PAPER

Genetic variability in tench (*Tinca tinca* L.) as revealed by PCR-RFLP analysis of mitochondrial DNA

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Abstract

Four mitochondrial DNA segments, ND1, ND6, cyt *b* and D-loop, were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in 14 tench (*Tinca tinca* L.) populations located in Europe and Asia; data on 5 Italian populations previously analyzed for the same mtDNA segments were also included in the study. All the considered segments were polymorphic and originated a total of 9 composite haplotypes which were clustered into 2 haplogroups, A and B, possibly corresponding to the Western and Eastern phylogroups previously described in tench. Nine out of 19 populations showed polymorphism, with haplotype diversity ranging from 0.246 to 0.643 and nucleotide diversity from 0.009 to 0.078. Seventy-five percent of the pairwise comparisons were significant, indicating a high between-population variability. The Neighbour-Joining tree revealed the presence of 3 clusters, including *pure* populations, with only a A or B haplogroup, and *mixed* populations, with both haplogroups. The possibility of identifying populations with different haplotypes has practical implications for both conservation and supportive stocking.

Introduction

For the last twenty years, the genetic research in aquaculture has been exponentially increasing, but genetic information on tench (*Tinca tinca* L.) is still limited compared to other fish species. In fact, apart from some studies carried out over the past decades by means of protein markers (Valenta *et al.*, 1978; Šlechtova *et al.*, 1995; Kohlmann and Kersten, 1998), only recently have tench specific microsatellite loci been described (Kohlmann and Kersten, 2006) and used to characterize many European and Asian populations (Kohlmann *et al.*, 2007, 2010; Lo Presti *et al.*, 2010b). Also, the complete mitochondrial DNA (mtDNA) sequence has only recently been made available (Saitoh *et al.*, 2006). The subsequent analysis of the mitochondrial cyt *b* gene, together with 3 nuclear genes, has helped clarify the molecular phylogeography of the tench with the discovery of 2 geographical clades (Eastern and Western), possibly developed in response to recurrent isolation in glacial refugia during the Pleistocene (Lajbner *et al.*, 2007). Within the Eastern phylogroup, the analysis of cyt *b* also allowed the identification of populations distinct from the major Eastern clade in the Anzalee lagoon of the Caspian Sea in Iran and in the Iskar River of the Danube River drainage in Bulgaria (Lajbner *et al.*, 2011). Only one study was devoted to analyzing the polymorphism of different mitochondrial segments as a tool to detect tench genetic variability (Lo Presti *et al.*, 2010a).

The aim of this paper is to extend the study of the mtDNA polymorphisms to a larger number of tench populations distributed in a wide geographical area, in order to obtain a more comprehensive picture of the within-population and between-population variability in tench.

Materials and methods

A total of 126 individuals were analyzed. These belonged to 14 wild and cultured populations located in different European and Asian countries. Data on the 5 Italian populations previously studied for the same mtDNA segments (Lo Presti *et al.*, 2010a) were also included in order to cover a larger geographical area (Table 1). All the populations had been already analyzed by microsatellite markers (Kohlmann *et al.*, 2010; Lo Presti *et al.*, 2010b).

Total genomic DNA was extracted from muscle or fin using the NucleoSpin Tissue kit (Macheray-Nagel, Düren, Germany). PCR reactions to amplify ND1, ND6, cyt *b* and D-loop segments were performed as described by Lo Presti *et al.* (2010a). Each amplicon was digested with 4 restriction enzymes which were selected on the basis of the previous results (Lo Presti *et al.*, 2010a) and in consideration of the expected restriction pattern derived from virtual digestion of the reference

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sequence with Webcutter 2.0 software (Heiman, 1997). Some of the enzymes were used to digest different amplicons (Table 2) so that a total of 7 endonucleases were used: *Alu*I, *Sau3A*I (Sigma, St Louis, MO, USA), *Ase*I, *Hae*III, *Msp*I (New England BioLabs, Beverly, MA, USA) *Hind*III, *Hinf*I (Fermentas, Burlington, ON, Canada). The digested fragments were resolved on 2% agarose gels, stained with ethidium bromide and visualized under UV light. The size of the fragments was estimated by comparison with a 100 bp size ladder (Sigma, St Louis, MO, USA) and each different pattern produced by each enzyme was identified by a single letter code, with A assigned to the pattern expected on the basis of the reference sequence. Composite haplotypes were designed by a 16-letter code representing the pattern for each restriction enzyme.

