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PCR–RFLP analysis of mitochondrial DNA in tench *Tinca tinca*

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Polymorphism was detected at ND1, ND6, D-loop and cyt *b* segments of mtDNA in 105 tench (*Tinca tinca* L.), using the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique and five composite haplotypes were identified. The diversity indices and the results of the population comparisons revealed that the identified markers provide a powerful tool for further studies on this species.

Key words: genetic variability; mtDNA; *Tinca tinca*.

Despite the increasing interest in fish genetics, few data exist on tench *Tinca tinca* (L.) Until 1990s, its genetic characterization has been mainly carried out by means of protein markers and the variability detected both within and between populations was rather low (Valenta *et al*., 1978; Šlechtová *et al*., 1995; Kohlmann & Kersten, 1998). Only recently, microsatellite markers have been described in *T. tinca*, showing a much higher level of genetic variation (Kohlmann *et al*., 2007). Also mitochondrial DNA (mtDNA) has been widely used for genetic studies in aquaculture. The polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique applied to mtDNA proved to be very effective in detecting variability within and between populations in a great number of fish species, including brown trout *Salmo trutta* L. (Hansen & Loeschcke, 1996), rainbow trout *Onchorhynchus mykiss* (Walbaum) (Sajedi *et al*., 2003), Atlantic herring *Clupea harengus* L. (Hauser *et al*., 2001), common carp *Cyprinus carpio* L. (Gross *et al*., 2002) and skipjack tuna *Katsuwonus pelamis* (L.) (Menezes *et al*., 2006) but, as far as is known, no information was available on mtDNA polymorphism in *T. tinca* hitherto. This study was aimed at investigating the variability of mtDNA in *T. tinca*, applying the PCR–RFLP technique to the analysis of ND1, ND6, cyt *b* and D-loop segments.

A total of 105 dorsal fin samples were collected from *T. tinca* living in different areas of Italy (Fig. 1), in order to include as much variability as possible. Fifty-seven individuals were taken from Pianalto Ponds (PI), where aquaculture was practised

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FIG. 1. Geographical location of the sampled populations with in Italy. PI, Pianalto; VA, Valagola Lake; TR, Trasimeno Lake; BO, Bolsena Lake; AL, Alcantara River.

since 17th century; all the others belong to wild populations, meaning that they live and reproduce in natural conditions, regardless of their native or captive origin: 13 *T. tinca* from Valagola Lake (VA), 21 from Bolsena Lake (BO), nine from Trasimeno Lake (TR) and five from Alcantara River (AL).

Total genomic DNA was extracted from frozen dorsal fin using the NucleoSpin Tissue kit (Macherey-Nagel; www.mn-net.com). PCRs to amplify ND1, ND6, cyt *b* and D-loop segments were performed using the following primers, designed on the basis of the reference sequence for *T. tinca* complete mitochondrial genome (GenBank accession no. NC_008648): ND1: forward, 5′-CCCAGTTCATGCTAAACACTT-3′; reverse, 5′-AAAGTGGTCCCTAGGCATT-3′; ND6: forward, 5′-CCATAACCCTGGCGATTCTAT-3′; reverse, 5′-CGGTTAAAGTCCGAGCAAGA-3′; cyt *b*: forward, 5′-AACAATAATGGCAAGCCTACGA-3′; reverse, 5′-GCTCATTTCAATGCTTTATTTTCC-3′; D-loop: forward, 5′-CGCCCAGAAAAAGGAGATT-3′; reverse, 5′-TTGGACTTTTTAGCATTAAGAAATTG-3′.

