Zoological Studies **56:** 3 (2017)
doi:10.6620/ZS.2017.56-03

Zoological Studies

Phylogeographic Identification of Tench *Tinca tinca* (L., 1758) (Actinopterygii: Cyprinidae) from the Northern Balkans and Adjacent Regions and its Implications for Conservation

Jelena Lujić[1,*], Klaus Kohlmann[2], Petra Kersten[2], Zoran Marinović[1,3], Miroslav Ćirković[4], and Vladica Simić[5]

[1]*Department of Aquaculture, Szent István University, 2100 Gödöllő, Hungary. E-mail: lujicjelena@gmail.com; zor.marinovic@gmail.com*
[2]*Department of Ecophysiology and Aquaculture, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, 12587 Berlin, Germany. E-mail: kohlmann@igb-berlin.de; kersten@igb-berlin.de*
[3]*Department of Biology and Ecology, University of Novi Sad, 21000 Novi Sad, Serbia. E-mail: zor.marinovic@gmail.com*
[4]*Scientific Veterinary Institute "Novi Sad", 21000 Novi Sad, Serbia. E-mail: miroslavcirkovic@yahoo.com*
[5]*Institute of Biology and Ecology, University of Kragujevac, 34000 Kragujevac, Serbia. E-mail: simic@kg.ac.rs*

(Received 24 October 2016; Accepted 18 January 2017; Published 17 February 2017; Communicated by Ka Hou Chu)

Jelena Lujić, Klaus Kohlmann, Petra Kersten, Zoran Marinović, Miroslav Ćirković, and Vladica Simić (2017) The tench, *Tinca tinca*, is an endangered freshwater fish species in the Balkans. However, there are no management and conservation strategies developed for this species so far. In order to be able to develop such strategies, we first determined the phylogeographic identity of 70 tench individuals from four countries (Serbia, FYRO Macedonia, Hungary and Croatia) by PCR-RFLP analyses of two nuclear markers (*Act* and *RpS7*) and one mitochondrial marker (*Cytb*). All makers enabled the identification of two major geographic clades of tench (Western and Eastern), while nuclear markers additionally enabled the identification of hybrids between the two clades. Based on the mitochondrial marker *Cytb*, tench populations can be separated into two distinct areas: areas north of the Danube River with the dominant Western origin, and areas south of the Danube River with the dominant Eastern origin. Data obtained for the *Act* gene demonstrated Eastern origin for most individuals (88.23%) while data obtained for the *RpS7* gene demonstrated mixed origin with a high percentage of hybrids. The presence of high numbers of individuals with Western alleles for the *RpS7* gene in areas south of the Danube may indicate a natural invasion of this phylogroup. According to these results, areas north and south of the Danube are identified as two main management units. Additionally, we identified the rare western haplotype W2 based on the *Cytb* marker which clearly indicated human-aided dispersals of tench in the investigated region and since some individuals with W2 origin were cultured, attention must be given to the genetic structure and identity of the introduced individuals, whether during introduction or reintroduction since biological and ecological consequences of the hybridization between the two major clades are still unknown. Finally, we propose and discuss management and conservation strategies for tench of both management areas.

Key words: Tench, Phylogeographic clades, Conservation, Management strategy, Population genetics.

BACKGROUND

The tench (*Tinca tinca* L., 1758) is a widely distributed freshwater fish species with a native Eurasian distribution (Brylińska et al. 1999; Kottelat and Freyhof 2007). It has a great potential for aquaculture (Gela et al. 2006; Celada et al. 2007). The early research on tench genetics based on allozyme polymorphisms revealed only low variability of tench populations (Šlechtová et al. 1995; Kohlmann and Kersten 1998). However, later research based on species-specific microsatellites indicated considerably higher genetic variability both within and between tench populations

*Correspondence: Tel: +381646259819. E-mail: lujicjelena@gmail.com

(Kohlmann and Kersten 2006; Kohlmann et al. 2007, 2010).

Recent analysis of the mitochondrial Cytochrome *b* (*Cytb*) gene and the two nuclear genes Actin (*Act*) and Ribosomal Protein S7 (*RpS7*) have enabled the detection of two geographical clades within the Eurasian range of tench, the Western and the Eastern clade (Lajbner and Kotlík 2011). This divergence most likely occurred during the independent evolution in two separate refugia during the Pleistocene ice age, the Westeuropean and Ponto-Caspian (Lajbner et al. 2007). Although Lajbner et al. (2010) detected a lack of reproductive isolation between these two major phylogroups and the occurrence of hybrids between them, the authors consider that populations out of the contact zone are allopatric with low introgression which could further support the independent evolution of these two groups. However, human introductions of tench for aquacultural purposes may contribute to introgressions between these groups and thus disturb their independent evolution (Lajbner et al. 2011).

