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MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

PCR-RFLP assays to distinguish the Western and Eastern phylogroups in wild and cultured tench *Tinca tinca*

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Abstract

The tench *Tinca tinca* is a valued table fish native to Europe and Asia, but which is now widely distributed in many temperate freshwater regions of the world as the result of human-mediated translocations. Fish are currently being transplanted between watersheds without concern for genetic similarity to wild populations or local adaptation, and efficient phylogeographic markers are therefore urgently needed to rapidly distinguish genetically distinct geographical populations and to assess their contribution to the hatchery breeds and to the stocked wild populations. Here, we present a new method to distinguish recently discovered and morphologically undistinguishable Western and Eastern phylogroups of the tench. The method relies on PCR-RFLP assays of two independent nuclear-encoded exon-primed intron-crossing (EPIC) markers and of one mitochondrial DNA (mDNA) marker and allows the rapid identification of the Western and Eastern phylogroup and also of three geographical mtDNA clades within the Eastern phylogroup. Our method will enable researchers and fishery practitioners to rapidly distinguish genetically divergent geographical populations of the tench and will be useful for monitoring the introduction and human-mediated spread of the phylogroups in wild populations, for characterization of cultured strains and in breeding experiments.

Keywords: EPIC, exon-primed intron-crossing marker, mtDNA, stocking

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The tench *Tinca tinca* is a valued table fish, native to Europe and Asia between the British Isles and central Siberia (Brylińska *et al.* 1999), but it is now widely distributed in many temperate freshwater regions of the world as the result of human-mediated translocations (Welcomme 1988). Up until recently, there has been only limited information about population structure of the tench throughout its vast native range (Kohlmann *et al.* 2010), resulting in the absence of efficient phylogeographic markers to monitor the genetic and geographical origin of hatchery stocks, such that fish are being transplanted from one watershed to another without concern for genetic similarity to neighbouring wild populations or adaptation to local environment. Therefore, for successful fishery management and sustainable exploitation of tench, it is extremely important to develop easy markers that would enable researchers and fishery practitioners to rapidly distinguish genetically distinct geographical populations and to assess their contribution to the hatchery breeds and to the stocked wild populations.

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Recently, phylogeographic analysis of DNA sequences of several exon-primed intron-crossing (EPIC) markers and of a mitochondrial DNA (mtDNA) gene discovered within the Eurasian range of the tench two geographical clades, a Western phylogroup (clade W) found in Europe from the British Isles to Poland and an Eastern phylogroup (clade E) distributed from Europe throughout Asia to China (Z. Lajbner, O. Linhart and P. Kotlík, unpublished manuscript). Within the Eastern phylogroup, populations with mtDNA (but not nuclear markers) distinct from the major Eastern clade (EA) were found in a southern tributary of the Danube River in Bulgaria (clade EI) and in the southern part of the Caspian Sea basin in Iran (clade EC). Separation into the phylogroups most reasonably reflects long-term evolutionary isolation of populations in different parts of the native range, but we have shown that tench of both phylogroups can freely interbreed with one another when in contact in wild populations (Lajbner *et al.* 2010). The presence of both phylogroups in cultured breeds of different geographical origins and the occurrence of EA haplotypes at the western edge of the native range (e.g. in Spain and Britain) showed that extensive admixture

has occurred both in wild populations and in hatcheries, largely as a consequence of fishery practices that ignore the genetic structure among and within populations.

To facilitate the assessment of admixture among wild and cultured tench, we have developed a rapid and efficient method that distinguishes between the nuclear and mtDNA genomes of the phylogroups. The method further enables distinguishing between the three mtDNA clades (EA, EI and EC) in the Eastern phylogroup. The method relies on polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) assays of two independent nuclear-encoded EPIC markers, the second intron of the actin gene (*Act*) and the first intron of the gene coding for the S7 ribosomal protein (*RpS7*), and of one mtDNA marker, the cytochrome *b* gene (*Cytb*). Examination of DNA sequences of all known haplotypes for each of these three markers found in a survey of 225 tench from a wide range of geographical regions (GenBank accession nos HM167935–HM167938, HM167941–HM167965) indicated that the Western and Eastern phylogroup could be reliably distinguished by cleavage of *Act* by *Eco*52I restriction endonuclease, of *RpS7* by *Nde*I endonuclease and of *Cytb* by *Alu*I and *Mbo*I endonucleases. In addition, cleavage of *Cytb* using *Alu*I distinguishes the EI clade and cleavage with *Mbo*I the EC clade.

