, as the assignment to the Danube River basin was 83% for the individuals of Atlantic lineage and 88% for the individuals of Danubian lineage. Similar results were obtained for the different *LDH-C1** genotypes.

AMOVA displayed the highest θ_{CT} value in the country-based model, which showed statistically significant values for both mtDNA (θ_{CT} = 0.3714, $P \leq 0.0001$) and microsatellites (θ_{CT} = 0.0083, P = 0.0444). The components representing variance within populations (θ_{ST}) and among populations within groups (θ_{SC}) were significant in all models.

Within the Danube River basin, the mean frequency of Atlantic mtDNA lineage was significantly higher in the Czech and Slovak (85%) than in the Austrian (36%) populations (t = 6.199, d.f. = 34, $P \leq 0.0001$). It was

Table 4. Percentage of individuals assigned to each population with GENECLASS software (Piry *et al.* 2004). Each individual was assigned to the sample in which it has the highest probability of belonging

	KA	KW	IT	LI	UP	LO	ZP	LA	ZE	PP	JP	BI	OD	VP	PO	KV	CP	ME	LP	MP	MD	DY	JI	DE	VB	LT	BP	VA	TO	MU
KA	**13**															27							7				13	7		33
KW	4	**61**													4	4						7		4						18
IT		11	**57**																			5		3			3			22
LI				**36**											9								9					9		36
UP					**15**					5				5	5	5					5	5	10	5			5	5		30
LO	6					**6**	6									12						18		18	6		6			24
ZL							**30**							4		9						17	4		9		9	4		13
LA								**43**							5	14	5									5	5			24
ZP									**11**							21						5	6		11					47
PP									6	**11**				6		6	11						17		11	6		6		22
JP	4										**38**	4				8	4					21	8							13
BI											5	**37**				11						5	11							32
OD													**46**			4	4					8	4	4	8			4	4	13
VP														**35**								15	10	10				5		25
PO															**7**	20						27					27	7	7	7
KV															13	**27**						13			7		13	13		13
CP															4	4	**50**					4	4					13	4	17
ME															5	10		**57**									10	5		14
LP																24			**12**			8	4		4		4	4		40
MP																2	2			**67**			6					2		21
MD															6	6					**12**	18					12	6		41
DY															3							**38**					9	3	3	44
JI														7		7						20		13			7	13		33
DE	5													5	5	5						10		**24**	10		5	5	5	24
VB															7	20							7		**47**	7				13
LT															4							7				**54**		7		29
BP																15						30					**19**			37
VA																5						23						**41**	5	27
TO																6						13					7	31	**25**	19
MU																13						13	7				20	13	7	**27**

Assignment of individuals to the population where they were sampled is indicated in bold.

also significantly higher in the upper (76%) and central Danube (89%) populations in Czech Republic and Slovakia than the left-side tributaries of the Danube in Austria (49%) (t = 2.102, d.f. = 12, P = 0.029; t = 4547, d.f. = 16, $P \leq 0.0001$). No differences in Atlantic lineage frequency were found between the Czech and the Slovak populations within the Danube basin. When considering the entire area, the Czech populations exhibited significantly higher frequency of Atlantic mtDNA lineage than the Slovak populations (t = 4.421, d.f. = 23, $P \leq 0.0001$). The genetic diversity (h, π) was significantly higher in the Slovak than in the Czech populations in the entire area (t (h) = 3.039, P = 0.003; t (π) = 5.217, $P \leq 0.0001$) and in the Danube basin alone (t (h) = 3.330, d.f. = 10, P = 0.004; t (π) = 5.217, $P \leq 0.0001$). In addition, *LDH-C1*100* allele frequency was significantly higher in Slovakia than in Czech Republic, including all the populations (22 vs 4%) (t = 5.750, $P \leq 0.0001$) and the Danube populations alone (17 vs 6%) (t = 2.277, P = 0.023). Within the upper Danube basin, the frequency of *LDH-C1*100* was significantly higher in Germany (40%) than in Czech Republic (6%) (t = 4.442, d.f. = 6, P = 0.004). Based on the microsatellite data, significantly higher values of heterozygosity were observed in Slovak than in Czech populations in the whole area (t = 3.934, d.f. = 20, P = 0.002) and in the Danube basin alone (t = 4.089, d.f. = 20, P = 0.002). The mean AR was also significantly higher in Slovak than in Czech populations in both comparisons (t = 5.862, d.f. = 20, $P \leq 0.0001$ and t = 5.133, d.f. = 20, $P \leq 0.0001$).

Discussion

The genetic mixing of Atlantic and Danubian groups of brown trout was confirmed using three types of markers. In particular, an extensive contribution of the Atlantic basin brown trout in the Danube River basin populations was found. However, a level of differentiation between populations was still detectable. Moreover, some of the populations possess unique alleles and/or haplotypes, thus maintaining substantial genetic diversification.

During the past two centuries, anthropogenic transfers have resulted in gene flow between substantially distant populations, even between different river basins. Currently, the breeding stocks of Central Europe contain gene pools of various origins. Considering that stocking with hatchery-reared fish occurs in this region, the high haplotype diversity and intermixing between the Atlantic and Danubian lineages in many populations is not surprising. Extensive distribution of the Atlantic mtDNA lineage in the Danube River basin was previously reported in Austria (Weiss *et al.* 2001). The populations in the Czech and Slovak parts of the Danube River basin showed higher frequencies of Atlantic lineage than the Austrian populations. The frequencies were also significantly higher in the central as well as upper Danube populations in Czech Republic and Slovakia than the left-side tributaries of the Danube in Austria. The among-countries partitioning seems to be the most pronounced among-group pattern of genetic diversity. A more intensive stocking of Atlantic brown trout in the Czech and Slovak than in the Austrian part of the Danube basin could explain the differences in Atlantic lineage frequencies. The significant differences in haplotype frequencies, H_O and AR, also indicate the distinct effect of stocking in Czech Republic compared with Slovakia. Whereas stocking has probably caused a loss of the genetic structure of brown trout within the Danube River basin in Czech Republic, it may lead to an increase in genetic variability in the Slovak and Austrian populations. However, a loss of the among-population genetic diversity at least in Slovakia has probably occurred as the pairwise F_{ST} values were not significant in most cases, even between the hatchery and wild populations. The assignment of majority of the Elbe River individuals to the Danube River basin could also indicate significant changes in genetic structure within the central Danube basin in Slovakia and Czech Republic. It is likely that in this part of the basin the among-population genetic variability is almost homogenised as a result of long-term stocking with fish of various origins. This could be the reason why the less introgressed populations in the Elbe basin were assigned to the Danube River basin.

The occurrence of the six Atlantic mtDNA haplotypes found exclusively in the Danube River basin and the substantial distinctiveness of Mlýnský potok population are difficult to explain. These could be the results of ancient introgression, as well as human-mediated transfers from stocks not analysed so far. A Pleistocene contact between the Atlantic and upper Danube populations was reported for the perch *Perca fluviatilis* L. (Nesbø *et al.* 1999) and European grayling *Thymallus thymallus* L. (Gum *et al.* 2005) and has also been proposed for brown trout (Bernatchez 2001; Weiss *et al.* 2001). However, the upper Danube populations of brown trout have been further affected by a recent gene flow from the Atlantic basin in Germany, where stocking has a very long tradition and where strongly introgressed populations are found (Riffel *et al.* 1995).

The A3 haplotype was revealed as one of the most frequent in hatchery stocks in Spain, Norway (Cortey

& García-Marín 2002; Cortey *et al.* 2004), Austria (Duftner *et al.* 2003), Czech Republic and Slovakia (this study). It was also found in wild populations from Denmark, Norway, Spain (Cortey & García-Marín 2002; Cortey *et al.* 2004), Austria (Duftner *et al.* 2003), Slovakia, as well as in the Danube and Oder River basins in the Czech Republic (this study). Therefore, the absence of A3 in the Elbe River basin could indicate a low impact of stocking with non-indigenous trout in this basin. The substantial differentiation of the strongly domesticated population (IT) revealed by microsatellite data also supports this suggestion. Nevertheless, the result of Bayesian analysis in STRUCTURE and the occurrence of the *LDH-C1*100* allele in the Elbe River basin indicate penetration of the Danube basin trout to the Elbe River basin.

Although the Atlantic mtDNA haplotypes dominated in each of the analysed populations, a high proportion of the Danube mtDNA phylogenetic group was found in the Vistula River basin. It corresponds with the hypothesis of post-glacial contact and colonisation of the upper tributaries of Vistula River basin from the Danube River basin (Konopinski *et al.* 2007).