Although widespread and considered as a least concern species by the IUCN, tench is currently facing decreases in population numbers in some parts of Europe. Such decreases have been reported in Italy (Pompei et al. 2012), Poland (Wołos et al. 2009) and throughout the Balkans (Simic et al. 2013). In the Italian Red list, tench is listed as nearly threatened (Zerunian 2007, cited in Pompei et al. 2012). Reasons of this decline in populations are introductions of invasive fish species, degradation of spawning areas and habitats as a whole, eutrophication and other anthropogenic factors.

Although a steep decline in population number of tench in the Balkans has been detected over the last decade (Simic et al. 2013), neither specific conservation units nor conservation strategies have been established for this species. Furthermore, phylogeographic identification or analyses of population structure have not been conducted in tench from the Balkans. This information is needed to identify the tench origin, delineate distinct management units for conservation, develop management strategies and identify source populations for future translocations.

Here, we aimed to (1) determine the phylogeographic identity of the tench populations from the Balkans and surrounding areas based on molecular markers and methods which proved to be successful in recent studies, (2) identify management units for conservation and (3) suggest management strategies which could be applied in practice in order to conserve the remaining tench populations in the studied area.

MATERIALS AND METHODS

Sample collection

A total of 70 tench individuals from four countries (Serbia, FYRO Macedonia, Hungary and Croatia) were analysed (Fig. 1, Table 1). Samples included both wild (63 individuals) and cultured tench (7 individuals). Fin clips were taken and fixed in 96% ethanol.

PCR-RFLP analysis

Total genomic DNA was extracted from the fin clips with the peqGOLD Tissue Mini Kit (Peqlab Biotechnologie). The molecular markers used in this study were the second intron of the actin gene (*Act*) and the first intron of the gene coding the S7 ribosomal protein (*RpS7*) as nuclear markers and the cytohrome *b* gene (*Cytb*) as a mitochondrial marker. PCR reactions to amplify the selected genes were performed as described by Lajbner and Kotlík (2011). Furthermore, digestion of the PCR amplicons was also performed as described by Lajbner and Kotlík (2011). In short, *Mbo*I and *Alu*I endonucleases were used for the cleavage of the *Cytb* amplicons, *Nde*I was used for the digestion of the *RpS7* amplicons and *Eco52*I was used for the cleavage of the *Act* amplicons. The restriction fragments were separated on 2% agarose gels containing Roti®-GelStain (Carl Roth GmbH) and visualized under UV light. The size of fragments was estimated by comparison to a 100 bp ladder (GeneRuler 100 bp Plus DNA Ladder, Fermentas). Different patterns produced by each enzyme were identified according to Lajbner and Kotlík (2011).

Sequencing

In order to enable a comparison with already published *Cytb* haplotypes, two randomly chosen samples from each of the three haplotypes (E, W, and W2) detected by the previous PCR-RFLP analyses were sequenced. Because of its length and technical restrictions of the sequencer, the *Cytb* sequence had to be split into two overlapping segments. PCR amplification of both segments

was performed as described by Lo Presti et al. (2014). Before sequencing, the PCR fragments were purified with the peqGOLD Cycle-Pure Kit (Peqlab Biotechnologie). DNA concentrations were determined with a BioPhotometer (Eppendorf). The cycle sequencing was performed using the CEQ DTCS-Quick Start Kit (Beckman Coulter) according to the manufacturer instructions. The forward and reverse sequences of both segments were aligned and edited manually using the Genetic Analysis System v7.0, CEQuence Investigator module (Beckman Coulter). The resulting segments for each individual were manually assembled using the MEGA v6.0 software (Tamura et al. 2013). Furthermore, the MEGA v6.0 software was used for manual alignment of the six complete *Cytb* sequences in order to identify variable nucleotide positions. When all *Cytb* haplotypes were detected, a Megablast search for highly similar sequences was conducted in NCBI GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

Lajbner et al. (2011, in their fig. 2) described a broad contact zone of the Western and Eastern phylogeographic clades of tench in Central Europe including parts of the Danube River drainage. Moreover, a major north (from Estonia) to south (to Bosnia) barrier divided the tench distribution into a western part and an eastern part (Figure 4 in Lajbner et al. 2011). Therefore, the wild-caught tench individuals from the present study were arranged into two groups, north of the Danube (sampling locations 1 and 16; n = 15) and south of the Danube (sampling locations 4, 5, 9, 10-13, 15, 17, 18; n = 46), respectively, to test the observed distribution of *Cytb* Western and Eastern

