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ISSN 0792 - 156X

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PUBLISHER:
Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL

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Use of PCR-RFLP Analysis of mtDNA Cytochrome-b Gene to Determine Genetic Differences in *Capoeta* spp.

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(Received 1.4.07, Accepted 3.5.07)

Key words: *Capoeta* spp., Cytochrome-b, MtDNA, PCR, RFLP

Abstract

Genetic differences between *Capoeta capoeta capoeta*, *Capoeta capoeta umbla*, and *Capoeta tinca* were determined using PCR-RFLP of mtDNA cytochrome-b (Cyt-b) by amplifying approximately 400-500 bp of this region from each of the three subspecies. The restriction enzymes *SpeI* and *HinfI* did not indicate genetic differences but *AluI* and *HpaII* did. Thus, PCR-RFLP of the mtDNA was used to distinguish between closely related subspecies without having to analyze the entire DNA sequence of specimens. Use of this technique demonstrated that the Cyt-b regions of the three subspecies have different base sequences. The similarity between *C. c. capoeta* and *C. c. umbla* is 71.4% while *C. tinca* is more distant (50% for *C. c. umbla* and 33.3% for *C. c. capoeta*). The Cyt-b similarity is consistent with morphological and taxonomical similarities. PCR-RFLP can serve as a tool for genetically identifying subspecies of fish in nature and in aquaculture.

Introduction

Capoeta are widely distributed throughout southern China, northern India, Turkmenistan, Lake Aral, the Middle East, and Anatolia, inhabiting gravel and stony areas of fast flowing rivers. There are five species and six subspecies of this genus in the inland waters of Turkey (Geldiay and Balik, 1996).

Fish species can be identified by analyzing proteins by isoelectric focusing (Pineiro et al., 2001) or immunoreaction (Carrera et al., 1997; Ochiai and Watabe, 2003). However, in most cases, these methods of analysis are inapplicable to thermally processed products

as a result of protein denaturation. In such cases, species can be identified using DNA nucleotide sequencing (Quinteiro et al., 1998), RFLP (Borgo et al., 1996; Quinteiro et al., 1998), or single strand conformation polymorphism (SSCP; Marklund et al., 1995; Rehbein et al., 1995). Analysis of nucleic acids, such as mitochondrial and nuclear DNAs, has advantages over protein-based analyses, as the former method is not dependent on tissues and the age of the individual. Further, mitochondrial DNA (mtDNA) has several advantages over nuclear DNA in the diagno-

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sis of fish products. For example, the former has several times higher copy number and, therefore, is abundant in various tissues (Brand et al., 1994; Zhang and Hewitt, 2003). As mtDNA is haploid and a maternally inherited sequence, ambiguities as a result of heterozygous genotypes are theoretically avoided (Lockley and Bardsley, 2000).

DNA analysis for the identification of closely related fish species has already been reported (Rehbein et al., 1995; Borgo et al., 1996; Birstein and Desalle, 1998; Sezaki et al., 2001). In this study we established an alternative method for identifying *Capoeta* spp. by PCR-RFLP application and concluded

that PCR-RFLP can serve as a tool to genetically identify subspecies of fish both in nature and in aquaculture (Quinteiro et al., 1998; Céspedes et al., 2000; Cocolin et al., 2000; Hisar et al., 2006).

Materials and Methods

Sample collection and DNA extraction. A cast net (1-2 km) was used to collect five adults (fork length > 19 cm) from each population: *Capoeta capoeta capoeta* (Fig. 1) from the Aras River, *Capoeta capoeta umbla* (Fig. 2) from the Karasu River, and *Capoeta tinca* (Fig. 3) from the Coruh River, all in eastern Anatolia, Turkey. The subspecies were identi-



Fig. 1. *Capoeta capoeta capoeta* from the Aras River (39°59'13"N 41°42'55"E).



Fig. 2. *Capoeta capoeta umbla* from the Karasu River (39°54'58"N 40°49'38"E).

fied by methods proposed by Geldiay and Balik (1996).