Each PCR consisted of 1× PCR buffer, 1·5 mM $MgCl_2$, 200 μM dNTPs, 0·2 μM of each primer, 1 unit of RedTaq DNA polymerase (Sigma; www.sigmaaldrich.com), *c*. 100 ng of genomic DNA and sterile water up to a final volume of 25 μl. The amplification was carried out under the following conditions: an initial denaturation step at 97° C for 5 min, followed by 30 cycles of denaturation at 94° C for 30 s, annealing for 30 s at 56° C for ND1, 60° C for ND6 and cyt *b* and 59° C for D-loop, extension

at 72° C for 1 min and a final extension at 72° C for 5 min. All the amplicons were digested with five enzymes: *Alu*I (Sigma), *Hinf*I (Fermentas; www.fermentas.com), *Ase*I, *Hae*III and *Msp*I (New England BioLabs; www.neb.com); in the absence of any previous information, these endonucleases were selected on the basis of their easily recognizable expected restriction pattern, derived by virtually digesting the reference sequence with Webcutter 2·0 (Heiman, 1997). Three microlitres of PCR product were digested with 5 (*Ase*I, *Hae*III, *Hinf*I), 6 (*Msp*I) or 8 (*Alu*I) units of enzyme according to the manufacturer's instructions and the digested fragments were resolved on 2% agarose gel, stained with ethidium bromide and visualized under UV light. The size of the fragments was estimated in comparison to a 100 base pair (bp) size ladder (Sigma) 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 (accession no. NC_008648). Composite haplotypes were designed by a 20 letter code representing the pattern for each restriction enzyme.

The relationships among haplotypes were analysed by calculating the mean number of substitutions per site between all pairs of haplotypes, from restriction site data, using the maximum likelihood method of Nei & Tajima (1983), suitable when enzymes with different numbers of recognition nucleotides are used. Arlequin ver. 3.1 program (Excoffier *et al*., 2005) was used to evaluate the variability within populations by haplotype and nucleotide diversity (Nei & Tajima, 1981), as well as to test the population differentiation by the pair-wise exact test (Raymond & Rousset, 1995). Significance levels for multiple comparisons were adjusted using the sequential Bonferroni correction (Rice, 1989).

The proposed PCR methods proved to be very effective in amplifying products of the expected size: a 1019 bp fragment for ND1, a 576 bp fragment for ND6, a 1146 bp fragment for cyt *b* and a 998 bp fragment for D-loop. On the whole, the amplified fragments represented *c*. 25% of *T. tinca* mitochondrial genome.

Of the five enzymes used, only *Alu*I, *Hae*III and *Hinf*I detected variability. Polymorphism was found at all the amplicons (Table I). As for ND1, two variants were observed, derived from the digestion with *Alu*I and *Hinf*I, and respectively due to the presence and the absence of a restriction site compared with the A pattern. One variant was detected for ND6 with *Alu*I, due to the absence of a restriction site. Three polymorphisms were observed for cyt *b*: two were revealed by *Alu*I and were due to the presence (B) and the absence (C) of a restriction site, whereas the other was detected by *Hae*III and was due to the presence of an additional restriction site. The alignment of the sequences available for cyt *b* (GenBank accession nos. DQ841176, Y10451, AJ555552, AJ388426) with the reference sequence revealed a base substitution A > G in three of them (nt 861 in Y10451 and DQ841176, nt 839 in AJ555552), which could introduce a restriction site for *Alu*I, originating a pattern consistent with the B one observed in the present investigation. Only one variant was found in D-loop region, for the presence of a fourth restriction site after digestion with *Alu*I.

The polymorphism at the four segments originated a total of five composite haplotypes, named H1 to H5, with H1 corresponding to the reference sequence (Table II). This haplotype, even though with a very different distribution (0·31–0·86), was observed in all the studied populations (overall frequency of 0·74) and, therefore, it could represent the ancestral type. The H3 haplotype resulted the second most spread haplotype and was shared by the central and southern Italian populations, suggesting either the occurrence of gene flow between them or a recent time of divergence. The