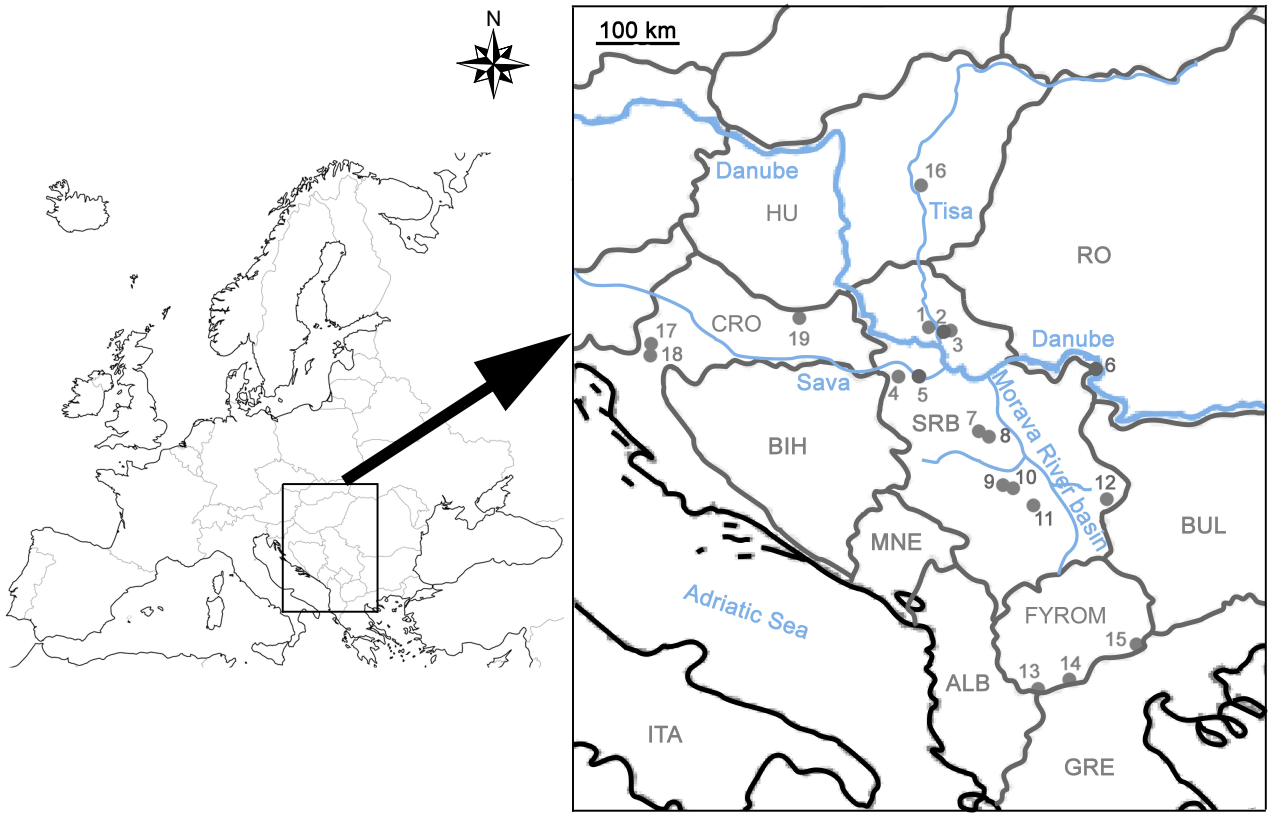

Fig. 1. Map of the sampling sites. 1 – Jegrička River (N = 5), 2 - Mošorin (N = 1), 3 - Ečka (N = 1), 4 - Zasavica (N = 1), 5 - Trskovača ponds (N = 1), 6 – Danube River (Kladovo) (N = 1), 7 - Šumarice (N = 1), 8 - Kragujevac (N = 2), 9 - Podunavačke ponds (N = 4), 10 - Grabovačke ponds (N = 5), 11 - Lake Blace (N = 18), 12 - Krupačko Lake (N = 11), 13 - Lake Prespa (N = 1), 14 - Žabeni (N = 2), 15 - Lake Dojran (N = 1), 16 - Tisa River (N = 10), 17 – Mrežnica River (N = 2), 18 - Dobra River (N = 2), 19 - Grudnjak (N = 1). SRB - Serbia, HU - Hungary, CRO - Croatia, FYROM - Former Yugoslav Republic Of Macedonia, RO - Romania, BIH - Bosnia and Herzegovina, MNE - Montenegro, BUL - Bulgaria, ALB - Albania, GRE - Greece, ITA - Italy. Coastline is marked by black lines while the country borders are highlighted by grey lines.