The stocking with farm-reared brown trout has been performed to compensate for population decline as a result of exploitation or environmental changes. Nevertheless, stocking practices have various effects on wild populations. The farm population of Italian origin showed reduced genetic variability and other indications of domestication, such as enormously fast growth, willingness to accept granulated food and low survival of the offspring in a new environment (Pokorný *et al.* 2000). In addition, considering its late spawning (December and January; Pokorný *et al.* 2000), which might cause digging up of eggs of early-spawned fish (Ferguson 2007), stocking with this trout should be avoided. The remaining hatchery populations, especially those from the Danube River basin, were highly variable, which could be explained by the multiple origin of these populations. In any case, possible negative effects on wild populations such as exceeding carrying capacity, outbreeding depression and spreading of diseases and parasites should be prevented. A rapid decrease in brown trout densities in Czech Republic and Slovakia has been reported, although stocking is intensive (e.g. Spurný *et al.* 2006; Vítek & Spurný 2006; Stráňai & Andreji 2008). It corresponds with the poor performance of stocked fish reported in Austrian (Weiss & Schmutz 1999) and German rivers (Baer & Brinker 2010). Considering the genetic structure of brown trout is strongly affected, stocking practices should be re-evaluated. Populations cannot adapt to a specific environment if there is a continual addition of non-adapted fish. Supportive breeding appears to be a more suitable strategy for the management of brown trout, as it would help to increase trout densities without the risk of interbreeding with non-native stocks (Ryman & Laikre 1991; Ferguson 2007 and citations therein). On the other hand, the potential risk of inbreeding and loss of genetic variation must not be underestimated (Ryman & Laikre 1991; Hansen *et al.* 2000). The causes of population decline should be examined in particular cases. Importantly, the long-term stabilisation of trout densities is often not possible without environmental improvement strategies (e.g. habitat restoration, removing migration barriers, hydrological regime enhancement), which are generally more effective than stocking (Cowx 1994; Fjellheim *et al.* 2003; Oosterhout *et al.* 2005; Ferguson 2007). Cessation of the import of non-indigenous fish and stocking with strongly domesticated brown trout is essential for effective fishery management and conservation in open waters.

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CHAPTER 3

GENETIC DIVERSITY AND PHYLOGENETIC ORIGIN OF BROWN TROUT *SALMO TRUTTA* L. POPULATIONS IN EASTERN BALKANS

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GENETIC DIVERSITY AND PHYLOGENETIC ORIGIN OF BROWN TROUT *SALMO TRUTTA* L. 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.

Keywords: *Danube, microsatellites, mitochondrial DNA, stocking, introgression*

1. 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 and 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) linages. 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 R. basin indicating an existence of another lineage. Geographically restricted mtDNA lineage has been recently suggested also in the Duero R. 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 R. 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 (R.) was stocked in its Greek part about 30 years ago by fish from Acheloos R. (Apostolidis et al., 1997), which belongs to the Adriatic-Ionian Ichthyogeographical zone (Economidis and 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 R. have also been released into the Venetikos R., a tributary of the Aliakmon R., and a high number of individuals with the Acheloos haplotype was detected in the Venetikos R. by Apostolidis et al. (2008a). Impact of stocking with exogenous fish was indicated using microsatellite markers in the Axios R. 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. 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. belongs to the most intensely 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.

2. MATERIALS 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 from a tributary of Zamanti R. (Seyhan R. 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 Figure 1.

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) R. basin and populations 3 and 4 belong to the Mesta (Nestos) R. basin.*

		No. of individuals assessed		mtDNA haplotype																						
	Population	mtDNA	msat	**AdAE 3**	ADcs 1	**AdAE 2**	**AdZA 1**	ADcs 20	**AdAE 1**	H 13	H 1	Da 1A	Da 26	**DaDA 2**	Da 2	**DaDA 1**	DaBS 9	**DaBS 3**	**DaBS 4**	**DaBS 2**	**DaBS 5**	**DaBS 6**	**DaBS 7**	**DaBS 8**	**DaBS 1**	MAcs 1
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																			

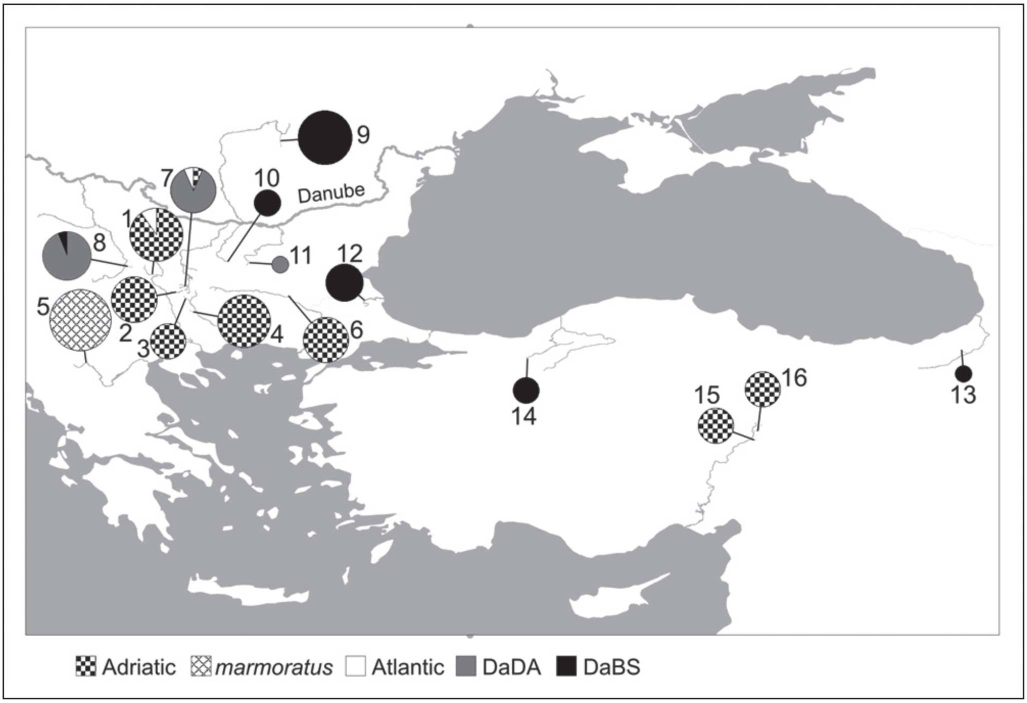

Figure 1. *Map showing the geographic origin of the samples. Pie charts display the frequencies of haplotype groups for each sample. The size of the circle corresponds to the number of analysed individuals from each sampling site. The numbers of sampling sites correspond to the numbers of populations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

Fin clips were preserved in 96% ethanol and stored at 4 °C. 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 and Danzmann, 1993) with PCR conditions: 94 °C for 5 min, 35 cycles at 94 °C for 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 and 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. 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 and 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 and 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 and 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 and 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 and Smouse, 2006) was used to determine private alleles in each population and in the three basins (Black Sea, Aegean Sea, the Zamanti R.). The analysis of molecular variance (AMOVA) performed by 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 10000 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 100000 burn-in and 500000 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 R. basin populations and the Aliakmon R. population were excluded from this analysis.

3. 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 (Table 1 and 2). The haplotype diversity within the whole sample set was 0.851 ± 0.011 and the nucleotide diversity 0.0063 ± 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 R. 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 ± 0.013, the nucleotide diversity 0.0045 ± 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 ± 0.029, the nucleotide diversity 0.0028 ± 0.0001 and the number of polymorphic sites ten.

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).*

Haplotype	GenBank No.	Variable sites																					
		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

The median-joining network (Figure 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 R., the Džepska R. (except for one haplotype), the Vidima R., as well as almost all published Danubian lineage haplotypes from Austria (Weiss et al., 2001; Duftner et al., 2003), Serbia (Marić et al., 2006), Switzerland (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 R., Beli Vit R., one haplotype from the Džepska R. (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 R. 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 R. in Serbia (Marić et al., 2006). The average Tamura-Nei distance between the DaDA and DaBS haplotypes was 0.007.

