



## Molecular Identification of *Astacus leptodactylus* and *Austropotamobius torrentium* Using mtDNA-RFLP Method

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### Abstract

mtDNA-RFLP, a technique commonly used for molecular identification of species, was used to distinguish between morphologically similar crayfish species *Austropotamobius torrentium* and *Astacus leptodactylus* in this study. The mitochondrial COI gene was amplified and digested using 15 restriction endonucleases to establish a restriction map. The COI gene sequence contained recognition sites for three of the restriction endonucleases (*SspI*, *KpnI*, and *RsaI*) and produced two fragments and three genotypes for *Austropotamobius torrentium*. However, the *Astacus leptodactylus* COI gene did not have restriction sites for these three enzymes. Thus, a mtDNA-RFLP using these three endonucleases can be used to successfully distinguish between these two species of crayfish.

**Keywords:** crayfish, species identification, mtDNA, COI.

### *Astacus leptodactylus* ve *Austropotamobius torrentium* Türlerinin mtDNA-RFLP Metodu ile Moleküler Teşhisi

#### Özet

Bu çalışmada, canlı türlerinin moleküler olarak teşhisinde yaygın olarak kullanılan mtDNA-RFLP tekniği, morfolojik olarak birbirine benzeyen *Austropotamobius torrentium* ve *Astacus leptodactylus* kerevit türlerinin ayırımında kullanılmıştır. Mitokondrial COI gen bölgesi polimeraz zincir reaksiyonu ile çoğaltılarak 15 farklı endonükleaz enzimi ile kesilmiştir. Bu kesim enzimlerinden üç tanesi (*SspI*, *KpnI*, and *RsaI*) çoğaltılan COI gen bölgesini keserek *Austropotamobius torrentium* türü için iki parçacık ve üç genotip üretirken, *Astacus leptodactylus* türü için kesim bölgesi bulamamıştır. Çalışma sonuçları bu üç kesim enziminin mtDNA-RFLP tekniği kullanılarak bu iki kerevit türün ayırımında başarılı bir şekilde kullanılabileceğini göstermiştir.

**Anahtar Kelimeler:** Kerevit, tür teşhisi, mtDNA, COI.

#### Introduction

The freshwater crayfish belongs to the order Decapoda which is the largest group of crustaceans (Tool *et al.*, 2009). There are more than 640 widely distributed species of freshwater crayfish. The taxonomists discover nearly 10 new species every year (Crandall and Buhay, 2008). Crayfish are represented by six indigenous and five non-indigenous species in Europe and by two species (*Astacus leptodactylus* and *Austropotamobius torrentium*) in Turkey (Harlıoğlu and Güner, 2006; Akhan *et al.*, 2014).

Crayfish species are naturally abundant in most of natural freshwater bodies of Turkey. Among these

species, *A. leptodactylus* distributes in most of Turkish freshwater; while *Austropotamobius torrentium* distributes only in Trace region of Turkey. It is well known that crayfish have been transferred between water bodies due to anthropogenic effects (Akhan *et al.*, 2014) such as introduction of crayfish into newly constructed dam lakes by fisherman or government fisheries officials. Monitoring of the crayfish species in water bodies is essential for their successful stock management and conservation of genetic diversity (Souty-Grosset *et al.*, 2003), which in turn requires fast methods of species identification. Conventional species identification depends on morphological features; however, morphological identification can be very difficult in certain cases such as during the larval stage (Soroka, 2008).

Additionally, an accurate species description may not be possible using conventional methods, and this especially true in the case of processed crayfish products. Molecular methods are currently being widely used for the identification of many species. Besides protein-based methods, more reliable DNA-based methods are also being used (Agerberg, 1990; Fevolden *et al.*, 1994). Single nucleotide polymorphism (SNP) (Akhan *et al.*, 2014), amplified fragment length polymorphism (AFLP) (Chiesa *et al.*, 2011), restricted fragment length polymorphism (RFLP) Grandjean *et al.*, 1997; Gouin *et al.*, 2003) and single sequence repeat (SSR) Yue *et al.*, 2010; Gouin *et al.*, 2011; Iorgu *et al.*, 2011, Vorburger *et al.*, 2014; Ming *et al.*, 2014) are some of the very useful methods that have been used for analyzing both population genetics and systematic classification of crayfish.

These DNA-based methods provide rapid and accurate results that facilitate species identification and distinction between closely related species and subspecies of fish, crayfish and other organisms. The PCR-RFLP method is based on the restriction digestion of DNA amplified from highly conserved regions, such as the mitochondrial genome (Ming *et al.*, 2014). This method is easier, more practical and cheaper than other molecular tools used for species identification, such as microsatellites, sequence analysis and molecular barcoding and it has been successfully used for the identification of crayfish species (Soroka, 2008), fish species (Teletchea, 2009; Sumathi *et al.*, 2015), shrimp species (Khamnamtong *et al.*, 2005; Pascoal *et al.*, 2011), and other aquatic organisms (Chow *et al.*, 2006; Guerao *et al.*, 2011; Jagadeesan *et al.*, 2009).