Table 1. Proportions of western (W and W2), eastern (E), and hybrid (H) tench individuals in different countries and sampling locations based on PCR-RFLP analysis

Country	Sampling location	Genetic status	Sample size (n)	*Cytb – MboI*	*Cytb – AluI*	*RpS7 – NdeI*	*Act – Eco52I*
Serbia	Lake Blace	wild	18	W = 0.00 E = 1.00	W = 0.00 E = 1.00	W = 0.72 E = 0.00 H = 0.28	W = 0.00 E = 1.00 H = 0.00
Serbia	Krupačko Lake	wild	11	W = 0.00 E = 1.00	W = 0.00 E = 1.00	W = 0.64 E = 0.09 H = 0.27	W = 0.00 E = 1.00 H = 0.00
Serbia	Grabovačke ponds	wild	5	W = 0.00 E = 1.00	W = 0.00 E = 1.00	W = 0.40 E = 0.40 H = 0.20	W = 0.20 E = 0.80 H = 0.00
Serbia	Jegrička River	wild	5	W = 0.60 E = 0.40	W = 0.60 E = 0.40	W = 0.20 E = 0.00 H = 0.80	W = 0.20 E = 0.80 H = 0.00
Serbia	Podunavačke ponds	wild	4	W = 1.00 E = 0.00	W = 0.75 W2 = 0.25 E = 0.00	W = 0.50 E = 0.00 H = 0.50	W = 0.00 E = 1.00 H = 0.00
Serbia	Trskovača ponds	wild	1	W = 0.00 E = 1.00	W = 0.00 E = 1.00	W = 0.00 E = 0.00 H = 1.00	W = 0.00 E = 0.00 H = 1.00
Serbia	Lake Šumarice	wild	1	-	-	-	W = 0.00 E = 1.00 H = 0.00
Serbia	Danube River	wild	1	W = 1.00 E = 0.00	W = 1.00 E = 0.00	W = 1.00 E = 0.00 H = 0.00	W = 0.00 E = 1.00 H = 0.00
Serbia	Zasavica	wild	1	W = 0.00 E = 1.00	W = 0.00 E = 1.00	W = 0.00 E = 1.00 H = 0.00	W = 0.00 E = 1.00 H = 0.00
Hungary	Tisa River	wild	10	W = 0.80 E = 0.20	W = 0.80 E = 0.20	W = 0.30 E = 0.20 H = 0.50	W = 0.00 E = 1.00 H = 0.00
Croatia	Mrežnica River	wild	2	W = 0.50 E = 0.50	W = 0.50 E = 0.50	W = 0.00 E = 0.00 H = 1.00	W = 0.00 E = 0.50 H = 0.50
Croatia	Dobra River	wild	2	W = 0.50 E = 0.50	W = 0.50 E = 0.50	W = 0.00 E = 0.50 H = 0.50	W = 0.50 E = 0.50 H = 0.00
Macedonia	Lake Prespa	wild	1	W = 0.00 E = 1.00	W = 0.00 E = 1.00	W = 0.00 E = 1.00 H = 0.00	W = 0.00 E = 0.00 H = 1.00
Macedonia	Lake Dojran	wild	1	W = 0.00 E = 1.00	W = 0.00 E = 1.00	W = 1.00 E = 0.00 H = 0.00	W = 0.00 E = 1.00 H = 0.00
Serbia	Kragujevac	cultured	2	W = 0.50 E = 0.50	W = 0.50 E = 0.50	W = 0.50 E = 0.00 H = 0.50	W = 0.00 E = 1.00 H = 0.00
Serbia	Ečka	cultured	1	W = 1.00 E = 0.00	W = 1.00 E = 0.00	W = 0.00 E = 1.00 H = 0.00	W = 1.00 E = 0.00 H = 0.00
Serbia	Mošorin	cultured	1	W = 1.00 E = 0.00	W = 1.00 E = 0.00	W = 0.00 E = 0.00 H = 1.00	W = 1.00 E = 0.00 H = 0.00
Croatia	Grudnjak	cultured	1	W = 0.00 E = 1.00	W = 0.00 E = 1.00	W = 0.00 E = 0.00 H = 1.00	W = 0.00 E = 1.00 H = 0.00
Macedonia	Žabeni	cultured	2	W = 1.00 E = 0.00	W = 0.00 W2 = 1.00 E = 0.00	W = 1.00 E = 0.00 H = 0.00	W = 0.50 E = 0.50 H = 0.00

haplotypes in both regions against the null-hypothesis of equal distribution expected for a contact zone with complete lack of reproductive isolation by the Chi^2-test implemented in MS Excel 2010. The wild individual from the Danube (sampling location 6) was excluded from this analysis as well as the single tench from Šumarice (sampling location 7) for which no *Cytb* data could be obtained.