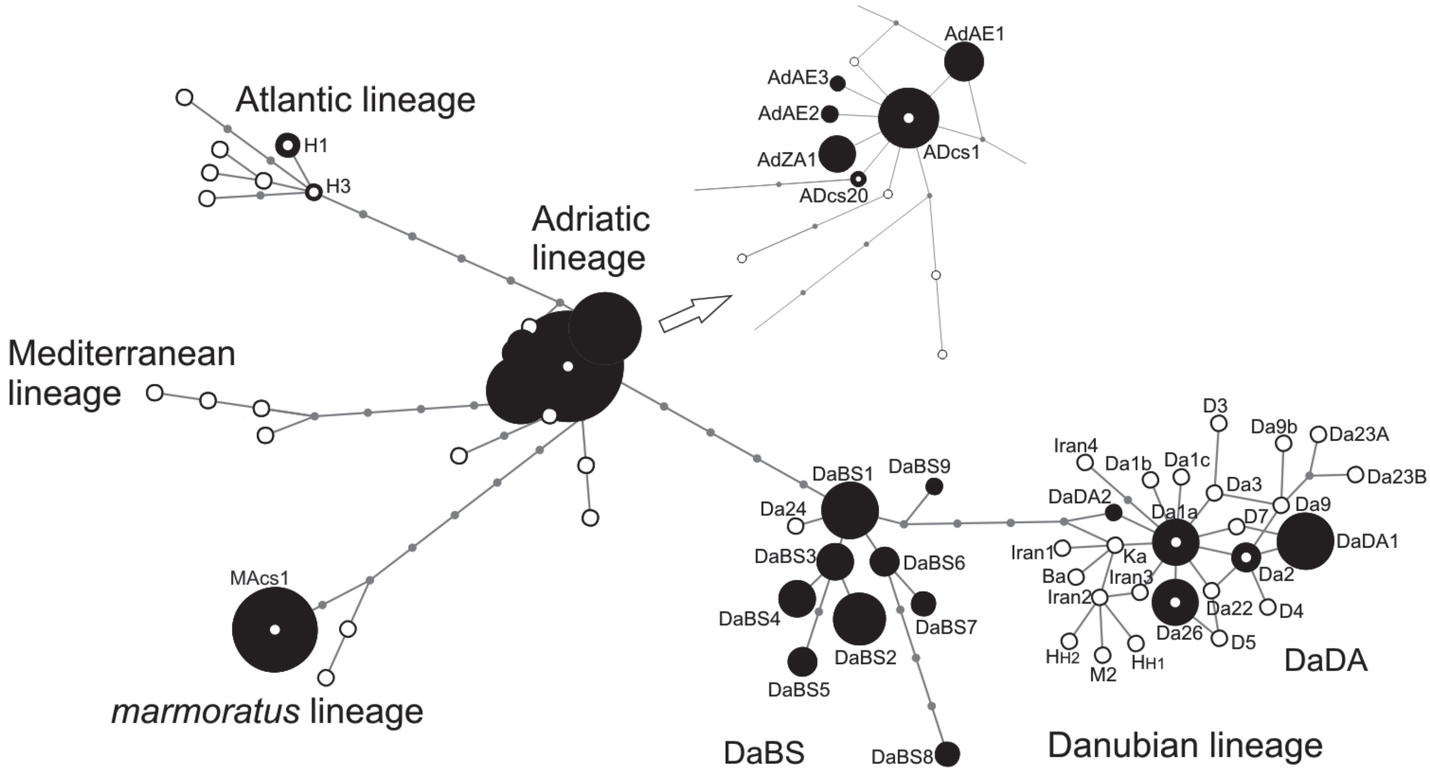

Figure 2. *Median-joining network of brown trout control region haplotypes. Original data (Tables 1 and 2), as well as previously published haplotypes are included: H1, H3 (Cortey and García-Marín, 2002); Da1a, Da1b, Da2, Da22, Da23a, Da23b, Da3, Da9, Da24 (Duftner et al., 2003); ADcs1, ADcs20, MAcs1 (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, HH1, HH2, M2 (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.*

More than half of the individuals from the Aegean Sea basin (excluding the Aliakmon R.) 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 R. 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 R. 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 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 R. and the Treklianska R. ($\alpha = 0.05$). A significantly positive value of F_{IS} in the Džepska R. (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 R. basin 13 private alleles. The F_{ST}

value across all eastern Balkan samples with more than 15 individuals excluding exogenous Aliakmon R. 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 R. vs. the Cerni Iskar R.) to 0.32 (the Treklianska R. vs. the Džepska R.). Since no genetic differentiation was revealed between the two samples from Seyhan R. basin, these samples were further considered as one (F_{IS} for the grouped sample was not significant). Values of F_{ST} for the Seyhan R. 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 R. 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 R. basin and the Aliakmon R.. After excluding the Aliakmon and Seyhan R. populations and including the three Atlantic basin populations from the Czech Rep. three clusters were revealed (Figure 3). The clusters corresponded to: 1. North and Baltic Sea basins samples including hatcheries 2. samples from the Danube R. 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 R. basin. The Cerni Iskar R. population had the highest membership of both alien clusters (0.099 and 0.084, respectively).

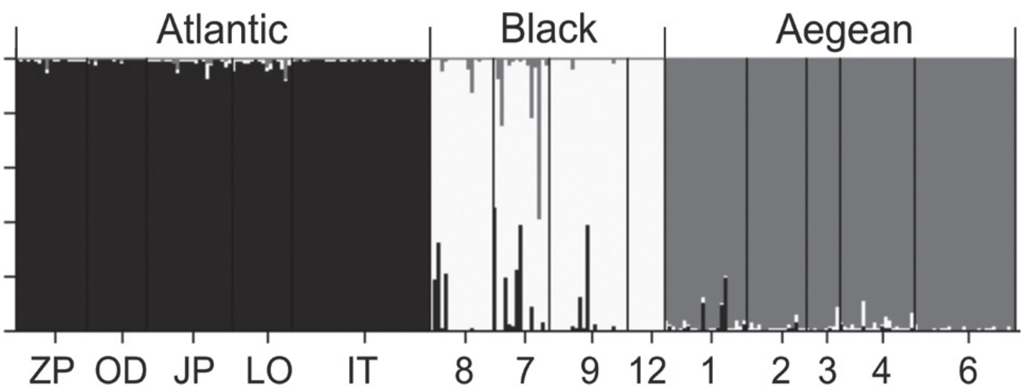

Figure 3. *Individual membership of the samples from the Atlantic (including North and Baltic Seas), Black Sea and Aegean Sea basins in each cluster (K = 3) estimated using STRUCTURE. Each individual is represented by a vertical line. The letter codes correspond to the Atlantic origin populations analysed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

4. DISCUSSION

Black Sea basin

Analyses of mtDNA revealed the new haplotypes in the Danube R. 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 R. 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 R. and found also in the Beli Vit R. population (Danube R. 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) R. 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 R. 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 R. basin (Figure 4). Moreover, the DaBS and DaDA haplotypes have been found together in only one population (Džepska R.). It can be speculated that the occurrence of the distinct haplotypes in the Danube R. 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, 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.

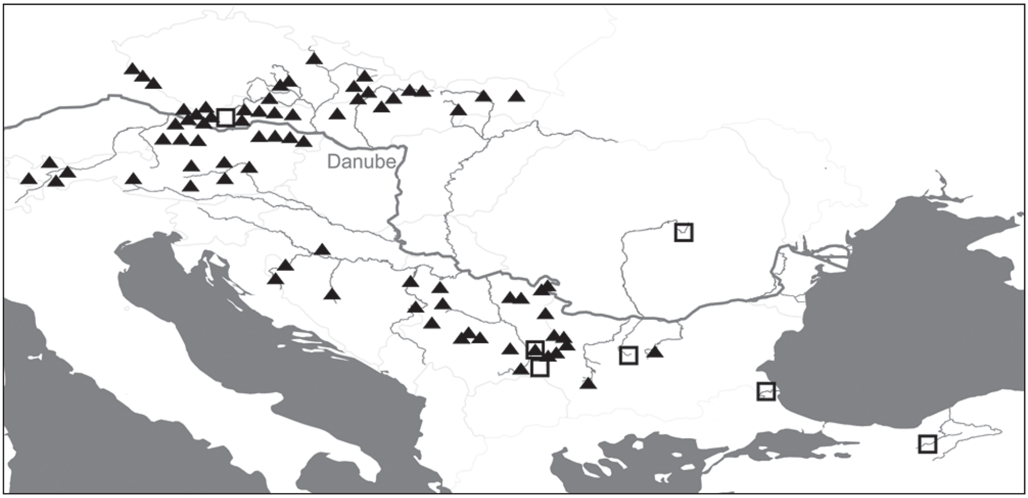

Figure 4. *Distribution of the DaDA (triangles) and DaBS (squares) haplotypes in the western part of the Black Sea basin using the data of Weiss et al. (2001), Duftner et al. (2003), Marić et al. (2006), Kohout et al. (2012), this study and our unpublished data. Some sample locations in Austria were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

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 R. 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 R. (tributary of the Aliakmon R.), 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 R. Analysis of the two Seyhan R. 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 R. 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 R. basin was indicated. The introgression was most pronounced in the Cerni Iskar R., 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 R. 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 R. basin. Future management and conservation strategies should avoid such activities to prevent disruption of unique genetic structure of local populations.

