Botskaris Kalafatis Stavros, Markou Kyriaki, Karaiskou, Gagavouzis, Minoudi, Antonopoulou Euthymia, Triantafyllidis

Genetic analysis based on mitochondrial DNA in tench populations (*Tinca tinca* L., 1758) from different European countries

Abstract

In order to investigate the phylogenetic structure of tench *Tinca tinca* (L., 1758), three mitochondrial DNA segments, D-loop, COXI and *Cyt-b* were analyzed in 50 individuals from five European populations. The analysis split tench species in two phylogroups, Western and Eastern, indicating a hybridization zone in the region of the Danube River. A simple PCR-RFLP analysis was also conducted on additional individuals which identified cases of mixture of the two phylogroups in the Romanian, Czech and English populations. Our results reveal the unique characteristics of *T. tinca*'s genetic structure that makes this species a valuable model for applied genetic research, supporting future aquaculture breeding practices.

Key words: mtDNA, genetic variability, *phylogroups*

The tench, *Tinca tinca* (L., 1758) is a freshwater species, native to Eurasian boarders (Brylinska *et al.*,1999; Kottelat & Freyhof 2007). However, due to human-mediated translocations, it is now widely distributed across several freshwater regions of the world (Lajbner & Kotlik, 2011). Tench has a great potential for aquaculture (Gela *et al.*, 2006; Celada *et al.*, 2007; Kohlmann *et al.*, 2009), but there is limited information on its genetic structure compared to other fish species (Presti *et al.*, 2012). So far, genetic diversity within and between tench populations relied on enzyme variability, microsatellite markers (Kohlmann 1998, 2005, 2007, 2008), nuclear and mitochondrial DNA (mtDNA) (Presti 2009, 2012, 2013; Lajbner 2009, 2010; Lajbner & Kotlik 2010; Lujic 2017), as well as genes of growth hormones (Kocour & Kohlmann, 2014). The analysis of polymorphisms on nuclear markers and mtDNA regions is verified as an exceptional tool in the detection of genetic variability among tench species (Briolay 1998; Presti 2009, 2012) hence, the complete mtDNA sequence of *T. tinca* has already been published (Saitoh *et al.*, 2006).

Similarly to many widely distributed freshwater species, tench presents deep phylogeographic subdivisions (Van Houdt *et al.*, 2005; Hanfling *et al.*, 2009; Lajbner *et al.*, 2010*)*. Recent evidence separates tench in two deeply divergent phylogroups, the Western phylogroup (W) and the Eastern (E) (Presti *et al.*, 2012; Lajbner & Kotlik 2011; Lujic *et al.*, 2017). Hybridization zone between the two phylogroups have been reported in Central Europe across the Danube River while the phylogroup E is partitioned into three subclades (Lajbner *et al.*, 2011). Specifically, tench species in the Anzalee Lagoon of the Caspian Sea (Iran) and in the Iskar River of the Danube River (Bulgaria), are genetically divergent compared to the rest of phylogroup E. Furthermore, human-aided translocations of *T. tinca* populations have contributed to introgressions between the two phylogroups, disturbing their independent evolution (Lajbner *et al* 2011; Lujic *et al.*, 2017).

The present study aims to investigate the variability of five wild European tench populations (England, Greece, Spain, Romania and Czech Republic), by analyzing three mtDNA segments, D-loop, COXI, and *Cytb*, and reconstructing phylogeny to unveil the evolutionary relationships within and between different tench populations. The COXI gene has never been used before in tench phylogeographic research. A total of 50 tench individuals collected from five European countries, England (Portsmouth), Greece (Pamvotida), Spain (Zamora), Romania (Costant) and Czech