RESULTS

PCR-RFLP analysis

After a successful DNA isolation, the PCR method yielded a total of three products (*Cytb*, *Act* and *RpS7* genes) which could be subjected to RFLP analysis. All endonucleases detected restriction fragment length polymorphisms. Based on the different patterns produced, all individuals could be classified into the phylogeographic clades according to Lajbner and Kotlík (2011) (Table 1).

The digestion of *Cytb* amplicons by *Alu*I resulted in three distinct patterns. The first two patterns were identified as the major eastern (E) and western (W) phylogroups, respectively. The third pattern was identified as haplotype W2 belonging to the western phylogroup. The digestion with *Mbo*I yielded similar results as *Alu*I. The only difference was the lack of W2 individuals (which were now identified as major western W), thus this digestion gave only two distinct patterns, the eastern and western. The correctness of classification based on the PCR-RFLP analysis was verified by sequencing. Comparisons to highly similar sequences in NCBI GenBank by Megablast analysis displayed in all six cases 100% identity at coverage rates of 99-100% with already existing sequences for these three *Cytb* haplotypes of tench (Table 2).

The digestion of *Act* amplicons by *Eco52*I resulted in three distinct patterns: eastern, western and hybrids (H) between the two.

The digestion of *RpS7* amplicons by *Nde*I also resulted in three distinct patterns: eastern, western and hybrids between the two.

Geographic distribution of haplotypes and phylogroups

Cytb gene: Individuals with patterns for the eastern phylogroup E were found in all four countries and were dominant in the southern Balkans. Three Serbian populations (Lake Blace, Krupačko Lake and Grabovačke ponds localities) were 100% eastern. Individuals with the western pattern W were dominant in the northern parts with 89% of Hungarian individuals belonging to this phylogroup. Only three individuals (two from the

Table 2. NCBI GenBank BLAST results for tench cytochrome *b* sequences from two randomly chosen samples from each of the identified haplotypes (accession date: 8 November 2013)

Sequences of samples from present study	GenBank sequences			Source of GenBank sequences
	Accession number	Identity (%)	Cover (%)	
LSB 1 and LSK 20 (E haplotype)	JX974523	100.0	100.0	Haplotype H2a, Felchowsee, Germany (Lo Presti et al. 2014)
	HM167941	100.0	99.0	Haplogroup EA, haplotype EA1, Ukraine (Lajbner and Kotlík 2011)
	HM560230	100.0	99.0	Trebisnjica River, Bosnia/Herzegovina (Perea et al. 2010)
	DQ841176	100.0	99.0	Romania, unpublished direct submission (Luca and Costache 2006)
LHD 56 and LSP 58 (W haplotype)	JX974520	100.0	100.0	Haplotype H1a, Felchowsee, Germany (Lo Presti et al. 2014)
	AB218686	100.0	100.0	Saone River, France (Saitoh et al. 2006)
	HM167950	100.0	99.0	Haplogroup W, haplotype W1, France (Lajbner and Kotlík 2011)
LMR 53 and LSP 61 (W2 haplotype)	JX974522	100.0	100.0	Haplotype H5, Valagola Lake, Italy (Lo Presti et al. 2014)
	HM167951	100.0	99.0	Haplogroup W, haplotype W2, Sweden (Lajbner and Kotlík 2011)

Žabeni fish ponds in Macedonia and one individual from the Podunavačke ponds locality in Serbia) were identified with pattern W2. The Chi^2-test for distribution of *Cytb* haplotypes among wild tench north and south of the Danube revealed a highly significant dominance of the Eastern phylogroup south of the Danube whereas the prevalence of the Western phylogroup north of the Danube was only close to being significant (Table 3).

Act gene: The eastern pattern E was the most dominant with 88.23% of the total samples displaying this pattern. Four populations (Tisa River in Hungary and Lake Blace, Krupačko Lake and Podunavačke ponds localities in Serbia) displayed 100% eastern pattern. The other individuals were identified as western (W) or hybrid (H).