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CHAPTER 4

GENETIC CHARACTERISATION OF BROWN TROUT (*SALMO TRUTTA* L.) POPULATIONS IN HEADWATERS OF THE OTAVA RIVER

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GENETIC CHARACTERISATION OF BROWN TROUT (*SALMO TRUTTA* L.) POPULATIONS IN HEADWATERS OF THE OTAVA RIVER

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1. INTRODUCTION

The Otava River is a tributary of the Vltava River and arises from the confluence of two tributaries, the Vydra and Křemelná Rivers. Basins of these two tributaries cover an area of 224 km^2 and consist of oligotrophic streams located in the Šumava national park and protected landscape area. One stream of the Vydra River basin, Švelský potok is isolated by 2.5m-high natural barrier preventing an upstream migration of fish (Závorka et al., 2013). The brown trout populations occurring in this area are considered as native. However, several hatcheries for salmonid fish in the Otava River basin were built in the 19th century and the history of rearing and stocking is not well documented. In last years, the populations of brown trout in the Otava River tributaries as well as in other drainages of Šumava are supplementary stocked with trout from Borová Lada hatchery (www.npsumava.cz). Nevertheless, substantial variability in individual growth among the brown trout populations in the headwaters of Otava River basin was found (Závorka et al., 2013). It may reflect a genetic substructuring within 'Šumava trout'.

The aims of this study were to reveal genetic variability and verify the origin of brown trout in headwaters of the Otava River based on microsatellite data. The impact of migration barrier and geographic distance on the genetic structure of brown trout was evaluated. The genetic relationships of the wild populations and the hatchery population of Borová Lada were analysed. Results of this study could bring important information for future conservation and management strategies in the Šumava national park and protected landscape area.

2. MATERIALS A METHODS

In total, 216 individuals from 22 locations in headwaters of the Otava River and one hatchery stock from Borová Lada were sampled (Table 1, Figure 1). Eight previously analysed populations from the Elbe River basin were included in the statistical analyses (Kohout et al., 2012). The wild samples from headwaters of the Otava River were grouped on the basis of preliminary genetic analyses and analyses of individual's abundance growth rate into four populations: the Vydra population, the Švelský potok population, the Křemelná population and the Jezerní potok population.

Table 1. *Number of samples (N), observed heterozygosity (HO), expected heterozygosity (HE), FIS values, allelic richness based on the rarefaction method (ARR) and private allelic richness (ARP) for the brown trout populations from the Elbe River basin. Codes of the populations from the Otava River headwaters and Borová Lada hatchery are underlined. *P < 0.05.*

code	population	reference	N	H_O	H_E	F_{IS}	AR_R	AR_P
VYD	Vydra	this study	127	0.586	0.625	0.0661*	3.39	0.09
SVE	Švelský potok	this study	27	0.538	0.563	0.0635	2.95	0.09
KRE	Křemelná	this study	33	0.665	0.657	0.0024	3.58	0.02
JEZ	Jezerní potok	Kohout et al., 2012	21	0.582	0.587	0.0323	3.23	0.14
BOR	Borová Lada	this study	29	0.640	0.680	0.0771*	3.83	0.08
ZLA	Zlatý potok	Kohout et al., 2012	24	0.694	0.663		3.67	0.13
KAM	Kamenice	Kohout et al., 2012	15	0.659	0.639		3.89	0.26
UPA	Úpa	Kohout et al., 2012	20	0.567	0.593		3.39	0.06
ZEL	Zelenský potok	Kohout et al., 2012	19	0.637	0.637		3.61	0.18
PRA	Pramenský potok	Kohout et al., 2012	18	0.599	0.598		3.48	0.12
JIR	Jiřetínský potok	Kohout et al., 2012	24	0.593	0.600		3.31	0.13
BIL	Bílý potok	Kohout et al., 2012	19	0.637	0.605		3.36	0.08

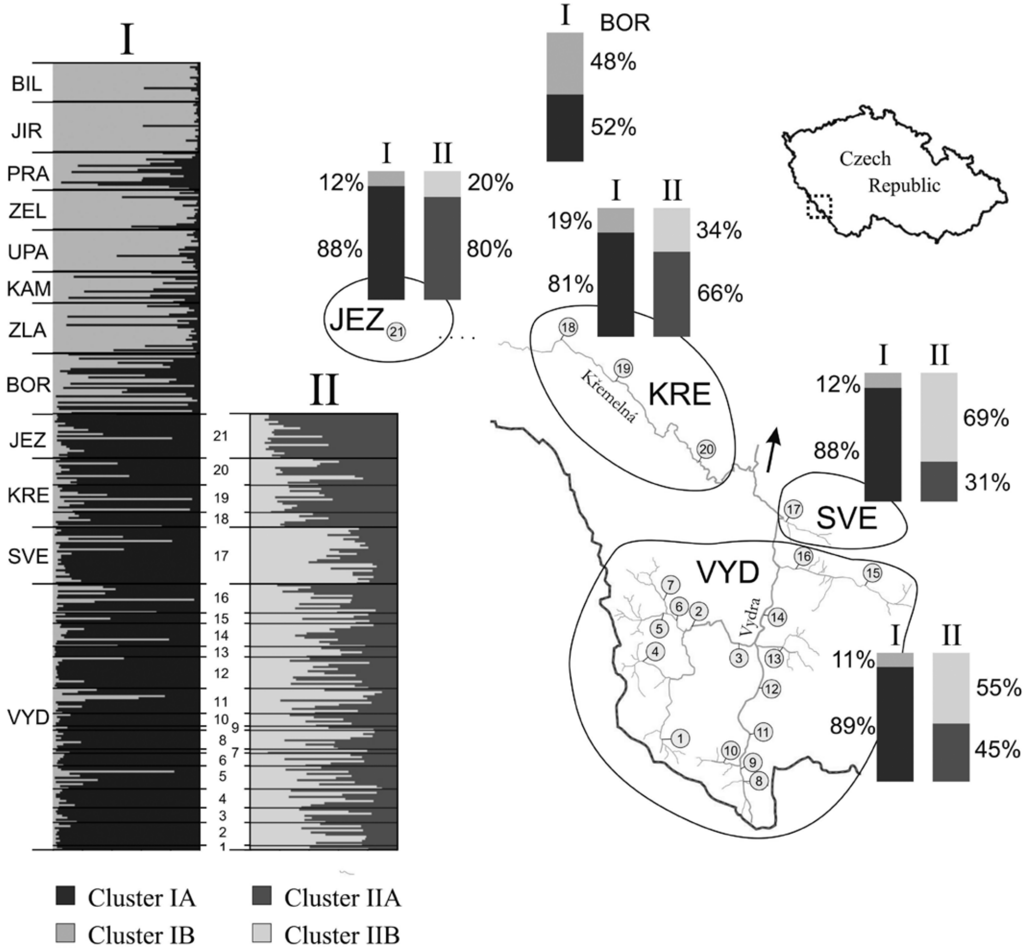

Figure 1. *Map of sampling sites and distribution of the clusters revealed by STRUCTURE analyses of the Elbe River basin samples (I) and the samples from Otava River headwaters only (II).*

The samples were analysed using nine microsatellite loci (*SsaD190*, *SsaD71*, *SSsp2213*, *SsoSL438*, *Str60*, *Ssa85*, *SSsp2216*, *Str73*, *SsoSL417*), which were used in previous study (Kohout et al., 2012). Genetic variability within populations was evaluated by observed heterozygosity and expected heterozygosity in GENALEX 6 software (Peakall and Smouse, 2006) and by allelic richness and private allelic richness computed using the rarefaction method in HP-RARE v1.0 (Kalinowski, 2005). Pairwise genetic differentiation (F_{ST}) and its statistical significance was tested using 10000 permutations in Arlequin v3.5 software (Excoffier et al., 2005). The STRUCTURE v2.3.3 (Pritchard et al., 2000) software was applied to reveal genetic structuring among the populations using an admixture model. 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 each *K*

(1–12), five Markov Chain runs, each consisting of 600 000 iterations with the first 100000 iterations discarded as a burn-in, were applied. Proportions of clusters were ploted for individuals and populations. Assignment test in GENECLASS v2.0 (Piry et al., 2004) based on the Bayesian method of Rannala and Mountain (1997) was performed to estimate genetic homogeneity of the populations and detect effects of migration and recent stocking. A neighbour-Joining tree based on pairwise chord distances (D_{CE}; Cavalli-Sforza and Edwards, 1967) was generated in POPULATIONS v1.2.32 (Langella, 2002) and visualised in TREEVIEW (Page, 1996).

3. RESULTS

All pairwise F_{ST} values were significant. Jezerní potok and Švelský potok populations were most genetically distinct. Borová Lada hatchery population was substantially differentiated from all wild populations. Statistically significant values of F_{IS} were found in the Borová Lada and Vydra River populations (Table 1). Among the samples from Otava River headwaters, allelic richness based on the rarefaction method ranged from 2.95 in Švelský potok to 3.58 in the Křemelná River, whereas it was considerably higher in Borová Lada hatchery population (3.83). The gene diversity (H_E) varied from 0.563 in Švelský potok to 0.657 in the Křemelná River and it was 0.680 in Borová Lada hatchery stock (Table 1).