To validate the new assays, tench genomic DNA was extracted from ethanol-preserved muscle tissue or fin clips using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). A 335-bp amplicon containing the *Act* intron was PCR-amplified with the EPIC primers described by Atarhouch *et al.* (2003), and a 923–927-bp amplicon (928 total bp of the alignment) containing the *RpS7* intron with the EPIC primers published by Chow & Hazama (1998). A 1226-bp part of the mitochondrial DNA containing the entire *Cytb* was amplified with the primers located in flanking tRNAs as described by Machordom & Doadrio (2001). Primer names and sequences are listed in Table 1. The 25 μL PCRs contained 0.5× concentration of PPP Master Mix (2× stock solution: 150 mM Tris-HCl [pH 8.8], 40 mM $(NH_4)_2SO_4$, 0.02% Tween 20, 5 mM $MgCl_2$, 400 μM of each dNTP, 100 U/mL Taq-Purple DNA polymerase, stabilizers, additives; Top-Bio, Prague, Czech Republic), 10 pmol of each primer, 0.2 μg of template DNA and demineralized water to the final volume. To facilitate high-throughput analysis, the amplification in a PTC 200 Peltier thermal cycler (MJ Research, Watertown, MA, USA) was performed using the same PCR program for all markers, which contained 5 min of initial denaturation at 95 °C, six cycles of a touch-down profile of 1-min denaturation at 94 °C, 1 min 30 s at 60–56 °C with the annealing temperature lowered by 2 °C after every two cycles and 2-min elongation at 72 °C, followed by 30 cycles with the annealing temperature held at 54 °C. For the RFLP analyses, 4 μL of the PCR products were digested for 10 h at 37 °C in 10 μL reaction volumes containing 4 μL of demineralized water and 1 μL of endonuclease buffer with 1 μL of the endonuclease *Eco*52I, *Alu*I, *Mbo*I (Fermentas, Vilnius, Lithuania) or *Nde*I (New England Biolabs, Ipswich, MA, USA) and then deactivated at 65 °C for 20 min. Restriction fragments were separated on 2% agarose gels containing 2 μL of GoldView (SBS Genetech, Shanghai, China).

Digesting the amplicons of individuals carrying all known haplotypes for each of the three genes, including heterozygotes for each nuclear gene, yielded the predicted RFLP profiles (Fig. 1). The restriction digestion of the 1226-bp amplicon of *Cytb* by *Mbo*I at four cleavage sites (345, 642, 876, 946 bp) resulted in five fragments (70, 234, 280, 297, 345 bp) in the Eastern phylogroup, except in clade EC, which does not have the 642-bp and 946-bp sites but has a 132-bp site and had a four-band pattern (132, 213, 350, 531 bp). The Western phylogroup also has the 132-bp site but does not have the 946-bp site and had a unique five-band pattern (132, 213, 234, 297, 350 bp). The digestion of *Cytb* by *Alu*I at three cleavage sites (184, 891, 1168 bp) resulted in four fragments (58, 184, 277, 707 bp) in the Eastern phylogroup, except in the EI clade, which does not have the 1168-bp site and had a three-band pattern (184, 335, 707 bp). The Western phylogroup does not have the 891-bp site and had a different three-band pattern (58, 184, 984 bp), except in one of the haplotypes (W2), which only has the 1168-bp site and had a two-band pattern (58, 1168 bp).

Table 1 Primers and their sequences

Marker (amplicon size)	Primer	Sequence (5′ to 3′)	Reference
Cytb (1226 bp)	GluF	AACCACCGTTGTATTCAACTACAA	Machordom & Doadrio (2001)
	ThrR	ACCTCCGATCTTCGGATTACAAGACCG	Machordom & Doadrio (2001)
RpS7 (923–927 bp)*	S7RPEX1F	TGGCCTCTTCCTTGGCCGTC	Chow & Hazama (1998)
	S7RPEX2R	AACTCGTCTGGCTTTTCGCC	Chow & Hazama (1998)
Act (335 bp)	Act-2-F	GCATAACCCTCGTAGATGGGCAC	Atarhouch *et al.* (2003)
	Act-2-R	ATCTGGCACCACACCTTCTACAA	Atarhouch *et al.* (2003)

*Length variation because of gaps.