TABLE I. Approximate fragment size of the restriction morphs observed by digesting tench *Tinca tinca* mtDNA segments with five endonucleases

mtDNA segment	ND1							ND6					
Endonuclease	*Alu*I		*Ase*I	*Hae*III	*Hinf*I		*Msp*I	*Alu*I		*Ase*I	*Hae*III	*Hinf*I	*Msp*I
Restriction morph	A	B	A	A	A	B	A	A	B	A	A	A	A
Fragment size (bp)			1019	964									
	570	570				383	496			576	576		
	317				328	328	346					287	316
					308	308			266			275	260
		185			226			239					
		132			157		177	183	183				
	74	74						73	73				
	58	58		55				54	54				
								27				14	

mtDNA segment	cyt *b*								D-loop					
Endonuclease	*Alu*I			*Ase*I	*Hae*III		*Hinf*I	*Msp*I	*Alu*I		*Ase*I	*Hae*III	*Hinf*I	*Msp*I
Restriction morph	A	B	C	A	A	B	A	A	A	B	A	A	A	A
Fragment size (bp)	985		1146	1146	1146	1059		1146						998
		707					495		634	634	758	596	568	
		278					480					270	375	
	161	161					129		240	~220	119	132		
						87			90	90	86			
							42		34	34	35		55	
										~20				

TABLE II. Frequencies of composite haplotypes, haplotype and nucleotide diversity of the sampled *Tinca tinca* populations. Composite haplotypes are denoted by capital letters in the following order: ND1, ND6, cyt *b*, D-loop, each digested with *Alu*I, *Ase*I, *Hae*III, *Hinf* I, *Msp*I. The sample sizes are in brackets

Haplotype	Composite	PI (57)	VA (13)	TR (9)	BO (21)	AL (5)
H1	AAAAA AAAAA AAAAA AAAAA	0·86	0·31	0·78	0·76	0·40
H2	BAABA BAAAA BAAAA AAAAA	0·14	0	0	0	0
H3	AAAAA AAAAA AAAAA BAAAA	0	0	0·11	0·24	0·60
H4	AAAAA AAAAA AABAA BAAAA	0	0	0·11	0	0
H5	AAAAA AAAAA CAAAA AAAAA	0	0·69	0	0	0
	Haplotype diversity	0·246 ± 0·067	0·462 ± 0·110	0·417 ± 0·191	0·381 ± 0·101	0·600 ± 0·175
	Nucleotide diversity	0·025 ± 0·019	0·011 ± 0·012	0·015 ± 0·015	0·009 ± 0·010	0·015 ± 0·016

PI, Pianalto; VA, Valagola Lake; TR, Trasimeno Lake; BO, Bolsena Lake; AL, Alcantara River.

TABLE III. Pair-wise test for homogeneity of haplotype frequencies between *Tinca tinca* populations

	PI	VA	TR	BO	AL
PI	—				
VA	***	—			
TR	ns	*	—		
BO	**	***	ns	—	
AL	**	*	ns	ns	—

PI, Pianalto; VA, Valagola Lake; TR, Trasimeno Lake; BO, Bolsena Lake; AL, Alcantara River.
***, $P < 0{\cdot}001$; **, $P < 0{\cdot}01$; *, $P < 0{\cdot}05$; ns, not significant.

other three were private haplotypes: H2 occurred only in the PI population, mainly in individuals collected from one historical pond; H4 was the rarest haplotype, as it was found only in one individual from TR population, whereas H5 was the predominant one in VA population.

The pair-wise nucleotide divergence between haplotypes varied from 0·0038 to 0·0244; the highest values were obtained for the comparisons involving the H2 haplotype, which differed from the others for four to six restriction sites.

The within-population variability resulted quite high (Table II): the haplotype diversity ranged from 0·246 to 0·600, with the highest values observed for the wild populations. It is noteworthy that the variability detected is comparable with that revealed in *T. tinca* by microsatellites, which usually are more powerful markers (Kohlmann *et al*., 2007). On the other hand, the highest diversity at nucleotide level was found in PI population, for the presence of the most divergent haplotype (H2), while quite similar values were observed in the other populations.

The mtDNA polymorphism was very helpful in the analysis of the population differentiation as well. Significant *P* values were obtained for most of the comparisons including VA and PI, consistently with the presence of private haplotypes, whereas BO, TR and AL had a high degree of genetic similarity (Table III).

These first data on the within and between-population variability revealed that mtDNA markers provide a quite high level of discriminatory power, even at a small geographic scale, therefore being effective for population studies in *T. tinca* too, as already demonstrated for other fish species.

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