Although there are many reports on the identification of *Astacus leptodactylus* and *Austropotamobius torrentium* crayfish species based on morphological features, molecular identification of these two species has not yet been attempted. Thus, the objective of this study was to develop a simple, practical and precise DNA-based method to distinguish between these two crayfish species found in Turkey, apart from defining restriction sites that could be used as markers for identifying *Astacus leptodactylus* and *Austropotamobius torrentium* based on the RFLP profile of the mitochondrial COI gene.

## Materials and Methods

*Astacus leptodactylus* specimens were obtained from Lake Manyas in Balıkesir, Turkey (N=16) (N=16) (40°14'25.35"N; 27°57'19.33"W) and *Austropotamobius torrentium* specimens were collected from the Balaban Creek in Kırklareli, Turkey (N=6) (41°46'25.35"N; 27°57'19.33"W) using fyke net. Specimens were stored at -20 °C till DNA extraction. DNA was extracted from the abdominal muscle using a commercial DNA isolation Kit (Promega, Madison, USA) according to

manufacturer's instructions, DNA concentration was measured on a Shimadzu 1700 spectrophotometer (Shimadzu, Kyoto, Japan) and purified DNA was stored at -20 °C until use. PCR amplification of the mtCOI gene was performed using universal primers a previously described protocol (Folmer *et al.*, 1994). Briefly, 50 µL of a final reaction volume contained 10 ng template DNA, 5 µL of 0.2 mM dNTPs, 1 U *Taq* DNA polymerase (Promega Madison, WI, USA), 10 µL 5X reaction buffer, 10 pmol of each primer and 50 mM MgCl<sub>2</sub>. A thermal cycler (Techne TC-3000G, Bibby Scientific, Staffordshire, UK) was used for the PCR under the following conditions: 3 min at 94 °C for initial denaturation, 45 sec at 94°C for denaturation, 45 sec at 48°C for annealing, 1 min at 72°C for extension and finally 5 min at 72°C for elongation. The PCR products were separated on a 1% agarose gel by electrophoresis and stained with ethidium bromide for visualization. To determine the restriction sites on the partially amplified COI gene, 15 four- and six-base cutting enzymes (*SspI*, *KpnI*, *RsaI*, *XbaI*, *HinfI*, *EcoRI*, *BclI*, *BsuRI*, *BamHI*, *XhoI*, *HindIII*, *MslI*, *XcmI*, *BsrGI*, *NdeI*) were used and the amplified COI gene sequence was digested using these restriction endonucleases in a 13 µL volume mixture which contained 1.5 µL PCR products, 5 U restriction enzyme, 1.3 µL of 10X reaction buffer, and 9.2 µL sterile distilled water. After incubating the digestion mixture at 37 °C for 2 h, the products were separated by electrophoresis on a 2% agarose gel with 1X TAE buffer (pH 8), the gel stained with ethidium bromide and visualized on a Quantum-Capt ST4, UV illuminator system (Vilber Lourmat, France). The amplified COI gene was also sequenced by an external laboratory (Macrogen Inc., Seoul, Korea) on an automatic sequencer (ABI 3730 XLs) to confirm species identification and the restriction sites of the endonucleases used. These sequences were subjected to the BLAST algorithm at the NCBI GenBank for further verification of species identification. The sequences were also aligned using the Clustal X software (Larkin *et al.*, 2007) to determine restriction sites.

## Results

The two freshwater crayfish species were distinguished based on RFLP analysis of the mitochondrial COI gene. The universal COI primers used for amplification yielded an approximately 681 bp fragment. Amplified fragments from both species were digested with three restriction endonuclease enzymes. Those enzymes (*SspI*, *KpnI* and *RsaI*) had restriction sites in the amplified sequence for *Austropotamobius torrentium* and yielded two fragments. However, no such sites were found on the *Astacus leptodactylus* COI gene sequence. This fact enables distinction between these two morphologically similar species of crayfish (Table 1). Restriction digestion using *SspI* produced 240 bp and

441 bp fragments (Figure 1), *RsaI* yielded 331 bp and 350 bp fragments (Figure 2), and *KpnI* produced 329 bp and 352 bp fragments (Figure 3); four different genotypes (A, B, C, and D) could be identified based on this RFLP pattern (Table 1).

The PCR products of both *A. torrentium* and *A. leptodactylus* were also sequenced to confirm the presence of restriction sites, and their sequence aligned using CLUSTAL X software (Figure 4).