RpS7 gene: This was the only gene for which the eastern pattern E was not so dominant. Only 11.76% of the total samples displayed the eastern pattern, while 47.06% displayed the western pattern W and 41.18% were hybrids (H). Although few in numbers, individuals with the eastern pattern for this gene were found in open waters of all four countries. Hybrids were the most dominant in the Danube and Sava River Basins (Tisa River in Hungary, Dobra River and Mrežnica River in Croatia and Jegrička River in Serbia).

Combining the results of the three different genetic markers, a high degree of hybridization between the western and eastern phylogroups became evident. In the whole data set of 69 tench individuals for which data for all markers were available, only five were of pure origin: one cultured Macedonian tench belonged to the western phylogroup and four wild tench to the eastern phylogroup (one from Hungary and three from Serbia). All others showed either contrasting patterns (i.e. eastern origin for one or two markers and western for the other or vice versa) or were directly identified as hybrids by at least one of the two nuclear markers. Interestingly, the single individual from the Danube (sampling location 6) also turned out to be a hybrid.

DISCUSSION

The results of the present study confirm that PCR-RFLP analysis of nuclear and mitochondrial markers is an effective method in phylogeographic studies of tench. This analysis was reliable in detecting the major western and eastern phylogeographic clades of the tench, but also in identification of the rare W2 haplotype of the *Cytb* gene as well as hybrids between clades by the two nuclear markers or combinations of mitochondrial and nuclear markers.

The relatively weak relationship between the nuclear and mitochondrial variability indicates that this combination can prove very useful in analysis of the population structure of tench. The reason behind this weak relationship, which was also supported by Lo Presti et al. (2012) for the tench is the different genetic background and mode of inheritance of these markers. Furthermore, the differential results observed for *Act* and *RpS7* as nuclear markers indicate that the loci for these two genes are found on separate chromosomes and are able to recombine freely. Usage of these two nuclear markers can also prove very significant in analysis of the population structure of tench.

According to Lajbner et al. (2011), the territory of Serbia and the rest of the Balkans should be dominantly inhabited by individuals of the eastern phylogroup. This is mostly because the Ponto-Caspian region and the River Danube basin were the glacial refugia of this phylogroup. However, we have found numerous individuals belonging to the western phylogroup or hybrids which points to intensive hybridization of the two phylogroups and not just their coexistence. The high percentage of western phylogroup individuals (according to the nuclear marker *RpS7*) may indicate a natural invasion of this phylogroup, with the Danube and its left tributary, the Tisa River, as the potential migratory routes. Results acquired for the mitochondrial marker (*Cytb* gene) further corroborate this hypothesis since most of the wild individuals belonging to the western phylogroup for

Table 3. Observed numbers of wild tench with western (W) and eastern (E) *Cytb* haplotypes north and south of the River Danube and *p*-values of the Chi^2-test for significant deviation from equal distribution.

	Observed number of wild tench with		Chi^2-test
Region	W	E	*p*-value
North of Danube	11	4	= 0.07
South of Danube	6	40	< 0.01

Cytb are found north of the Danube, and most of the individuals belonging to the eastern phylogroup are found south of the Danube.

In only two samples from Macedonia and in one sample from Serbia (Podunavačke ponds locality), we have detected the haplotype W2 for the *Cytb* gene. This haplotype is different from the major western haplotype in only one base pair (Lajbner and Kotlík 2011; Lo Presti et al. 2014). Since the W2 haplotype was found only in Sweden, France, Czech Republic (Lajbner et al. 2011) and Italy (Lo Presti et al. 2012) so far, individuals with this haplotype detected in the present study were most likely introduced to these localities. The two Macedonian tench individuals are cultured, thus they were most likely introduced to the fishponds. We suspect that the remaining individual from the Podunavačke ponds locality was also introduced to this site. This claim is further supported by the results of *Cytb*, since individuals from this locality are the only ones that have western haplotypes of *Cytb* among the Serbian localities south of the Danube.

When analysing the genetic structure of the tench populations, special attention must be given to the human-aided dispersals (Lajbner et al. 2011). In the last century, little attention was given to the genetic identity and origin of the introduced individuals. During the late 20th century, individuals originating from the Czech Republic were cultivated in the Ečka and Mošorin fish ponds. We have determined that these individuals and their offspring mostly belong to the western phylogroup. When considering that fish escapes into the open waters can occur, it is necessary to assess the genetic structure of the individuals which are being introduced.