Table 2. *Pairwise FST values for the brown trout populations from the Elbe River basin (below diagonal) and statistical significance after the Bonferroni correction (above diagonal). NS – not significant; *P < 0.05; **P < 0.01.*

	VYD	SVE	KRE	JEZ	BOR	ZLA	KAM	UPA	ZEL	PRA	JIR	BIL
VYD		**	**	**	**	**	**	**	**	**	**	**
SVE	0.0223		**	**	**	**	**	**	**	**	**	**
KRE	0.0204	0.0418		**	NS	**	*	**	**	**	**	**
JEZ	0.0780	0.1312	0.0462		**	**	**	**	**	**	**	**
BOR	0.0285	0.0535	0.0142	0.0645		NS	**	**	**	*	**	**
ZLA	0.0667	0.0982	0.0462	0.1005	0.0195		**	**	**	**	**	**
KAM	0.0442	0.0873	0.0328	0.0874	0.0385	0.0640		**	**	**	**	**
UPA	0.0915	0.1076	0.0904	0.1628	0.0382	0.0437	0.0881		**	**	**	**
ZEL	0.0927	0.1152	0.0743	0.1199	0.0612	0.0587	0.0554	0.0702		**	**	**
PRA	0.0574	0.0726	0.0612	0.1219	0.0218	0.0750	0.0710	0.0489	0.0976		**	**
JIR	0.1170	0.1525	0.1074	0.1693	0.0820	0.0963	0.1071	0.1001	0.1056	0.1325		*
BIL	0.1132	0.1446	0.1090	0.1915	0.0656	0.0659	0.1085	0.0706	0.1001	0.1144	0.0327	

Bayesian analysis in STRUCTURE proposed two genetic clusters (Figure 1). Cluster IA predominated in the samples from Otava River headwaters. Cluster IB predominated in the remaining samples from Elbe River basin, except for the Pramenský potok sample (Ohře River tributary), in which both clusters had similar proportions. The analysis using only the samples from Otava River headwaters revealed also two genetic clusters (Figure 1). Cluster IIA predominated in the Jezerní potok and Křemelná River samples, whereas cluster IIB had its highest proportion in the Švelský potok sample and predominated also in the Vydra River basin samples.

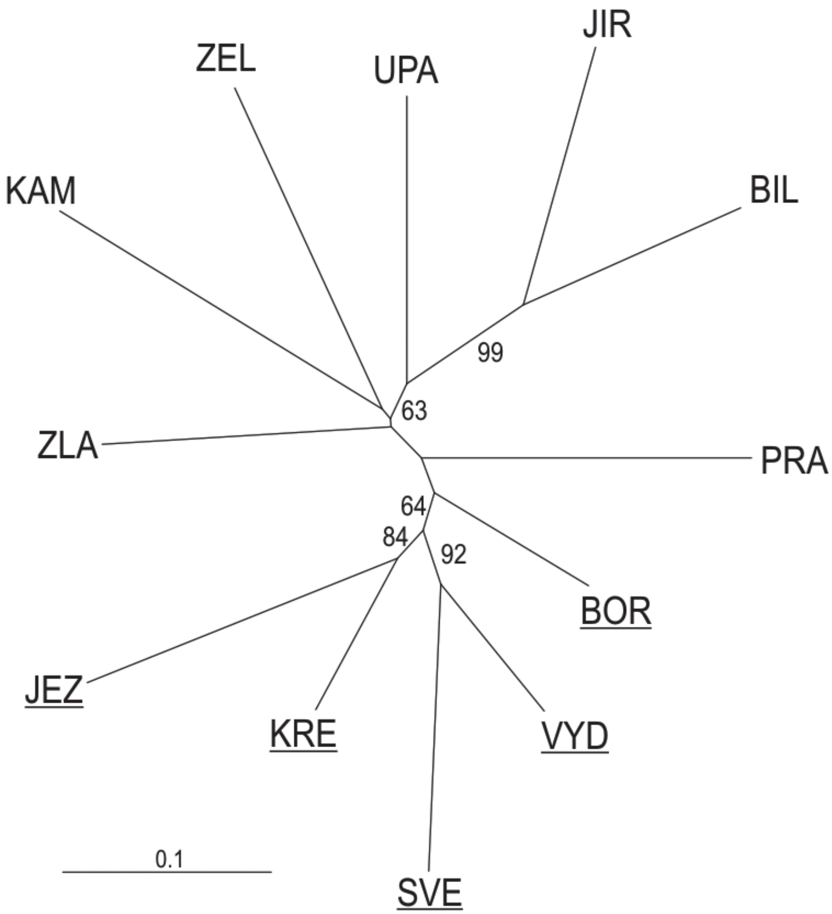

Figure 2. *Neighbor-Joining tree based on the DCE genetic distance (Cavalli-Sforza and Edwards, 1967) showing the genetic relationship between the brown trout populations from the Elbe River basin. Bootstrap values higher than 50% are indicated on the branches.*

Neighbor-Joining tree showed close relationships between the populations from headwaters of the Otava River (Figure 2), since these populations created a statistically supported clade. Moreover, two subclades, corresponding to the two basins (Vydra and Křemelná), were detected.

4. DISCUSSION

Although we have limited data for testing geographic pattern of genetic variability, apparent relationship between proportion of the clusters and geographic distance can be recognised. Samples from the upper streams had higher proportion of the cluster IA compared to the samples from Křemelná River. Cluster IA may represent native trout of the Otava River headwaters, whereas cluster IB may reflect gene flow from other parts of the Elbe River basin due to a natural migration and/or stocking. The statistically significant values of F_{IS} in the Vydra River population support this suggestion. The high proportion of cluster IIB in Švelský potok could be a result of reproductive isolation of this population due to the migration barrier. Moreover, the spatial pattern of genetic diversity seems to be related with geographic distance.

The phylogenetic analysis indicated that the Borová Lada population is more closely related to the populations from Otava River headwaters compared to the other Elbe River populations. Nevertheless, the values of F_{ST} revealed that this hatchery stock is genetically divergent from the wild populations, although the results for pairs with Křemelná River and Zlatý potok were not statistically significant after the Bonferroni correction. Bayesian clustering showed that the Borová Lada population had considerably higher proportion (48%) of the cluster IB compared to the four populations from headwaters of the Otava River (12–19%). The hatchery population had also substantially higher allelic richness and expected heterozygosity compared to the wild populations. Based on these results, it seems that the Borová Lada strain originated from several sources in Šumava. Therefore, it cannot be considered as native in particular streams of the Otava River basin. Stocking this trout may cause genetic homogenisation of local populations across the drainages. The stocking practises thus should not be performed preventively but should be restricted to reasonable cases, where a local population is seriously declined or threatened by extinction. If possible, supportive breeding of native trout instead of supplemental stocking with hatchery trout should be performed.

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CHAPTER 5

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLODGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING STUDY
CURRICULUM VITAE

GENERAL DISCUSSION

Our results confirmed the expectation that the genetic structure of brown trout in the Czech Republic and Slovakia is strongly affected by long-term stocking. The stocking activities caused extensive changes of brown trout genetic diversity leading to the drastic loss of among-population variability. The genetic homogenisation is apparent especially in the analysed populations from the middle Danube River basin in Slovakia and Moravia, whereas the populations from the Elbe River basin keep certain level of among-population variability. Differential impact of stocking is associated with differences in observed heterozygosity and polymorphism, which is higher in the Danube River populations and corresponds to higher level of introgression from non-native fish (Riffel et al., 1995; García-Marín et al., 1999; Berrebi et al., 2000; Almodóvar et al., 2006). There are several explanations for such contrasted results. It should be mentioned that analysed populations from the middle Danube River basin have been repeatedly stocked with hatchery-reared trout. These fish usually originate from substantially abundant broodstocks of multiple native/non-native origin, which have been kept in hatcheries or in small streams. Eggs have been hatched under controlled conditions and fingerlings distributed across wide area and released into the wild. A domestication changes resulting from inbreeding, accidental selection and genetic drift is therefore rather slow. Since stocking have been performed annually for many decades, the differences in haplotypes and allele frequencies are eliminated. It corresponds with results from Mediterranean slopes of French Pyrenees, where the accumulation of non-native genes due to repeated stocking with trout of admixed native/non-native origin resulted in homogenisation of genetic structure, although the annual increase of introgresion rate was small (Berrebi et al., 2000). In both middle Danube and Mediterranean areas, the hybridization between trout of different origins has taken place already in hatcheries. The artificial spawning associated with interbreeding, absence of mate choice and relaxed selection due to a greater survival under hatchery conditions resulted in fast intermixing of gene pools. As a substantial part of native genes is maintained, a loss of local adaptations may be relatively low. Stocking these hatchery-reared trout is probably more successful and may substantially contribute to the wild populations, because natural selection against stocked fish is probably lower compared to the selection against strongly domesticated fish reported in some studies. Nevertheless, fitness of hybrids is usually lower compared to native trout (Poteaux et al., 1998; Almodóvar et al., 2001).