Muscle tissues were preserved immediately in 95% ethanol and stored at ambient temperature in the field and -20°C in the laboratory. Sampled tissues were incubated overnight at 55°C in 500 µL of DNA extraction solution (400 mM NaCl, 10 mM Tris, 2 mM EDTA, 1% SDS). Total genomic DNA was isolated according to Asahida et al. (1996) with minor modifications.

Polymerase chain reaction (PCR) amplification. Approximately 400-500 bp of mitochondrial Cyt-b gene were amplified with Cyt1 (5'-cca tcc aac atc tca gca tga tga aa-3') and Cyt2 (5'-ccc ctc aga atg ata ttt gtc ctc a-3') primers (Barlett and Davidson, 1991; Cocolin et al., 2000). Each 100-µl reaction contained 50-100 ng DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1-5 mM MgCl₂, 2-5 units Taq DNA polymerase, 150 mM of each dNTP, and 0-3 mM of each primer, and was amplified with a cycling profile of 1 min at 94°C, 1 min at 53°C, and 2 min at 72°C for 35 cycles.

Restriction fragment length polymorphism (RFLP) analysis. The PCR products were digested with a restriction enzyme (AluI, HinfI, SspI, or HpaII) and the fragments were resolved on 1.5% agarose gels using a Tris/Borate/EDTA (TBE) buffer. The size of the restriction fragments was estimated by comparison to a 10,000 bp ladder.

Statistical analysis. Differences among *Capoeta* subspecies in genetic and proximal

matrix relationships were tested using the statistical package SPSS for Windows (1999), version 10.0 (Cluster analysis, Jaccard's model, Nearest Neighbor).

Results

Results of the PCR-RFLP analysis are shown in Fig. 4. The estimated molecular lengths of the fragments in each restriction morph are summarized in Table 1. A dendrogram of the results is presented in Fig. 5.

Discussion

Capoeta species are widespread in lakes, rivers, and other freshwater bodies in Turkey. While there are distinguishing taxonomic features among the species and subspecies, it is difficult and even impossible to distinguish them in some small fish. Without a method of morphological determination, analytical methods to distinguish between *C. c. capoeta*, *C. c. umbla*, and *C. tinca* are of great importance in fish biology. In this work, we focused on part of the mitochondrial Cyt-b gene for identifying *Capoeta* spp., using this DNA-based method to identify the *Capoeta* subspecies without having to analyze the entire DNA sequence.

MtDNA can be used as a marker in species identification instead of nuclear DNA (nDNA). MtDNA has advantages in that it is easy to isolate, has a simple genetic structure, exhibits a straight-forward mode of genetic transmission, and, in vertebrates, has a high mutation rate (Unselde et al., 1995; Hisar et al.,



Fig. 3. *Capoeta tinca* from the Coruh River (40°33'52"N 41°35'35"E).