## Discussion

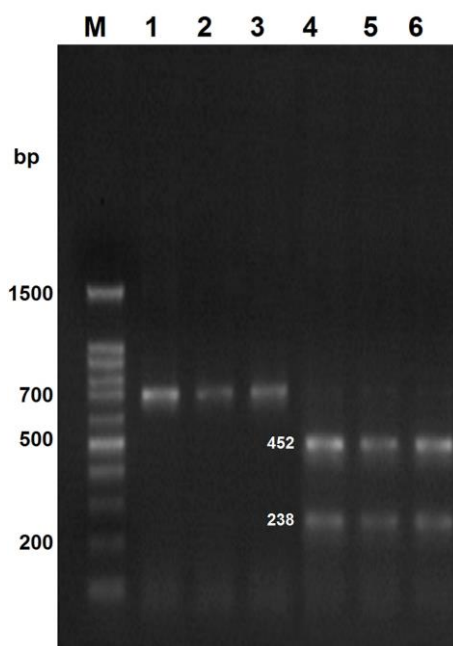
Research on the taxonomic classification of various crayfish species is of interest to scientists and genetic methods are being widely used for accurate identification and distinction between close species or systematic categories. The restriction analysis of PCR-amplified fragments using restriction endonucleases (RFLP-PCR) is a practical alternative

to gene sequencing for determining differences in gene sequences, as it is a low cost, easy, and fast method (Ming *et al.*, 2014). The mitochondrial COI gene is used most frequently for DNA-based species identification (Soroka, 2008). Mueller *et al.*, (2015) developed a fast complementary method for the generation of PCR-RFLP patterns using a novel 439 bp amplicon within the established COX1 barcoding gene fragment that contains restriction sites for the fast restriction enzymes *MluCI* and *HpaII*.

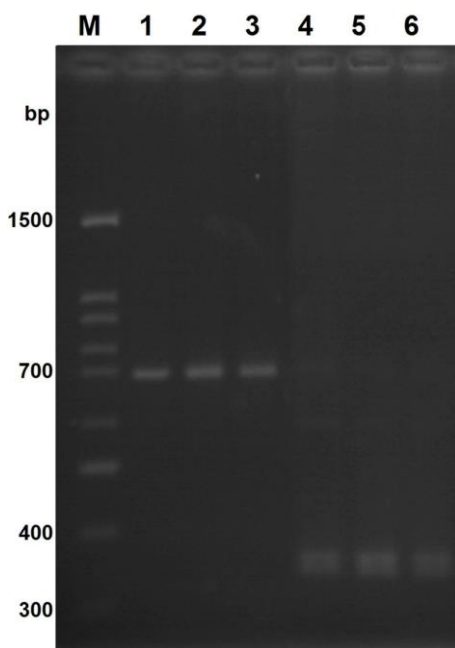
The sequence of the mitochondrial COI gene is sufficiently variable for identifying and distinguishing between even closely related species (Lefébure *et al.*, 2006; Bucklin *et al.*, 2009; Darling *et al.*, 2008); and therefore, the COI gene was used to distinguish between the crayfish species *Austropotamobius torrentium* and *Astacus leptodactylus* using PCR-RFLP analysis. According to Annex II of the EU Habitats Directive, *Austropotamobius torrentium* is a

**Table 1.** Size (bp) of digested mitochondrial COI gene fragments by three restriction enzymes and genotypes

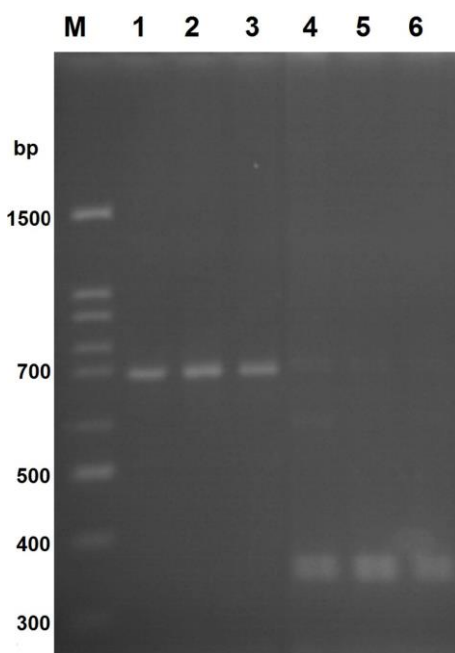
Restriction endonuclease	<i>Astacus leptodactylus</i>	<i>Austropotamobius torrentium</i>
	Genotype	Genotype
SspI	A	B
	681 bp	240 bp
		441 bp
RsaI	Genotype	Genotype
	A	C
	681 bp	331 bp
KpnI	Genotype	Genotype
	A	D
	681 bp	329 bp
		352 bp



**Figure 1.** PCR-RFLP analysis of COI gene after digestion of SspI. *Astacus leptodactylus* (1,2,3); *Austropotamobius torrentium* (4,5,6); (M: 100 bp DNA ladder).