The analysed populations from the Elbe River basin usually inhabit small streams where stocking activities are limited and/or stocked trout come from local hatcheries. However, an origin of the hatchery strains is not clear due to a lack of information on their founding and transfers between the hatcheries. Unfortunatelly, it was not possible to analyse all hatchery populations in the Czech Republic and Slovakia. Therefore, we were not able to evaluate impact of stoking in a local scale. Nevertheless, the values of genetic differentiation between the populations indicate that the transfers and stocking have not blurred the genetic structure of brown trout in the Elbe River basin. The analyses of *LDH-C1** evidenced slight introgression by non-indigenous trout in northern tributaries of the Elbe River, probably as a result of transfers from hatcheries in the Danube River basin. However, the absence of haplotype A3 and the significant genetic differentiation indicate that there is no or very little contribution of domesticated trout imported in the past from other countries. The lower effect of stocking could be explained to some degree by different environmental conditions. A positive correlation between the introgression rate and stream productivity was observed in Iberian populations of brown trout (Almodóvar et al., 2006). Introgression is also lower in streams and rivers with less stable flow conditions, since irregularity of discharge decreases survival of hatchery-reared trout (Almodóvar et al., 2006). The effect of stocking thus could be lower in small upper streams of the Elbe River basin compared to the larger streams and rivers sampled in the Danube River basin.

There is also another explanation for the differential genetic pattern in the two basins. It was found that populations in Mediterranean drainages are more introgressed by farm trout of Atlantic origin compared to wild populations in the Atlantic basin, although stocking intensity is comparable. Almodóvar et al. (2006) hypothesised that it is due to a poor adaptation of farm trout to migratory behaviour, which is quite common in Atlantic basin populations. Nevertheless, it could be also explained by a competitive advantage of Atlantic trout resulting in exclusion of Mediterranean trout. Stocking Atlantic trout thus may be in its effects similar to introducing non-native species (Ferguson, 2007). If Atlantic trout is advantageous also in competition with Danube basin trout, this advantage may be responsible for the high introgression from Atlantic trout in the Danube River basin populations and very low introgression from the Danube trout in the Atlantic basin populations. If this suggestion is true, successive exclusion of native alleles and haplotypes in the Danube River basin may be expected. Assuming the genetic similarity of stocked trout and wild trout in the Atlantic basin, stocking may not result in competitive advantage of hatchery-reared trout. In this case, genetic changes due to hybridisation between hatchery and native trout and subsequent selection against hybrids are the main consequences of stocking. This may be the reason why stocking has low impact on the Atlantic basin populations. Nevertheless, in both basins, stocking hatchery-reared trout may have participated in the decrease in population density due to reduced fitness of hybrids and outbreeding depression.

It is doubtful if some pure native populations persist in the Czech Republic and Slovakia. We are not able to reconstruct genetic variability of brown trout prior to stocking taking place, since we have no historical samples from this region. It is very difficult to find molecular markers, which can evaluate introgression rate and reliably distinguish native and stocked trout and their hybrids. However, estimating the extent of recent hybridisation between different gene pools due to importing allochtonous trout should be possible, if appropriate markers are used. Frequencies of *LDH-C1** alleles have been widely used for detecting effects of hatchery brown trout stocking on wild populations. Due to its simple screening and cost effectiveness, this marker is a useful primary estimator of stocking effects on wild trout (Sanz et al., 2009). Nevertheless, majority of populations analysed using this marker originated from Mediterranean and south Atlantic drainages, where the **90* allele is alien and represents a contribution of stocked trout originating from northern Atlantic drainages. It has limited value in Central and Northern Europe, where the **90* allele is native in the Atlantic drainages and may be native also in the Danube River basin due to the Pleistocene contact between the basins. It was found that besides the **90* allele, the **100* allele occurs in the hatchery stocks in Slovakia, whereas it is almost absent in the Elbe River basin. This marker therefore could serve as an indicator of introgression from Danube trout to the populations of Atlantic basin rather than the estimator of introgression from domesticated Atlantic trout.

Microsatellites are particularly useful in evaluating introgression in cases where genetic differentiation between stocked and wild trout is low (Sanz et al., 2006; Hansen et al., 2009; Hansen and Mensberg, 2009). Microsatellites are therefore most frequently used markers for evaluating genetic changes due to stocking in northern European Atlantic basin, where populations were established after the last glaciation and hatchery strains used for stocking were derived from local populations in the last two centuries (Hansen, 2002; Sønstebø et al., 2007; Hansen and Mensberg, 2009; Thaulow et al., 2013). Nevertheless, the application of commonly used methods is greatly difficult in the Czech Republic and Slovakia, where some of the hatchery strains are of local origin, others are of non-native origin and most of them are of multiple origins. Moreover, transfers and stocking have been performed regardless of the hydrogeographic division. The microsatellite alleles, private for particular populations, were found in very low frequencies and they are therefore inapplicable for evaluating the extent of hybridization. The identification of hybrids thus must be based on differences in allele frequencies (Sanz et al., 2009). There are individual assessment methods based on Bayesian approaches in STRUCTURE or NEW HYBRIDS programs, which can separate hybrid and pure individuals. However, the efficiency of these methods decreases with decreasing genetic differentiation (F_{ST}) between parental populations and it depends on number of analysed loci. Moreover, Vähä and Primmer (2006) estimated that accurate identification of F2 hybrids and backcrossess requires using at least 48 microsatellite loci, although this number is usually not reached in population genetic studies. Considering rather low F_{ST} values observed between populations from the Czech Republic and Slovakia, the number of microsatellite loci used for the analyses is too low for accurate individual assessment and detecting hybrids and pure individuals. Nevertheless, our study indicated that the method implemented in STRUCTURE is usable for estimating the introgression rate between wild trout and domesticated trout recently imported from Italy. The extent of intermixing between other captured populations and wild populations is poorly detectable. There are differences in proportion of clusters between the wild and hatchery trout in Šumava, which may indicate heterogeneous origin of the hatchery stock. Our results showed that partially Bayesian assignment test in GeneClass software is not usable for detecting hybrids and evaluating introgression, if large number of populations of multiple origins is analysed together. It may be applicable in cases, where non-native, substantially differentiated trout introgresses wild population. Since Sanz et al. (2009) found that this approach is less reliable in detecting hybrids compared to fully Bayesian approaches (STRUCTURE, NEW HYBRIDS, BAPS), its application for Central European brown trout populations has limited value.

Although the mitochondrial DNA alone is not suitable for estimating the introgression rate, it may increase the accuracy of detecting extent of introgression in combination with other markers. Frequency of the haplotype A3 can be used as a primary indicator of introgression from imported domesticated trout in the Elbe River basin. This haplotype corresponds to the haplotypes 4, At1c and ATcs4 reported in previous studies (Cotey and García-Marín, 2002; Duftner et al., 2003; Cortey et al., 2004; respectively) and is one of the most frequent haplotypes in farm strains in Europe (Table 1).

Table 1. *Frequency of A3 haplotype in hatchery and farm samples analysed so far using mtDNA control region of at least 904bp.*

Hatchery	Country	Proportion of A3	Reference
Jakta	Norway	10/10 (100%)	Cortey and García-Marín, 2002
Uña	Spain	12/29 (41%)	Cortey and García-Marín, 2002
Carballedo	Spain	2/14 (14%)	Cortey and García-Marín, 2002
Bagà	Spain	17/23 (74%)	Cortey et al., 2004
Hatchery	Austria	6/22 (27%)	Duftner et al., 2003
Turnov-Kolowrat	Czech Rep.	13/21 (62%)	Kohout et al., 2012
Turnov-Italian	Czech Rep.	0/22 (0%)	Kohout et al., 2012
Nedošín	Czech Rep.	2/6 (33%)	Kohout et al., 2012
Biely Potok	Slovakia	4/27 (15%)	Kohout et al., 2012
Východná	Slovakia	3/28 (11%)	Kohout et al., 2012

The A3 haplotype was not found in domesticated brown trout imported from Italy. The two haplotypes found in this population indicate that this trout is derived from other domesticated farm populations and the absence of A3 may be a result of genetic drift. The A3 haplotype is common in wild populations in Denmark and Norway. Since hatchery stocks in Spain, where the high proportion of A3 was found, are of German origin (Cortey et al., 2009), high abundance of this haplotype can be assumed in at least some of German populations. Unfortunately, except for few individuals analysed by Bernatchez et al. (1992), no data are available based on the analysis of mitochondrial DNA in German populations, although they might bring important information concerning history and effects of stocking of brown trout in Europe. It might also contribute to resolving phylogeographic question regarding the colonisation of the upper Danube River basin.