Republic (Vodňany) were analyzed. Total genomic DNA was extracted from muscle using the CTAB method (hexacytrimethymmonium bromide) (Hillis *et al.,* 1996). PCR amplification of D-loop was accomplished using primers designed by Primer3 software (D-loopR, 5'-TTCTCAGGGCCCATCTTAAC-3' and D-loopL, 5'- CGCCCAGAAAAAGGAGATTT-3'), while for the amplification of COXI and *Cytb* already published primers were used (Briolay *et al.,* 1998; Ward *et al.,* 2005). Overall, 2381 bp of DNA Triantafyllidis were amplified, 1067 bp for D-loop region, 650 bp for COXI and 664 bp for Cyt-b. The total volume of polymerase chain reaction was 25μl in which 100ng of genomic DNA was amplified, using 0.1 units of Qiagen Taq polymerase, 2mM dNTPs, 1 pmol/μl of each primer, 2.5 mM $MgCl_2$ and 2.5 μl of 10 X Reaction Buffer. Thermal cycling amplification conditions were as follows: initial denaturation at 94ºC for 3 min, followed by 33 cycles of strand denaturation at 94ºC for 1 min, annealing at 51ºC for 45 s and primer extension at 72ºC for 40 s and a final 3 min elongation time at 72ºC. The PCR products were purified using the Nucleospin Extra kit (Macherey-Nagel, Duren, Germany) and sequenced by Sanger method from Macrogen Inc. (Seoul, Korea) using an ABI 3730XL DNA Analyzer. The sequences were deposited in GenBank (GenBank Accession no. EU716086-EU716112, HQ600798-HQ600800). Sequences were aligned using ClustalW software and the reference sequence (RefSeq), (GenBank, accession n. NC08648), derived from an individual in Lake Saône, South France. Sequences of the three mtDNA markers were merged into a 1588 bp segment and analyzed using MEGA version 10.0.5 (Tamura, Dudley, Nei, and Kumar 2007). In particular, the phylogenetic analysis was performed under a maximum likelihood framework using Tamura 3-parameter model and the robustness of the trees was assessed by bootstrap resampling (1000 replicates; Felsenstein 1985). In addition, seventeen *Cytb* sequences (GenBank Accession no. HM167935-HM167965) were retrieved from (Lajbner *et al.*, 2011) and incorporated into our data in order to reconstruct a phylogenetic tree based on a single genetic marker. Since *T. tinca* is the only species belonging to Tincidae family, we used *Barilius bendelisis* as outgroup (GenBank accession no. AP011433). The lack of published data for D-loop and COXI restricted the comparative analysis only to the *Cytb gene.*

The nucleotide variation at three mtDNA regions originated a total of 29, 10 and 4 polymorphic sites in D-loop, COXI and *Cyt-b* regions, respectively. In particular, the polymorphisms are sub-divided into 25 translocations, 9 inversions and 4 insertions. The D-loop region logically presents higher number of polymorphisms due to the fact that unlike COXI and *Cyt-b*, it consists of non-coding area of the mitochondrial genome. (The polymorphic sites between 50 samples of *T. tinca* and RefSeq can be found in supplementary material).

The analysis revealed nine composite haplotypes (H1-H9) (Table 1). Higher frequency is reported for haplotypes H1, H6, H8 and H9, representing 80% of all samples. Greek, Czech and Romanian populations revealed higher haplotypic and nucleotide diversity compared to the other two populations of England and Spain; these were the most homogenous since each of them consisted by only one, though unique, haplotype.

The hypothesis of two distinct phylogroups related to tench populations is also established by the current phylogenetic analysis (Fig Ia). The English and Greek samples formed the North-West group. Taking into account that tench is not a native species in Greece, the results strongly suggests that tench populations in this region are of Western origin.

All Spanish, and most of the Romanian and Czech samples grouped together and composed the South-East group. However, the Czech and Romanian populations displayed mixed haplotype patterns (three samples grouped with the Western clade) implying the existence of tench hybrids across the region of the Danube River. In fact, it has been reported that admixed populations of this region could probably be a result of natural postglacial contact between the Eastern and Western lineages, rather than a cause of intentional translocations of tench populations by humans (Lajbner

et al., 2011). On the other hand, our findings reveal that tench in the Iberian Peninsula have been introduced from Eastern Europe or Asia and these populations are genetically divergent from the rest of the phylogroup, probably as a result of geographical isolation. The phylogeny verified that the discriminatory power of D-loop, COXI and *Cytb* markers was informative enough to clearly distinguish between the Western and Eastern phylogroups which has already been suggested in tench phylogeography (Lo Presti *et al.,* 2012; Kocour and Kohlmann, 2011; Kohlmann *et al.,* 2010; Lajbner *et al.,* 2007, 2011).