Based on the present results, we are able to identify two main management units for conservation. Those are the populations north from the River Danube where mostly individuals belonging to the western phylogroup are found, and populations south from the River Danube where mostly individuals belonging to the eastern phylogroup are found. Application of a suitable conservation program depends on conditions which prevail at a certain locality. If the conditions on a locality inhabited by tench are determined as suitable in terms of nutrition, spawning and ichthyofaunal structure, *in situ* conservation plans can be carried out. If these criteria are not met, *ex situ* conservation strategies must be conducted. During translocations, special attention must be given to the genetic structure and identity of the introduced individuals according to the National strategy for sustainable use of resources of Republic of Serbia (Službeni glasnik Republike Srbije 33/2012) since biological and ecological consequences of the hybridization between the two major clades are still unknown. Additionally, watercourses north and south of the Danube are ecologically different. Most of the watercourses north of Danube are typical plain watercourses, while the ones south of the Danube are mostly hilly watercourses, therefore this delineation might indicate certain adaptability of these haplotypes to these specific ecological conditions.

When establishing populations suitable for introduction or reintroduction into the sites south of the River Danube, it is necessary to create pure eastern populations. The procedure includes assessment of the phylogeographic identity of individuals, and then continuing to work only with broodstock belonging to the eastern clade according to all genetic markers. In this way all individuals from the F1 generation will belong to the same haplotype. If it is not possible to work with clear eastern individuals, then the process of selection should be continued in the following generations until the point of 100% of eastern individuals is reached. Only with these individuals it is possible to conduct repopulations. In addition, male spermatozoa and early stage germ cells can be collected and stored through cryopreservation (Rodina et al. 2007; Marinović et al., 2016; Lujić et al. 2017) in order to facilitate conservation efforts. Similar should be done when creating populations suitable for introduction or reintroduction into the sites north of River Danube where, according to the results for mitochondrial genetic markers from this study, most individuals belong to the western clade and thus that phylogroup should be favoured.

CONCLUSIONS

In the present study, we observed that most individuals south of the Danube belong to the eastern phylogroup, while most individuals north of the Danube belong to the western phylogroup of tench. Results obtained for *RpS7* and *Cytb* genes may indicate a natural invasion of individuals belonging to the western phylogroup with the Danube and its left tributary, the Tisa River, as potential migratory routes. Furthermore, the presence of major W and rare W2 haplotypes of *Cytb* in the southern Balkans indicate human-

aided dispersal of these phylogroups outside of their natural range. Thus, attention must be given to the genetic structure and identity of the introduced individuals, whether during introduction or reintroduction since biological and ecological consequences of the hybridization between the two major clades are still unknown. Furthermore, we have identified two major management units for conservation and discussed management strategies for conservation of this species in the Balkans.

Acknowledgments: This study was funded by the German Academic Exchange Service (DAAD; grant to JL), Ministry of Education, Sciences and Technological Development of Republic of Serbia [project no. TR 31011] and Provincial Secretariat for Science and Technological Development of Serbia [project no. 3181]. Authors want to express their gratitude to Dr. Stojmir Stojanovski, Dr. Stoe Smiljkov, Dr. Damir Kapetanović, Dr. Damir Valić, Dr. Ákos Horváth, Dr. Zoran Marković, Dr. Marko Stanković, Dr. Josip Barišić, Dr. Gergely Bernáth, Dr. Željko Popović and the whole team from the Aquarium "PMF KG" of the University of Kragujevac, Serbia, for aid in sample collection and technical assistance. We would also like to thank the anonymous reviewers for their valuable comments that helped improve the manuscript.

REFERENCES

Brylińska M, Bryliński E, Bnińska M. 1999. *Tinca tinca* (Linnaeus, 1758). In: Bănărescu PM (ed) The freshwater fishes of Europe. Cyprinidae 2: Part I: *Rhodeus* to *Capoeta*. AULA-Verlag, Wiebelsheim, p. 229-302.

Celada JD, Aguilera A, Carral JM, Sáez-Royela M, Melendre PM, Pérez JR. 2007. Effects of stocking density on survival and growth of juvenile tench (*Tinca tinca* L.). Aquacult Int **15:**461-465.

Gela D, Flajšhans M, Kocour M, Rodina M, Linhart O. 2006. Tench (*Tinca tinca*) broodstock management in breeding station under conditions in pond culture: a review. Aquacult Int **14:**195-203.

Kohlmann K, Kersten P. 1998. Enzyme variability in a wild population of tench (*Tinca tinca* L.). Pol Arch Hydrobiol **45:**303-310.

Kohlmann K, Kersten P. 2006. Microsatellite loci in tench: isolation and variability in a test population. Aquacult Int **14:**3-7.

Kohlmann K, Kersten P, Flajšhans M. 2007. Comparison of microsatellite variability in wild and cultured tench (*Tinca tinca*). Aquaculture **272:**S147-S151.