The relationships between composite haplotypes were analyzed by calculating the mean number of substitutions per site between all pairs of haplotypes from restriction site data (Nei and Li, 1979) which were used to construct a Neighbour-Joining tree as implemented in the PHYLIP version 3.5 package (Felsenstein, 1993); the reliability of the tree topology was tested by 1,000 bootstrap replicates.

The ARLEQUIN version 3.1 program (Excoffier *et al.*, 2005) was used to evaluate the variability within populations by haplotype and nucleotide diversity (Nei and Tajima, 1981), as well as to test the population differentiation by the pairwise exact test (Raymond and Rousset, 1995). Significance levels for

multiple comparisons were adjusted using the sequential Bonferroni correction (Rice, 1989). The genetic distances between populations were also estimated as the pairwise net nucleotide divergence (Nei and Li, 1979), followed by the construction of the Neighbour-Joining tree, using the MEGA 4 software (Tamura *et al.*, 2007).

Results

All the enzymes but *Msp*I detected restriction fragment length polymorphisms at some mtDNA segment (Table 2). The digestion of ND6 and ND1 revealed one and two variants, respectively, as previously reported (Lo Presti *et al.*, 2010a), while additional variation was observed for cyt *b* and D-loop. At cyt *b* the *Sau3A*I endonuclease detected a new variant the pattern of which does not seem to derive directly from the loss or gain of a restriction site with respect to the reference pattern. A more complex situation could, therefore, be hypothesized, such as concomitant loss and gain of restriction sites. For the D-loop, two new variants were found, one with *Ase*I and one with *Hae*III, respectively, due to the presence and absence of a restriction site. The polymorphisms at the 4 mtDNA segments gave rise to a total of 9 composite haplotypes, named H1 to H9, with H1 corresponding to the reference sequence (Table 3). An analysis of the overall frequencies indicated that H1 and H2 were the most frequent composite haplotypes with a cumulative frequency of 0.805, while the others were very rare, with frequencies lower than 0.05.

Table 1. Description of the populations.

Population	Code	N	Status	Geographical location
Alcantara°	ALC	5	Wild	River Alcantara, Italy
Badajoz	BAD	10	Cultured	Badajoz, Spain
Bolsena°	BOL	21	Wild	Lake Bolsena, Italy
Bracciano	BRA	4	Wild	Lake Bracciano, Italy
China	CHI	10	Cultured	Wuhan, China
Döllnsee	DÖL	10	Wild	Lake Döllnsee, Germany
Felchowsee	FEL	8	Wild	Lake Felchowsee, Germany
Golden	GOL	10	Cultured	Colour variety developed in Vodňany, the Czech Republic
Hungary	HUN	10	Cultured	Hungary, collected at Vodňany live gene bank
Iseo	ISE	3	Wild	Lake Iseo, Italy
Königswartha	KÖW	10	Cultured	Königswartha, Germany
Marianske Lazne	MAL	10	Cultured	Czech Republic, year class 1998
Pianalto°	PIA	57	Cultured	Poirino highland, Italy
Romania	ROM	10	Cultured	Romania, collected at Vodňany live gene bank
Trasimeno°	TRA	9	Wild	Lake Trasimeno, Italy
Turkey	TUR	11	Wild	Lake Sapanca, Turkey
Valagola°	VAL	13	Wild	Lake Valagola, Italy
Velke Mezirici	VEM	10	Cultured	The Czech Republic
Vodňany 1998	VOD	10	Cultured	The Czech Republic, year class 1998

°From Lo Presti *et al.*, 2010a.