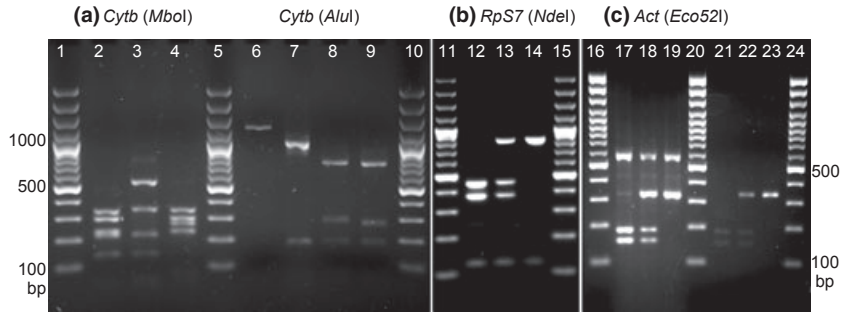

Fig. 1 Agarose gels showing diagnostic restriction fragment patterns. (a) *Cytb* assays. Lanes 1, 5 and 10: 100-bp ladder molecular weight standard (500- and 1000-bp bands highlighted); lane 2: Western phylogroup digested with *Mbo*I; lane 3: EC clade digested with *Mbo*I; lane 4: other Eastern clades digested with *Mbo*I; lane 6: W2 haplotype digested with *Alu*I; lane 7: other Western haplotypes digested with *Alu*I; lane 8: EI clade digested with *Alu*I; lane 9: other Eastern clades digested with *Alu*I. (b) *RpS7* assay with *Nde*I. Lanes 11 and 15: 100-bp ladder; lane 12: Western phylogroup; lane 13: Western/Eastern heterozygote; lane 14: Eastern phylogroup. (c) *Act* assay with *Eco52*I. Lanes 16, 20 and 24: 100-bp ladder (500-bp band highlighted; note different version of ladder than in panels a and b); lanes 17–19: native PCR product; lane 17: Western phylogroup; lane 18: Western/Eastern heterozygote; lane 19: Eastern phylogroup; lanes 21–23: target amplicon (335-bp) purified by gel-extraction prior to endonuclease treatment; lane 21: Western phylogroup; lane 22: Western/Eastern heterozygote; lane 23: Eastern phylogroup. Note that fragments with size under 100 bp are difficult to visualize but this does not affect scoring.

The digestion of the 923–927-bp amplicon of *RpS7* (size range is because of gaps) by *Nde*I yielded a two-band pattern for the Eastern phylogroup and a three-band pattern for the Western phylogroup (Fig. 1b). There is a cleavage site at 110 bp common to both phylogroups that produced a small fragment (110 bp) in both phylogroups and a second, large fragment (813–817-bp) in the Eastern phylogroup. An additional cleavage site at 478 bp that is absent in the Eastern phylogroup produced two fragments of an intermediate size (368, 446–448 bp) in the Western phylogroup.

The digestion of the 335-bp amplicon of *Act* by *Eco52*I at a single cleavage site (182 bp), which is absent in the Eastern phylogroup, resulted in two fragments (153, 182 bp) in the Western phylogroup (Fig. 1c).

The amplification of *Act* under the above-mentioned conditions produced a second major amplicon of a larger molecular size (approximately 580 bp) than our marker, plus a minor amplicon (approximately 430 bp). The genome of teleost fishes contains multiple paralogous actin genes (Venkatesh *et al.* 1996), and these additional amplicons thus were most probably derived from paralogous annealing of the EPIC primers. The extra amplicons were not cleaved with *Eco52*I, so that for reliable genotyping of our EPIC marker, they did not need to be removed (see Fig. 1c). Occasionally, the amplification of *RpS7* also yielded up to three minor amplicons of different molecular sizes in addition to the expected amplicon, some of which were present in multiple individuals (most notably amplicons of approximately 580 and 1600 bp), but these minor amplicons were not cleaved by *Nde*I and did not interfere with the genotyping.

All individuals with previously sequenced amplicons (*Cytb*: $n = 17$; *RpS7*: $n = 11$; *Act*: $n = 11$) were correctly genotyped by the new method, which demonstrates its accuracy. An additional 71 tench, originated from two lakes in Germany where the Western and Eastern phylogroup (EA clade) coexist (Lajbner *et al.* 2010), were screened by these assays (only 35 fish for *Cytb*) to verify the efficiency of the protocol. All specimens were unambiguously genotyped as either the Western phylogroup (*Cytb*: $n = 32$; *RpS7*: $n = 55$; *Act*: $n = 42$) or Eastern phylogroup (*Cytb*: $n = 3$; *RpS7*: $n = 4$; *Act*: $n = 1$) or heterozygotes (*RpS7*: $n = 12$; *Act*: $n = 28$). Thus, the new RFLP assays are a robust and rapid method to distinguish the Western and Eastern phylogroup and also the three mtDNA clades in the Eastern phylogroup of the tench. The assays will be useful for monitoring the human-mediated dispersal of the phylogroups among wild populations currently occurring via aquaculture, for characterization and identification of cultured strains, and in breeding experiments.

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