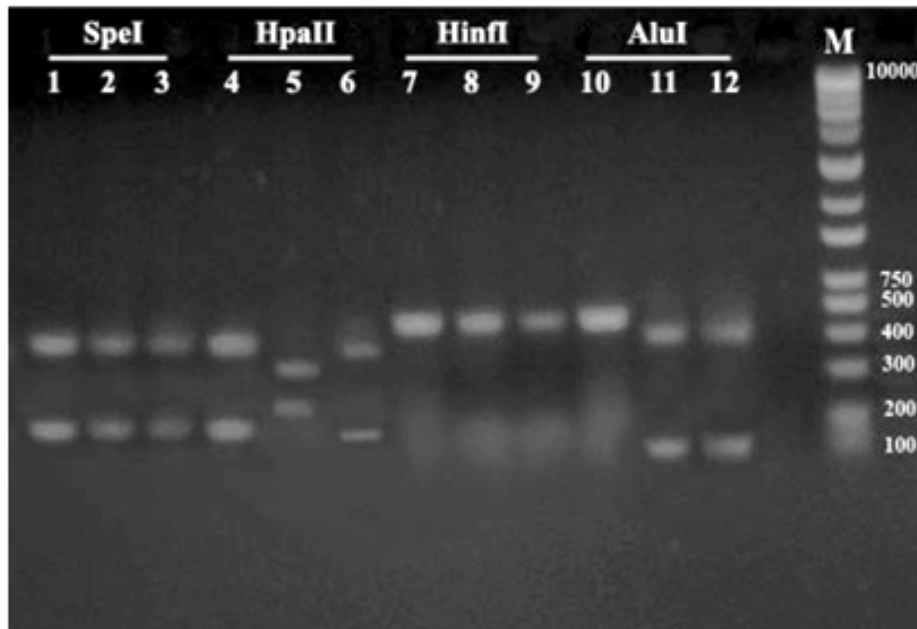


Fig. 4. Analysis by restriction fragment length polymorphism (RFLP; 400-500 bp PCR-amplikon) of *Capoeta capoeta capoeta* (lanes 1, 4, 7, 10), *C. tinca* (lanes 2, 5, 8, 11), and *C. c. umbla* (lanes 3, 6, 9, 12) using *SpeI*, *HpaII*, *HinfI*, and *AluI* for digestion and compared to M, a DNA marker (50-10,000 bp).

Table 1. Approximate size of fragment patterns obtained by PCR-RFLP analysis of the mtDNA of *Capoeta* spp.

Subspecies	Restriction enzyme			
	<i>SpeI</i>	<i>HpaII</i>	<i>HinfI</i>	<i>AluI</i>
<i>C. capoeta capoeta</i>	100-200 bp 300-400 bp	100-200 bp 300-400 bp	-	-
<i>C. capoeta umbla</i>	100-200 bp 300-400 bp	200-300 bp 200-300 bp	-	<100 bp 400-500 bp
<i>C. tinca</i>	100-200 bp 300-400 bp	100-200 bp 300-400 bp	-	<100 bp 400-500 bp

2006). The primers used in this study, Cyt1 and Cyt2, were used to identify tuna species (Barlett and Davidson, 1991) and to distinguish among flatfish (Céspedes et al., 1998) and have become universal primers. PCR-RFLP analysis of the mtDNA was selected due

to its simplicity, speed, and low cost (Céspedes et al., 2000). This method is applicable to the small PCR products that are available from highly processed materials and can, therefore, easily be adapted to distinguish among fish species in juveniles and larvae.

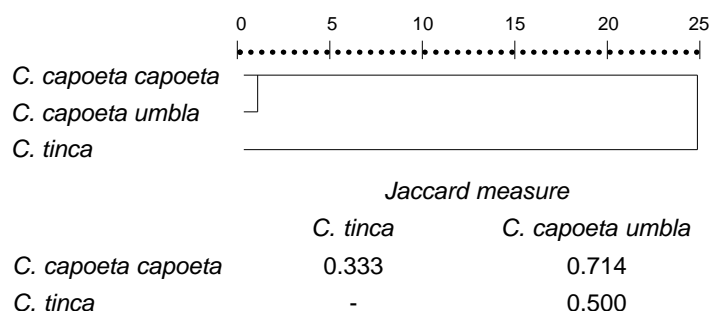


Fig. 5. SPSS dendrogram showing the similarity between *Capoeta capoeta capoeta*, *C. capoeta umbla*, and *C. tinca*.

The PCR-RFLP analysis revealed three mitochondrial genotypes. While the restriction enzyme *HinfI* did not cut the Cyt-b region, *AluI*, *SpeI*, and *HpaII* did. There are many studies involving RFLP techniques in other fish species (Szalanski et al., 2000; McDowell and Graves, 2002, Docker and Heath 2003), but none in *Capoeta*. Nor is there any genetic information about *C. c. capoeta*, *C. c. umbla*, and *C. tinca* in Genbanks. Therefore this study can become a reference for future research on these species.

The genetic relationships revealed by our PCR-RFLP analysis are similar to those suggested by authors using morphological characters (Geldiay and Balik, 1996) and to the taxonomical classification of the subspecies. Our study shows that these species can be identified safely and simply by using PCR-RFLP.

Acknowledgements

This work was all of the master thesis entitled *The Determination of Genetical Differences by Using PCR-RFLP Application of mtDNA Cytochrome-b Region of Capoeta sp. Captured from Aras, Karasu and Coruh Basin*, supported by the Ataturk University Scientific Research Project BAP 2005/45.

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