Table 2. Approximate fragment size of the restriction morphs observed by digesting 4 mtDNA segments with 7 different endonucleases.

mtDNA segment	ND1						ND6				
Endonuclease	*Alu*I		*Hae*III	*Hinf*I		*Msp*I	*Hind*III		*Hinf*I	*Msp*I	*Sau3A*I
Restriction morph	A	B	A	A	B	A	A	B	A	A	A
Fragment size, bp			964								
	570	570			383	496		576			
	317			328	328	346				316	361
				308	308		310		287		
		185		226			266		275	260	215
		132		157		177					
	74	74									
	58	58	55								
									14		

mtDNA segment	Cyt *b*								D-loop						
Endonuclease	*Alu*I			*Hae*III		*Hinf*I	*Sau3A*I		*Alu*I		*Ase*I		*Hae*III		*Hinf*I
Restriction morph	A	B	C	A	B	A	A	B	A	B	A	B	A	B	A
Fragment size (bp)	985		1146	1146	1059										
		707				495			634	634	758	659	596	596	568
						480	305	320						402	
		278					297	297							375
							224	234	240				270		
	161	161					213	225		~220	119	119			
						129						99	132		
					87			70	90	90	86	86			
						42			34	34	35	35			55
										~20					

The analysis of the pairwise nucleotide divergence between composite haplotypes led to a phylogenetic tree where 2 highly divergent haplogroups were identified: one, designated as haplogroup A, included the H1, H3, H4, H5 and H6 composite haplotypes, while the other, designated as haplogroup B, included the H2, H7, H8 and H9 composite haplotypes (Figure 1). The 2 haplogroups differed for polymorphisms at the ND1 and Cyt *b* segments: all the haplotypes belonging to the haplogroup A had the restriction morphs ND1/*Alu*I A, ND1/*Hinf*I A and Cyt *b*/*Sau3AI* A, while all the haplotypes of the haplogroup B had the restriction morphs B at the same sites (Table 3). The bootstrap value of 96% strongly supported the between-haplogroup differentiation, while the within-haplogroup relationships were less clear, with low to medium bootstrap values.

As for the composite haplotype distribution, H1 and H2 were present in 63 and 47% of the populations, respectively, whereas the others were limited to one or few populations (Table 4). H3 was observed only in the Central and Southern Italian populations (BOL, TRA, ALC); H4 and H6 were the rarest composite haplotypes, found in one individual only from Trasimeno (Italy) and Felchowsee (Germany) lakes, respectively. H5, H7 and H8 were private haplotypes for the wild VAL and TUR, and cultured MAL populations, respectively. Moreover, H8 seemed to be fixed in the latter population, so it might be used as a genetic tag if the data were to be confirmed on a larger sample (the present sample size is 10 individuals only). H9 was fixed in the GOL population (the golden color variety), but present also in one wild FEL individual.

Ten out of 19 populations showed no variability (Table 4). For ISE and BRA, the finding is possibly dependent on the low sample size (3 and 4 individuals, respectively), and, therefore, these 2 Italian wild populations were excluded from the subsequent analysis. On the contrary, the fixation of the haplotype H9 in GOL can be interpreted as a result of the founder effect (Kvasnika *et al.*, 1993). It is worth underlining the fact that the other monomorphic populations are cultured, except for the German DÖL which is wild. The absence of polymorphism in DÖL and KÖW is quite unexpected considering that these populations when analyzed by microsatellite markers showed a high variability (Kohlmann *et al.*, 2010).

In the polymorphic populations, the haplotype diversity ranged from 0.246 (PIA) to 0.643 (FEL), whereas the nucleotide diversity ranged from 0.009 (BOL) to 0.078 (FEL) (Table 4). As expected, the highest values for nucleotide diversity were observed in the populations where composite haplotypes of both evolutionary lineages were present. In particular, FEL showed the highest values for both indices, confirming its importance as a reservoir of genetic diversity, in agreement with the high variability detected by previous studies on microsatellite markers (Kohlmann *et al.*, 2010).