Kohlmann K, Kersten P, Panicz R, Memis D, Flajšhans M. 2010. Genetic variability and differentiation of wild and cultured tench populations inferred from microsatellite loci. Rev Fish Biol Fisher **20:**279-288.

Kottelat M, Freyhof J. 2007. Handbook of European freshwater fishes. Imprimerie du Démocrate SA, Delémond.

Lajbner Z, Linhart O, Kotlik P. 2007. Molecular phylogeography of the tench *Tinca tinca* (Linnaeus, 1758). *In*: Buj I, Zanella L, Mrakovcic M (eds) The 12th European Congress of Ichthyology, 2007 September 9-13, Cavtat, Paris (FRA): European Ichthyological Society.

Lajbner Z, Kohlmann K, Linhart O, Kotlík P. 2010. Lack of reproductive isolation between the Western and Eastern phylogroups of the tench. Rev Fish Biol Fisher **20:**289-300.

Lajbner Z, Kotlík P. 2011. PCR-RFLP assay to distinguish the Western and Eastern phylogroups in wild and cultured tench *Tinca tinca*. Mol Ecol Resour **11:**374-377.

Lajbner Z, Linhart O, Kotlík P. 2011. Human-aided dispersal has altered but not erased the phylogeography of the tench. Evol Appl **4:**545-561.

Lo Presti R, Kohlmann K, Kersten P, Gasco L, Lisa C, Di Stasio L. 2012. Genetic variability in tench (*Tinca tinca* L.) as revealed by PCR-RFLP analysis of mitochondrial DNA. Ital J Anim Sci **11:**103-108.

Lo Presti R, Kohlmann K, Kersten P, Lisa C, Di Stasio L. 2014. Sequence variability at the mitochondrial ND1, ND6, cyt *b* and D-loop segments in tench (*Tinca tinca* L.) J Appl Ichthyol **30:**15-21.

Lujić J, Bernáth G, Marinović Z, Radojković N, Simić V, Ćirković M, Urbányi B, Horváth Á. 2017. Fertilizing capacity and motility of tench *Tinca tinca* (L., 1758) sperm following cryopreservation. Aquacult Res **48:**102-110.

Marinović Z, Lujić J, Kása E, Bernáth G, Urbányi B, Horváth Á. 2016. Cryosurvival of isolated testicular cells and testicular tissue of tench *Tinca tinca* and goldfish *Carassius auratus* following slow-rate freezing. Gen Comp Endocrinol. doi:10.1016/j.ygcen.2016.07.005.

Perea S, Böhme M, Zupančič P, Freyhof J, Šanda R, Ozuluğ M, Abdoli A, Doadrio I. 2010. Phylogenetic relationships and biogeographical patterns in Circum-Mediterranean Subfamily Leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. BMC Evol Biol **10:**265.

Pompei L, Franchi E, Giannetto D, Lorenzoni M. 2012. Growth and reproductive properties of tench, *Tinca tinca* Linnaeus, 1758 in Trasimeno Lake (Umbria, Italy). Knowl Managt Aquatic Ecosyst **406:**07.

Rodina M, Gela D, Kocour M, Hadi Alavi SM, Hulak M, Linhart O. 2007. Cryopreservation of tench, *Tinca tinca*, sperm: Sperm motility and hatching success of embryos. Theriogenol **67:**931-940.

Saitoh K, Sado T, Mayden RL, Hanzawa N, Nakamura K, Nishida M, Miya M. 2006. Mitogenomic evolution and interrelationships of the Cypriniformes (Actinopterygii: Ostariophysi): the first evidence toward resolution of higher-level relationships of the world's largest freshwater fish clade based on 59 whole mitogenome sequences. J Mol Evol **63:**826-841.

Simic V, Lujic J, Kostic D, Cirkovic M, Bjelic-Cabrilo O, Simic S, Markovic G. 2013. Diversity characteristics of the fish species important for fishery in the waters of Serbia. Bulg J Agr Sci **19:**77-87.

Šlechtová V, Šlechtá V, Valenta M. 1995. Genetic protein variability in tench (*Tinca tinca* L.) stocks in Czech Republic. Pol Arch Hydrobiol **42:**133-140.

Službeni glasnik Republike Srbije br. 33/2012. Nacionalna strategija održivog korišćenja prirodnih resursa i dobara.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol **30:**2725-2729.

Wołos A, Zdanowski B, Wierzchowska M. 2009. Long term changes in commercial fish catches in Lake Mamry Północne (northeastern Poland) on the background of physical, chemical, and biological data. Arch Pol Fish **17:**196-210.