Bernatchez et al. (1992) found the Atlantic lineage haplotype in nine out of eleven individuals from two populations of the upper Danube River basin and hypothesised that the Atlantic lineage is native in this region. However, Weiss et al. (2001) pointed that stocking is a common practice in Austria and the hatchery strain of 'local' origin analysed by Bernatchez et al. (1992) thus may not represent native trout. Weiss et al. (2001) concluded that stocking is the main reason for extensive introgression by Atlantic trout in the Danube River basin. The authors aimed to detect the putative Pleistocene contact of the two lineages in the Danube River basin using frequencies of the Atlantic lineage haplotypes correlated with geographic data. They found that almost all Austrian populations possessed the Atlantic lineage haplotypes, with overall frequency 44%. However, the frequency of these haplotypes was significantly lower in southern slopes vs. northern slopes of the Alps and in the area that was glaciated in the past compared to the unglaciated area. Based on these results the authors supposed a late or post-Pleistocene penetration of the Atlantic lineage trout to the Danube River basin. This scenario is supported also by two Atlantic lineage haplotypes in Austrian streams, which were not found in hatchery populations. Moreover, we found another six Atlantic lineage haplotypes exclusively in the Danube River basin. Nevertheless, if the Atlantic lineage penetrated to the upper Danube River basin already at the end of Pleistocene, question is why it has not spread to the lower parts of this basin. It is evident that Atlantic trout has a high ability to introgress or even exclude trout from the Danube River basin. The Atlantic lineage haplotype was found in three individuals from three rivers of the Serbian and Bulgarian part of Danube River basin (Marić et al., 2006; Kohout et al., 2012) and our results of microsatellite analyses also evidenced a contribution of Atlantic trout to the Balkan populations. However, the rivers, where the Atlantic haplotype was found and where the proportion of Atlantic cluster was highest, had been stocked with hatchery trout, whereas most of the other rivers had not. Anthropogenic transfers and stocking can therefore be sufficient explanation for the observed introgression.

Based on morphological and ecological variation, brown trout populations in the Black, Caspian and Aral Sea basins were formerly recognized as distinct subspecies (Berg, 1948). Whereas any geographic pattern in the distribution of genetic diversity was revealed based on allozymes (Bernatchez and Osinov, 1995), the genetic discontinuity among the three sea basins was provided based on mitochondrial DNA data, especially using the nested clade analysis (Bernatchez and Osinov, 1995). The differences between the three basins was confirmed also in recent studies analysing mitochondrial DNA control region, since any of the found haplotypes was shared between basins (Griffiths et al., 2009; Vera et al., 2011; Segherloo et al., 2012). Nevertheless, our results based on the same mitochondrial DNA fragment indicate that the divergences among haplotypes within the Black Sea basin are even higher than the divergences among the three basins. It corresponds with the hypothesis that the Danubian lineage originated from the Black Sea basin (Bernatchez, 2001; Bardakci et al., 2006). The haplotypes from the upper and middle Danube River basin and the Caspian and Aral Sea basins created statistically well supported cluster. Assuming the results of demographic analyses provided by Bernatchez (2001) and our results, the Pleistocene expansion of this subclade across the Danube River basin, Caspian Sea basin and Aral Sea basin is apparent. Bernatchez (2001) suggested that it was enabled by the sea expansion and interconnection between the sea basins approximately 270000–290000 years ago (Arkhipov et al., 1995). The expansion was not statistically supported for the whole sample set including also the newly found haplotypes from the lower Danube and non-Danube Black Sea basin locations. This result may indicate long-term isolation of populations of brown trout in the southern part of Black Sea basin and supports the findings of sympatric, reproductively isolated groups of populations, based on which Turan et al. (2009) described the two new species. It seems that the pattern of brown trout diversity in the Black Sea basin is similar to the pattern found in the Mediterranean basin, where mosaic distribution of the evolutionary groups have been recognized and many taxa of brown trout have been described. Such 'southern species richness' has been observed in many freshwater fish genera and has been explained by the lower impact of cold periods during Pleistocene climate fluctuations (Bohlen and Ráb, 2001; Reyjol et al., 2007).

From the taxonomic point of view, trout from the upper Danube River basin is considered as *Salmo trutta* L. Remaining populations inhabiting the Danube River and northern Black Sea basins is currently listed as *Salmo labrax* Pallas, 1814 (Kottelat and Freyhof, 2007), although this name was formerly suggested only for migratory trout of the Black Sea and lower Danube River basins (Kottelat, 1997 and citations therein, p. 132). The results of the present and previous genetic studies support the distinctiveness of brown trout in the Black Sea basin from other European brown trout populations. Nevertheless, the extensive intermixing between Atlantic basin and Black Sea basin trout in the upper and middle Danube River basins has been revealed. On the other hand, the results may indicate the presence of at least two distinct groups in the middle and lower Danube River basins. The deep genetic divergence between the two groups has resulted from a long-term reproductive isolation. The taxonomic position of brown trout in the Danube River basin thus remains unclear and should be evaluated using relevant taxonomic methods.

The genetic variability of brown trout in the middle Danube River basin as well as in other basins in Central Europe is affected by continuous stocking of non-native fish. It was revealed that repeated stocking often result in a disruption of local adaptations and consequently a loss of fitness (Templeton et al., 1986; Cross et al., 1998). This process may be responsible for the decrease in brown trout densities, which have been reported in last decades. Majority of Austrian fish farmers do not consider rearing of non-native brown trout to be an important problem (Pinter, 2008). The farmers often overvalue an importance of some criteria (e.g. growth and colouration), whereas criteria, which are more important for the success of stocking programme, such as genetic integrity and adaptability to local environment, are neglected (Pinter, 2008). Similar views can be assumed in other central European countries, including Czech Republic and Slovakia. Fish breeders and managers awareness of possibilities to improve stocking strategies is very important presumption for sustainable fishery management. Therefore, I am suggesting several improvements of brown trout management:

1. First of all, it should be determined if supporting of wild population by stocking is necessary. Supportive breeding should be preffered instead of supplementary stocking, if possible.

2. Further transfers between basins and intermixing of trout of different origins should be avoided.

3. Stocked fish should originate from a basin or area into which they are released. The area should be specified for particular cases according to hydrogeographic divisions, local environmental conditions and results of genetic analyses, if available.

4. Hatchery breed should not be kept in captivity for many generations without a supplementation with wild local fish in order to prevent genetic drift and decrease of genetic variability (artificial bottleneck effect).

5. Stocked trout should be reared under conditions similar to the natural environment in order to reduce changes associated with domestication and selection.

6. Stocking strongly domesticated trout and trout imported from other areas should be prohibited.

7. Techniques of artificial spawning should be optimised to preserve genetic variability of populations. For example, a small number of individuals of one sex substantially reduces effective population size.

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ENGLISH SUMMARY

Brown trout is one of the most popular and valuable fish in terms of recreational and production fisheries in fresh waters. As a consequence, it has been transferred and artificially propagated for centuries. The artificial reproduction and hatchery rearing of brown trout started in the middle of 19th century in Central and Northern Europe. After the World War II, a production of hatcheries and farms increased rapidly and stocking and transfers of fingerlings, eggs and brood fish became much more extensive in Central Europe including Czechoslovakia. Between the 1950s and 1990s, brown trout catches increased more than threefold in Czechoslovakia. However, catches and abundances of many brown trout populations have decreased in last two decades, although the quality of water and other environmental conditions improved significantly. The decrease can be caused by many factors and it is very difficult to estimate their proportions. However, most serious threats may be associated with changes in genetic variability and weakening of local adaptations due to hybridisation of native and non-native or domesticated trout.