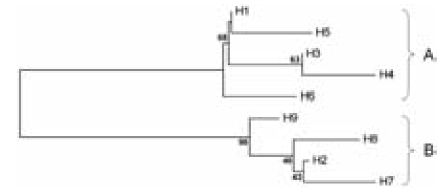

Figure 1. Neighbour-Joining tree of the composite haplotypes. Only bootstrap values higher than 40% are shown.

Table 3. Composite haplotypes and their overall frequency (P).

Haplotype	ND1	ND6	cyt *b*	D-loop	P
H1	AAAA	AAAA	AAAA	AAAA	0.498
H2	BABA	BAAA	BAAB	AAAA	0.307
H3	AAAA	AAAA	AAAA	BAAA	0.039
H4	AAAA	AAAA	ABAA	BAAA	0.004
H5	AAAA	AAAA	CAAA	AAAA	0.039
H6	AAAA	AAAA	AAAA	AABA	0.004
H7	BABA	BAAA	BAAB	AABA	0.017
H8	BABA	BAAA	BAAB	ABAA	0.043
H9	BABA	AAAA	BAAB	ABAA	0.048

Table 4. Within-population variability: haplotype frequency, haplotype (H) and nucleotide (π) diversity (mean value ± standard error).

POP	H1	H2	H3	H4	H5	H6	H7	H8	H9	H ± SE	π ± SE
BAD	-	1.000	-	-	-	-	-	-	-	-	-
CHI	-	1.000	-	-	-	-	-	-	-	-	-
TUR	-	0.636	-	-	-	-	0.364	-	-	0.509±0.101	0.014±0.014
FEL	0.625	0.125	-	-	-	0.125	-	-	0.125	0.643±0.184	0.078±0.052
KÖW	1.000	-	-	-	-	-	-	-	-	-	-
DÖL	1.000	-	-	-	-	-	-	-	-	-	-
ROM	0.200	0.800	-	-	-	-	-	-	-	0.356±0.159	0.058±0.040
HUN	-	1.000	-	-	-	-	-	-	-	-	-
GOL	-	-	-	-	-	-	-	-	1.000	-	-
MAL	-	-	-	-	-	-	-	1.000	-	-	-
VEM	0.300	0.700	-	-	-	-	-	-	-	0.467±0.132	0.076±0.049
VOD	-	1.000	-	-	-	-	-	-	-	-	-
ALC	0.400	-	0.600	-	-	-	-	-	-	0.600±0.175	0.014±0.015
BRA	1.000	-	-	-	-	-	-	-	-	-	-
BOL	0.762	-	0.238	-	-	-	-	-	-	0.381± 0.101	0.009±0.010
ISE	1.000	-	-	-	-	-	-	-	-	-	-
PIA	0.860	0.140	-	-	-	-	-	-	-	0.246±0.067	0.028±0.020
TRA	0.778	-	0.111	0.111	-	-	-	-	-	0.417±0.191	0.014±0.014
VAL	0.308	-	-	-	0.692	-	-	-	-	0.462±0.110	0.010±0.011

Table 5. Nucleotide divergence within population (diagonal) and between populations (above the diagonal); significance (*) of the exact test of Raymond and Rousset (1995) for population differentiation (below the diagonal).