Based on the nine distinct haplotypes of *Cytb* data (Figure 1b), the phylogeny clearly splits the wide-range data set into Western and Eastern phylogroups with four and five composite haplotypes, respectively. Only two and two haplotypes were found in our samples respectively. The internal structure of phylogroup E is partitioned into three subclades. Specifically, in accordance with Lajbner *et al., (*2011), C4 haplotype in the Anzalee Lagoon of the Caspian Sea (Iran) and C9 in the Iskar River of the Danube River in Bulgaria represent separate clades. It is yet unclear, why these two areas illustrate some kind of divergence from the rest of the phylogroups and introduce the necessity for a re-evaluation on the distribution of refugia between the Danube River and the Ponto-Caspian region.

The British samples are grouped exclusively into the Western phylogroup. In general, tench populations in UK are mainly of Western origin. However, breeds with Eastern genetic structure into the British Isle have been reported and could be a result of tench translocations for aquaculture purposes (Lajbner *et al.,* 2011). The concept of mixed populations consisting of both Western and Eastern haplotypes was tested using the *AseI* restriction enzyme to digest COXI fragment in additional samples on every population. The results show no mixed haplotype patterns in Greek and Spanish samples (30 individuals, respectively). However, in UK samples the PCR-RFLP analysis has permitted the identification of a single individual of Eastern phylogroup origin (out of 30 individuals analysed), in corroboration with previous results (Lajbner *et al.,* 2011). Equally, the analysis shows that the six out of fifty samples from Vodňany area (Czech Republic) and one out of fifteen samples from Costanta region (Romania) demonstrate Western haplotype patterns showing that breeds with admixed genetic structure occur across the region of Danube River. Apart from these areas, hybrids have been reported in Grosser Felchowsee and Kleiner Döllnsee Lakes in North-Eastern Germany in an area covered by the Scandinavian ice sheet during the Weichselian glaciation (Lajbner *et al.,* 2009). These lakes are located in a relatively short distance from Vodňany (approximately 550 kilometers) and could be an explanation of the distinct haplotype patterns that our analysis displayed on these samples.

In Turkey, tench is a native species in river drainages within the Black Sea basin but has also been translocated to the central and western parts of this country (Brylinska *et al.,* 1999). The results report that in Turkey, *T. tinca* displays Eastern haplotype patterns, suggesting that the non-native breeds are derived from an area within the range of the Eastern phylogroup. Unsurprisingly, the Chinese haplotype is also grouped into the Eastern phylogroup. This is fairly expected, since recently breeds from Vodňany have been imported in China as a source for stocking (Wang *et al.,* 2004). It is possible that this practice could induce introgression of tench populations with Western haplotypes into the native population of Asia, since tench in the Czech Republic is composed by mixed populations.

In conclusion, our study utilizes new sample and molecular data, never used before in tench phylogeography research and contributes to a better understanding of the genetic variability within as well as between fifteen tench populations. The data revealed that mtDNA markers provide a high level of discriminatory power in the application of population genetic research. The occurrence of tench hybrids across Danube River might be a valuable baseline to start selective breeding programs especially if mtDNA markers were to be combined with nuclear data (Presti *et al.,* 2012). The fact

that tench phylogroups can interbreed but still remain distinct has useful practical implications. Since no negative fitness consequence of mixed tench populations has been detected and tench is widely used in aquaculture, research on tench genetic structure may contribute to the identification of genes underlying crucial structural and physiological phenotypes (Allendorf *et al.*, 2001). Thus, the unique characteristics of *T. tinca*'s genetic structure makes this species a valuable model for applied genetic research.