The genetic structure of 25 wild populations and five hatchery stocks from Czech Republic and Slovakia were analysed using mitochondrial (control region) and nuclear DNA (microsatellites, *LDH-C1**) markers to elucidate the impact of stocking on central European populations of brown trout and to outline further management strategies. It seems that stocking practices have caused massive hybridisation between the Atlantic and Danube brown trout populations in the middle Danube basin and have led to a loss of among-population genetic variability in Slovakia and Moravia. Certain effect of stocking was detected also in the upper Danube, Vistula, Oder and Elbe River basins. However, the populations from the Elbe River basin keep certain level of among-population variability and seem to be less affected by stocking in comparison with the Danube River basin populations. The contribution of domesticated trout imported in the past from other countries seems to be very low. Comparison with studies from other Central European countries indicated that differences in stocking management and origins of breeding stocks may be crucial factors for the spatial variability of the genetic structure of brown trout. There are some indications of late or post-Pleistocene penetration of the Atlantic basin trout to the Danube River basin. However, it is not clear to which extent the natural contact participated to the present distribution of Atlantic haplotypes and alleles in the Danube River basin. Samples from lower parts of the Danube River basin were therefore analysed using the same mitochondrial and microsatellite markers. Samples from Aegean Sea basin were included in order to reveal genetic variability of eastern Balkan populations and to estimate an impact of stocking in this area. Very low levels of introgression from Atlantic and other non-indigenous trout were found in the eastern Balkan populations. The genetic differentiation among the populations is substantially higher in this area compared to the central European populations. It seems that these populations are less affected by human-mediated transfers and stocking compared to most European brown trout populations. The populations of eastern Balkans thus could be considered as native and represent a valuable information source for studying natural processes associated with genetic variability of brown trout. We conclude that the extent of introgression in the upper and middle Danube River basin is predominately a result of fishery management, because the Atlantic lineage is almost absent in lower part of the Danube River basin.

The populations in headwaters of the Otava River (Elbe River basin) was analysed using microsatellites in order to reveal origin of these population and evaluate the current management strategies of brown trout in Šumava National Park and Protected Landscape Area. The analysed populations were substantially differentiated from the remaining Elbe River basin populations and there was also certain level of genetic structure within trout from the headwaters of the Otava River associated with isolation by a migration barrier and geographic distance. However, stocking with hatchery trout also contributed to the pattern of genetic variability. The population of Borová Lada hatchery, which is used for stocking in Šumava exhibited higher genetic variability compared to the wild populations and it seems to be of heterogeneous origin. Regardless of the origin of the hatchery population, stocking this trout across Šumava National Park may result in genetic homogenisation of wild populations. Each stocking event therefore should be carefully evaluated.

Comparisons of the analysed populations with populations from other areas and results from other studies indicated that mtDNA haplotypes from the lower Danube River and southern Black Sea basins differ considerably from a subclade of the Danubian lineage consisting of haplotypes found so far in the most of the Danube River basin and in the Caspian and Aral Sea basins. The results thus evidence a complex evolutionary history of brown trout in the southern and western parts of the Black Sea basin.

CZECH SUMMARY

Pstruh obecný je vysoce ceněnou rybou, která od nepaměti přitahuje pozornost člověka a v současné době patří mezi nejvyhledávanější cíle sportovních rybářů v mnoha evropských zemích. Následkem toho se stává předmětem lidských aktivit spojených se snahou zvyšování produkce této ryby ve volných vodách. Umělý výtěr, líhnutí a odchov pstruha v umělých podmínkách má dlouhodobou tradici. Jeho počátky sahají do poloviny 19. století. V tomto období byly ve střední a severní Evropě vybudovány první umělé líhně. Ryby byly převáženy na značné vzdálenosti a vysazovány do různých povodí. Po druhé světové válce byly v Československu zbudovány velkokapacitní líhně a produkce násad pstruha a jeho vysazování do rybářských revírů mnohonásobně vzrostla. Následkem toho došlo mezi lety 1950 a 1990 k více než trojnásobnému zvýšení početnosti úlovků pstruha z volných vod. Avšak v posledních desetiletích jsou zaznamenávány poklesy úlovků odrážející snížení stavu pstruha v České republice, na Slovensku i v jiných částech Evropy, ačkoli kvalita vody a jiných ukazatelů životního prostředí se postupně zlepšuje. Pokles stavu může mít mnoho různých příčin a jejich přispění se bezpochyby liší v závislosti na konkrétních podmínkách daného toku nebo oblasti. Nicméně, změny v genetické struktuře divokých populací jako důsledek vysazování pstruha různého původu jsou pravděpodobně jedním z nejdůležitějších faktorů.

Za účelem vyhodnocení dopadu rybářského hospodaření na populace pstruha ve střední Evropě byla analyzována genetická struktura 25 divokých populací a pěti populací odebraných na líhních v České republice a na Slovensku. K tomu byly použity markery mitochondriální (kontrolní oblast) a jaderné (mikrosatelity, *LDH-C1**) DNA. Z výsledků je patrné, že vysazování ryb různého původu způsobilo rozsáhlé křížení populací atlantického a dunajského původu a vedlo ke ztrátě variability mezi populacemi v povodí středního Dunaje na Moravě a na Slovensku. Změny genetické variability v důsledku vysazování byly zaznamenány také v populacích povodí horního Dunaje, Visly, Odry a Labe. Nicméně, populace v povodí Labe mají zachovaný určitý stupeň genetické odlišnosti a zdají se být mnohem méně ovlivněny rybářským obhospodařováním než populace dunajské. Přispění domestikovaných pstruhů, dovezených v minulosti z jiných zemí, se zdá být zanedbatelné. Porovnání s výsledky prací z okolních zemí naznačuje, že rozdíly v rybářském hospodaření mohou vyústit v rozdílné vzory genetické struktury pstruha. Existují určité důkazy, že atlantický pstruh pronikl do povodí Dunaje již na konci Pleistocénu nebo bezprostředně po něm. Avšak není zřejmé, do jaké míry se přirozený kontakt odráží v současné úrovni promísení atlantické a dunajské linie v povodí Dunaje. Proto jsme analyzovali vzorky populací z dolních částí dunajského povodí za použití stejných mitochondriálních a mikrosatelitových markerů. Do analýz byly zahrnuty také populace z egejského úmoří, aby bylo možné vyhodnotit dopad rybářského hospodaření a porovnat jej s výsledky ze střední Evropy. Byla zjištěna pouze nízká úroveň introgrese z atlantických a jiných populací pstruha. Genetická diferenciace mezi populacemi východního Balkánu byla v porovnání s populacemi ze střední Evropy značně vyšší. Zdá se tedy, že populace východního Balkánu jsou v porovnání s populacemi ve střední Evropě mnohem méně ovlivněny převozem násad mezi povodími a vysazováním. Populace východního Balkánu tak mohou být považovány za původní a jsou cenným zdrojem informací pro studium přirozených procesů spojených s genetickou proměnlivostí a evolucí pstruha. Je možné usoudit, že rozsáhlá introgrese v populacích horního a středního Dunaje je především výsledkem opakovaných převozů a vysazování pstruha atlantického původu.

Na základě mikrosatelitů byly analyzovány také populace z horních přítoků řeky Otavy a populace z líhně Borová Lada s cílem ověřit původ divokých populací a vyhodnotit vhodnost současné strategie produkce a vysazování pstruha v oblasti Národního parku a chráněné krajinné oblasti Šumava. Analyzované populace byly geneticky odlišitelné od dříve analyzovaných populací z povodí Labe a byly také vzájemně diferencované v důsledku geografické vzdálenosti a přítomnosti migračních bariér. Vysazování pstruha z líhně v Borových Ladech však mohlo také ovlivnit genetickou variabilitu analyzovaných populací. Populace z této líhně se vyznačuje vyšší genetickou proměnlivostí a je geneticky odlišná od populací z horních přítoků Otavy, což by mohlo být způsobeno jejím nejednotným původem. Bez ohledu na její původ si je třeba uvědomit, že vysazování pstruha z této líhně do různých toků Šumavy může způsobit ztrátu genetické variability mezi populacemi. Tato ztráta by následně mohla vést až ke genetické homogenizaci divokých populací v celé oblasti. Proto by měla být každá aktivita spojená s vysazování předem důkladně promyšlena a aplikována pouze v případech, kdy došlo k silnému poklesu početnosti divoké populace, a předejít tak ohrožení její existence.

Porovnání analyzovaných populací s výsledky z jiných oblastí naznačuje, že mitochondriální haplotypy nalezené v populacích dolní části Dunajského povodí a jižní části úmoří Černého moře jsou značně odlišné od statisticky podpořené skupiny haplotypů z horního a středního Dunaje, povodí Kaspického moře a Aralského jezera. To dokazuje složitou evoluční historii pstruha v jižní a západní části úmoří Černého moře.

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Kohout, J., Šedivá, A., Jašková, I., Papoušek, I., Šlechta V., 2011. Genetic structure of brown trout in Central Europe is strongly affected by stocking with hatchery-reared fish. Symposium for European Freshwater Sciences 7, Girona, Spain, 27 June – 1 July 2011. (oral presentation)	2011
Kohout, J., Pekárik, L., Šedivá, A., 2012. Rapid and cost-effective molecular technique for discrimination of four species of invasive gobies. XIV European Congress of Ichthyology, June 27 – July 1, Liège, Belgium. (poster presentation)	2012

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