	BAD	CHI	TUR	FEL	KOW	DOE	HUN	ROM	GOL	MAL	VEM	VOD	PIA	VAL	BOL	TRA	ALC
BAD	*0.00*	0.00	0.11	3.43	6.00	6.00	0.00	0.13	2.00	1.00	0.40	0.00	4.42	6.46	6.05	6.03	6.30
CHI	.	*0.00*	0.11	3.43	6.00	6.00	0.00	0.13	2.00	1.00	0.40	0.00	4.42	6.46	6.05	6.03	6.30
TUR	.	.	*0.51*	3.45	6.11	6.11	0.11	0.24	2.11	1.11	0.51	0.11	4.53	6.57	6.16	6.14	6.41
FEL	*	*	*	*2.89*	0.18	0.18	3.43	1.71	3.43	4.18	1.05	3.43	0.10	0.64	0.23	0.21	0.48
KÖW	*	*	*	.	*0.00*	0.00	6.00	3.73	6.00	7.00	2.80	6.00	0.11	0.46	0.05	0.03	0.30
DÖL	*	*	*	.	.	*0.00*	6.00	3.73	6.00	7.00	2.80	6.00	0.11	0.46	0.05	0.03	0.30
HUN	.	.	.	*	*	*	*0.00*	0.13	2.00	1.00	0.40	0.00	4.42	6.46	6.05	6.03	6.30
ROM	.	.	.	*	*	*	.	*2.13*	1.73	1.13	-0.19	0.13	2.49	4.19	3.78	3.76	4.03
GOL	*	*	*	*	*	*	*	*	*0.00*	1.00	1.80	2.00	4.70	6.46	6.05	6.03	6.30
MAL	*	*	*	*	*	*	*	*	*	*0.00*	1.40	1.00	5.42	7.46	7.05	7.03	7.30
VEM	.	.	*	*	*	*	.	.	*	*	*2.80*	0.40	1.73	3.26	2.85	2.83	3.10
VOD	.	.	.	*	*	*	.	.	*	*	.	*0.00*	4.42	6.46	6.05	6.03	6.30
PIA	*	*	*	*	.	.	*	*	*	*	*	*	*1.47*	0.57	0.15	0.13	0.41
VAL	*	*	*	*	*	*	*	*	*	*	*	*	*	*0.46*	0.51	0.49	0.76
BOL	*	*	*	*	.	.	*	*	*	*	*	*	*	*	*0.38*	-0.03	0.06
TRA	*	*	*	.	.	.	*	*	*	*	*	*	*	*	.	*0.61*	0.06
ALC	*	*	*	.	*	*	*	*	*	*	*	*	*	*	.	.	*0.60*

Concerning the between-population differentiation, 75% of the pairwise comparisons were significant, indicating a high level of genetic variation at species level (Table 5). Going into more detail, GOL, MAL and VAL differed statistically from all the other populations, while most of the non-significant comparisons involved the Eastern populations (CHI, TUR, HUN, ROM, VEM and VOD) and the one from Spain (BAD). No differences were observed between the German populations (FEL, KÖW, DÖL) or between those of Central-Southern Italy (TRA, BOL, ALC).

The Neighbour-Joining tree, constructed on the basis of the pairwise net nucleotide divergence, separated 2 clusters, one including the Italian and German populations and one including all the others (Figure 2). The latter displayed lack of resolution, involving populations all fixed for the H2 composite haplotype (BAD, CHI, HUN and VOD). These populations had a nucleotide divergence of 0.248, while the Italian and German populations represented a more heterogeneous group with D_A of 0.711. The divergence between the two branches was much higher (D_A = 4.652) indicating a deep separation between the populations of the 2 groups. It is interesting to note that the populations with both haplogroups (FEL and PIA at one side, VEM and ROM at the other side) were located close to the principal node. Therefore, the tree can be subdivided into 3 clusters, corresponding to *pure* populations, with A or B haplogroup, and *mixed* populations, with both haplogroups.

Discussion

The PCR-RFLP analysis of ND1, ND6, cyt *b* and D-loop in 19 tench populations confirmed the effectiveness of these mtDNA markers for population genetic studies of this species. Of the 4 examined mtDNA segments, cyt b and D-loop showed the highest variability, with 4 and 3 variants, respectively. The quite high variability of the D-loop seems to be a peculiarity of the tench, which so far has not been observed in other teleosts; for example, no polymorphism was found in Danish brown trout (*Salmo trutta* L.) strains by digestion with 18 restriction enzymes (Hansen and Loeschke, 1996), nor in rainbow trout (*Onchorhyncuhus mykiss*) using 12 endonucleases (Sajedi *et al.*, 2003). These findings underline the fact that mtDNA segments which are more appropriate for population studies have to be chosen for each species individually. Nine out of 19 populations examined showed considerable haplotype as well as nucleotide diversity. However, the mtDNA markers generally revealed a lower power than microsatellite markers in detecting the within-population variability. In fact, the average haplotype diversity level of mtDNA (H_{mt}) observed in the present study (0.215) was lower than the average heterozygosity level of microsatellites (H_{ms}) deduced from the data of Kohlmann *et al.* (2010) and Lo Presti *et al.* (2010b) on the same populations (0.343). However, in some populations in which mtDNA was polymorphic, H_{mt} was even higher than H_{ms} (TUR, FEL, ALC, TRA, BOL). The absence of relationships between nuclear and mitochondrial variability,