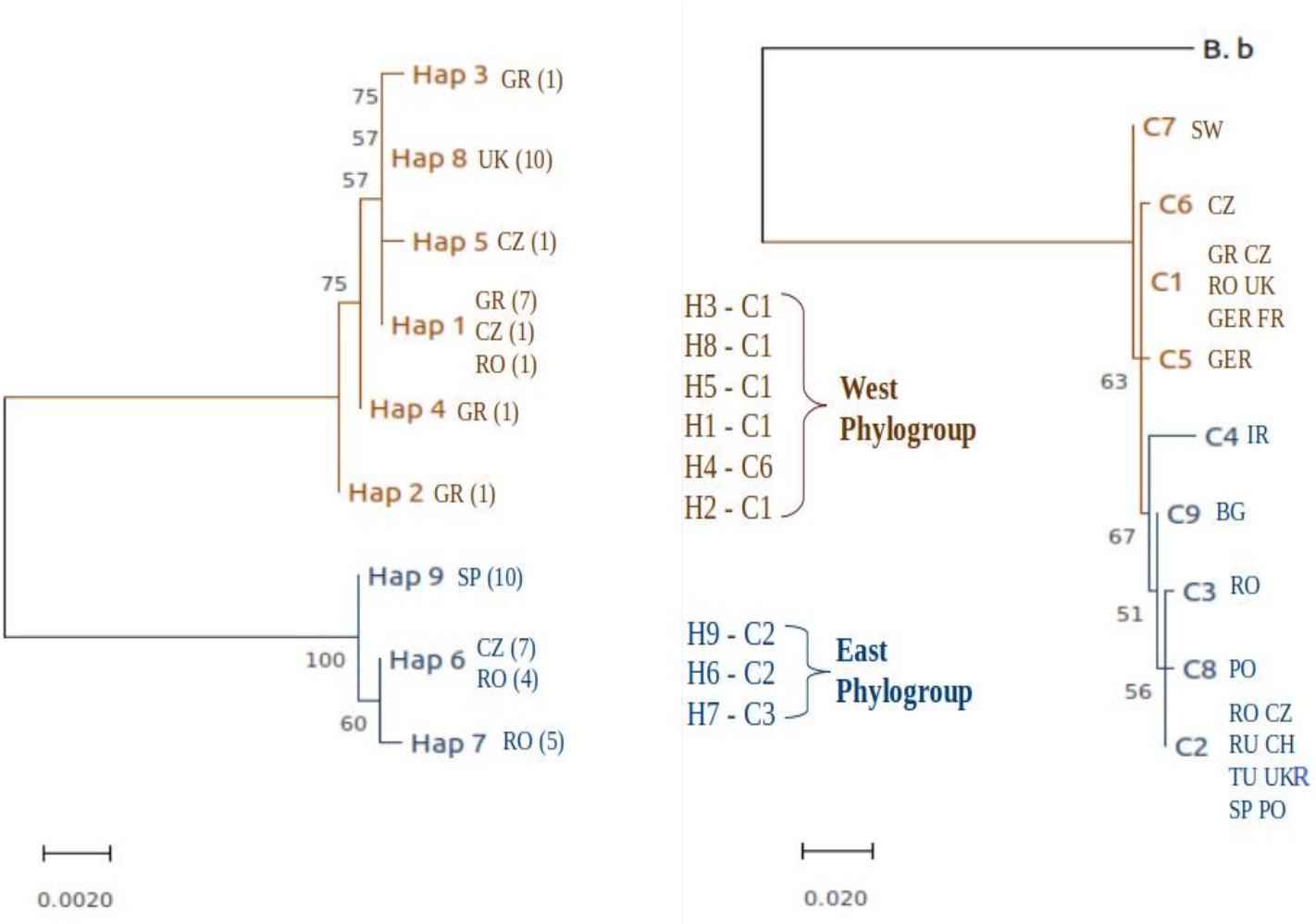
Table I. Distribution of the haplotype and nucleotide diversity values based on the analysis of 50 samples from five different European populations (indicated with country acronyms). The correspondence between haplotypes based on concatenated alignments (H) and haplotypes by *Cyt-b* (C) is shown in first row. Each halplotype is classified to (W) Western phylogroup or (E) Eastern phylogroup.