**Figure 2.** PCR-RFLP analysis of COI gene after digestion of RsaI. *Astacus leptodactylus* (1,2,3); *Austropotamobius torrentium* (4,5,6); (M: 100 bp DNA ladder).



**Figure 3.** PCR-RFLP analysis of COI gene after digestion of KpnI. *Astacus leptodactylus* (1,2,3); *Austropotamobius torrentium* (4,5,6); (M: 100 bp DNA ladder).

‘priority’ species that needs more efficient conservation and management systems across Europe (Iorgu *et al.*, 2011), and special conservation measures have been formulated to control the decreasing numbers of *A. torrentium*. Further, the International Union for Conservation of Nature (IUCN) has categorized this species in its red list with its population listed under the data deficient category. The annex II of the EU Habitats Directive had also emphasized that special conservation measures are

needed for this species of crayfish, it features on the German red list, and other countries have also listed this species as ‘under threat’ (Souty-Grosset *et al.*, 2006). Further, this species is included in Appendix III of the Bern Convention, and *A. leptodactylus* and *A. torrentium* haplotype identification is essential before they are restocked in water (Schrimp *et al.*, 2011).

RFLP studies on crayfish are limited and no restriction endonuclease maps are available for the



**Figure 4.** Aligned DNA sequences of COI gene and restriction sites of three restriction enzymes (SspI, RsaI and KpnI) for *Astacus leptodactylus* (Al), and *Austropotamobius torrentium* (At).

crayfish species found in Turkey. *Austropotamobius torrentium* and *Astacus leptodactylus* are morphologically similar crayfish species and we show that the restriction enzymes *SspI*, *RsaI* and *KpnI* can be used to identify or distinguish between them. Restriction digestion of the PCR product of the mt COI gene using *SspI*, *KpnI*, *RsaI* produced a fragmentation pattern unique to each enzyme (240/441bp for *SspI*, 331/350 bp for *KpnI* and 329/352 bp for *RsaI*) in *Austropotamobius torrentium*; however these endonucleases did not yield any fragment in *Astacus leptodactylus*. Similar to our study, Jadagesen, (2009) used an RFLP method with *EcoRI* and *HindIII* restriction enzymes to distinguish the calanoid copepod, *Paracalanus parvus*, from other morphologically similar copepods and concluded that RFLP-PCR clearly distinguishes *Paracalanus parvus* from the other morphologically similar copepods. We did not observe any intra-specific polymorphism in *Austropotamobius torrentium* with the restriction enzymes used. Similar results have been reported by Soroka, (2008) where a partial sequence of the COI gene was restriction digested with endonucleases and no intra-specific variation was observed among species obtained from a broad geographical range, and thus, our study further corroborates the observations made by Soroka, (2008). RFLP mtDNA variation within the *Austropotamobius* genus has been previously described (Gouin *et al.*, 2003) and only one haplotype has been reported in nine Irish *Austropotamobius* populations. However, the sequence alignment data in the current study show three haplotypes for *Austropotamobius torrentium* and one haplotype for *Astacus leptodactylus*.

RFLP-PCR techniques have been successfully used for species identification of aquatic animals.

Chow *et al.*, (2006) investigated genetic variation within and between 10 Indo-Pacific species of lobster using RFLP analysis of the mt COI gene and could successfully identify and distinguish between all the lobster species, including those of the *P. longipes* complex. (Khamnamtong *et al.*, 2005) have used RFLP-PCR technique for molecular analyses of five different penaeid species (*Penaeus monodon*, *Penaeus semisulcatus*, *Fenneropenaeus merguensis*, *Litopenaeus vannamei*, *Marsupenaeus japonicus*), and they reported that RFLP-PCR of the 16srDNA region enables precise distinction between *P. semisulcatus*, *P. monodon* and *L. vannamei*. (Guerao *et al.*, 2011) used RFLP-PCR to study four commercially important but morphologically similar *Maja* species belonging to the crab genus along the European coast, namely, *M. brachydactyla*, *M. squinado* and noncommercial *M. goletziana* and *M. crispata* and found that restriction combination for accurate discrimination of *Maja* species. We used the same primer pair and obtained a 681 bp amplicon.

RFLP-PCR has been used for first the time to clearly identify and distinguish between two morphologically similar crayfish species *Austropotamobius torrentium*, *Astacus leptodactylus*. The method developed and reported in this study is fast, easy and cheap and can be used to detect two crayfish species, even during the larval stages.

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