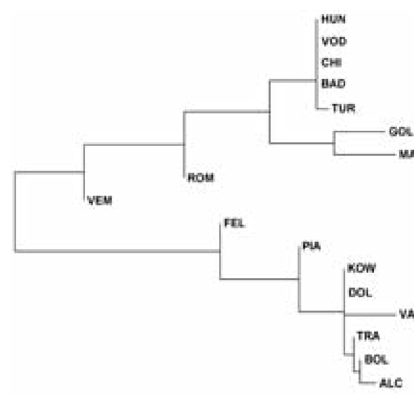

Figure 2. Neighbour-Joining tree of 19 tench populations.

already reported for other species (Palumbi and Baker, 1994), is not surprising given the different genetic background and mode of evolution of the 2 types of markers. Therefore, for the different information they provide, the complementary analysis of nuclear and mitochondrial markers represent a powerful strategy to clarify the population genetic structure. On the other hand, mtDNA markers were shown to be an excellent tool for revealing the between-population variability. The identification of 2 mtDNA haplogroups in the present study, along with the recent discovery of 2 major growth hormone gene classes in tench (Kocour and Kohlmann, 2011) further support the results of Lajbner *et al.* (2007, 2011) who investigated the molecular phylogeography of tench by the analysis of the cyt *b* locus and some nuclear markers. They showed that the

species is subdivided into deeply divergent Western and Eastern phylogroups which are not, however, distinct species (Lajbner *et al.*, 2010). Also in other freshwater species, including the chub (*Leuciscus cephalus*) (Durand *et al.*, 1999), perch (*Perca fluviatilis*) (Nesbø *et al.*, 1999) and barbel (*Barbus barbus*) (Kotlik and Berrebi, 2001), genetic lines related to the geographical location were observed which could indicate an evolutionary history common to different freshwater species.

On the basis of the haplogroup composition, composite haplotype distribution and population location, it can be inferred that the haplogroup A observed in the present study corresponds to the Western phylogroup reported by Lajbner *et al.* (2007, 2011) while the haplogroup B corresponds to the Eastern phylogroup.

It is worthy of note, for practical implications, that the mtDNA markers used in the present study allowed a good resolution for both phylogroups, compared to the markers used by Lajbner *et al.* (2011) and Lajbner and Kotlik (2011), that detected 3 subclades in the Eastern clade but showed only very little internal structure for the Western clade.

The Neighbour-Joining tree constructed on the basis of the pairwise net nucleotide divergence (Nei and Li, 1979) between populations showed some similarities with the tree obtained by Kohlmann *et al.* (2010) using the microsatellite markers: in both cases HUN, CHI, BAD and TUR clustered together and the German populations were located in the opposite branch. In particular, the results confirmed the genetic similarity between the Spanish and Chinese populations, which could be explained by human introduction of tench from the East to Spain.

Conclusions

The present PCR-RFLP based analysis of 4 segments of the tench mtDNA revealed considerable haplotype as well as nucleotide diversity in 9 out of 19 populations examined. Thus, these polymorphisms, which are easy and inexpensive to screen, might effectively be used for population genetic studies of this species, with implications for conservation and supportive stocking. For conservation purposes, these mtDNA markers might help to identify populations with different haplotypes within haplogroups and could thus help protect their genetic integrity. In case of stocking, donor and recipient populations should be as genetically similar as possible, i.e. they should at least belong to the same composite haplotype. On the other hand, mixed populations with higher mtDNA diversity (and also higher microsatellite variability) might be valuable baseline populations to start selective breeding programs, particularly if mtDNA and microsatellite information were to be combined with the recently described tench growth hormone gene polymorphisms.

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