Origin	W H1 (C1)	W H2 (C1)	W H3 (C1)	W H4 (C6)	W H5 (C1)	E H6 (C2)	E H7 (C3)	W H8 (C1)	E H9 (C2)	**SUM**	**Nucleotide Diversity %**	**Haplotype Diversity**
UK	-	-	-	-	-	-	-	10		10	0	0
GR	7	1	1	1	-	-	-	-	-	10	0.048 +/- 0.043	0.533 +/- 0.18
SP	-	-	-	-	-	-	-	-	10	10	0	0
RO	1	-	-	-	-	4	5	-	-	10	0.522 +/- 0.299	0.644 +/- 0.101
CZ	1	-	-	1	1	7	-	-	-	10	1.1417 +/- 0.627	0.533 +/- 0.18
SUM	9	1	1	2	1	11	5	10	10	50		

Fig I. The phylogenetic tree as produced by MEGA software, performed under a maximum likelihood framework using Tamura 3-parameter model. Fig I (a) displays the phylogenetic tree based on the concatenated alignments of

three mtDNA segments (D-loop, *Cyt-b*, COXI) for five European countries. The number of samples forming each haplotype is shown next to the origin of the haplotype. Fig I (b) illustrates the phylogeny based on a single genetic marker (*Cyt-b)* and represent fifteen different countries (all indicated with acronyms). The outgroup used for the reconstruction of the phylogeny (b) is *Barilius bendelisis.*

(a) ***(b)***



Acknowledgments

EA has been financially supported by EC grant of the 6th Framework program no. COOP-CT-2004-512575-PROTENCH.

References

Allendorf, F. W., Leary R. F., Spruell P., J. K. Wenburg . 2001. The problems with hybrids: setting conservation guidelines. Trends Ecology Evolution 16: 613-622.

Bataillon, P. E., & Republic, C. (2001). Phylogeography of the barbel (Barbus. *Molecular Ecology*, 2177–2185.

Briolay, Jérôme & Galtier, Nicolas & Brito, Miguel & Bouvet, Yvette. (1998). Molecular phylogeny of Cyprinidae infered from cytochrome b DNA sequences. *Molecular Phylogenetics and Evolution* 9, 100-8. 10.1006/mpev.1997.0441.

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, J. D., Aguilera, A., Carral, J. M., Sáez-Royuela, M., Melendre, P. M., & Pérez, J. R. (2007). Effects of stocking density on survival and growth of juvenile tench (*Tinca tinca* L.). *Aquaculture International*, *15*(6), 461–465. https://doi.org/10.1007/s10499-007-9111-4

Chein, I. (1980). Psychological, social, and epidemiological factors in juvenile drug use. *NIDA Research Monograph*, *30*, 76–82.

Durand, J. D., Persat, H., & Bouvet, Y. (1999). Phylogeography and postglacial dispersion of the chub (*Leuciscus cephalus*) in Europe. *Molecular Ecology*, *8*(6), 989–997. https://doi.org/10.1046/j.1365-294X.1999.00654.x

Felsenstein, Joseph. (1985). Confidence limits on phylogenies: An approach using the Bootstrap. *Evolution* 39, 783-791. 10.2307/2408678.

Gela, D., Flajšhans, M., Kocour, M., Rodina, M., & Linhart, O. (2006). Tench (*Tinca tinca*) broodstock management in breeding station under conditions of pond culture: A review. *Aquaculture International*, *14*(1–2), 195–202. https://doi.org/10.1007/s10499-005-9025-y

Hänfling, B., Dümpelmann, C., Bogutskaya, N. G., Brandl, R., & Brändle, M. (2009). Shallow phylogeographic structuring of *Vimba vimba* across Europe suggests two distinct refugia during the last glaciation. *Journal of Fish Biology*, *75*(9), 2269–2286. https://doi.org/10.1111/j.1095-8649.2009.02415.x

Hillis D, Moritz C, Mable BK. Molecular systematic. Nucleic acids IV: sequencing and cloning. Sinauer Associates, Sunderland, MA. 1996. p. 321–381.

Kocour, M., & Kohlmann, K. (2011). Growth hormone gene polymorphisms in tench, *Tinca tinca* L. *Aquaculture*, *310*(3–4), 298–304. https://doi.org/10.1016/j.aquaculture.2010.10.006

Kocour, M., & Kohlmann, K. (2014). Distribution of five growth hormone gene haplogroups in wild and cultured tench, *Tinca tinca* L., populations. *Journal of Applied Ichthyology, 30*(S1), 22–28. https://doi.org/10.1111/jai.12428

Kohlmann, K & Kersten, P. (1998). Enzyme variability in a wild population of tench (*Tinca tinca*). Polskie Archiwum Hydrobiologii. 45. 303-310.

Kohlmann, K., & Kersten, P. (2006). Microsatellite loci in tench: Isolation and variability in a test population. *Aquaculture International*, *14*(1–2), 3–7. https://doi.org/10.1007/s10499-005-9009-y

Kohlmann, K., Kersten, P., & Flajšhans, M. (2007). Comparison of microsatellite variability in wild and cultured tench (*Tinca tinca*). *Aquaculture*, *272*(SUPPL. 1), 147–151. https://doi.org/10.1016/j.aquaculture.2007.08.003

Kohlmann, K., Kersten, P., Panicz, R., Memiş, D., & Flajšhans, M. (2010). Genetic variability and differentiation of wild and cultured tench populations inferred from microsatellite loci. *Reviews in Fish Biology and Fisheries*, *20*(3), 279–288. https://doi.org/10.1007/s11160-009-9138-x

Lajbner, Z., Kohlmann, K., Linhart, O., & Kotlík, P. (2010). Lack of reproductive isolation between the Western and Eastern phylogroups of the tench. *Reviews in Fish Biology and Fisheries*, *20*(3), 289–300. https://doi.org/10.1007/s11160-009-9137-y

Lajbner, Z., & Kotlík, P. (2011). PCR-RFLP assays to distinguish the Western and Eastern phylogroups in wild and cultured tench *Tinca tinca*. *Molecular Ecology Resources*, *11*(2), 374–377. https://doi.org/10.1111/j.1755-0998.2010.02914.x

Lajbner, Z., Linhart, O., & Kotlík, P. (2011). Human-aided dispersal has altered but not erased the phylogeography of the tench. *Evolutionary Applications*, *4*(4), 545–561. https://doi.org/10.1111/j.1752-4571.2010.00174.x

Lo Presti, R., Gasco, L., Lisa, C., Zoccarato, I., & Di Stasio, L. (2010). PCR-RFLP analysis of mitochondrial DNA in tench *Tinca tinca*. *Journal of Fish Biology*, *76*(2), 401–407. https://doi.org/10.1111/j.1095-8649.2009.02495.x

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. *Italian Journal of Animal Science*, *11*(1), 103–108. https://doi.org/10.4081/ijas.2012.e19

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.). *Journal of Applied Ichthyology*, *30*(S1), 15–21. https://doi.org/10.1111/jai.12423

Lujić, J., Kohlmann, K., Kersten, P., Marinović, Z., Ćirković, M., & Simić, V. (2017). Phylogeographic identification of Tench *Tinca tinca* (L., 1758) (Actinopterygii: Cyprinidae) from the Northern Balkans and adjacent regions and its implications for conservation. *Zoological Studies*, *56*(February). https://doi.org/10.6620/ZS.2017.56-03

Saitoh, K., Sado, T., Mayden, R. L., 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. *Journal of Molecular Evolution*, *63*(6), 826–841. https://doi.org/10.1007/s00239-005-0293-y

Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599.

Van Houdt, J. K. J., De Cleyn, L., Perretti, A., & Volckaert, F. A. M. (2005). A mitogenic view on the evolutionary history of the Holarctic freshwater gadoid, burbot (*Lota lota*). *Molecular Ecology*, *14*(8), 2445–2457. https://doi.org/10.1111/j.1365-294X.2005.02590.x

Wang, J., W. Min, M. Guan, and S. Hu. 2004. Tench farming in China: present status and future prospects. in S. Sakowicz, ed. Ivth International Workshop on Biology and Culture of the Tench, *Tinca tinca* (L.), p. 32. Wierzba, September 20-23 2004. Programme and Abstracts, Inland Fisheries Institute in Olsztyn, Olsztyn.