CYTOCHROME *B* GENE BASED CLASSIFICATION OF SPECIES: EXAMPLE OF *APODEMUS SYLVATICUS* (LINNAEUS, 1758) (MAMMALIA: RODENTIA)

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ABSTRACT: This research is based on the analyses of Cytochrome *b* gene of *Apodemus sylvaticus* in Kırıkkale Province between August 2009 and September 2010. Diagnostic characters of the species were also recorded. The DNA isolation of tissue sample which was obtained from auricle was carried out and mitochondrial Cytochrome *b* gene was amplified via Polymerase Chain Reaction for the first time in Turkey and 139 and 154 base pair were obtained respectively. Results were compared with that of Gene Bank and the Cytochrome *b* gene sequence is 96% identical with that of *Apodemus hermonensis* (*A. witherbyi*). Although the base pair obtained for comparison is short, it is understood that the Cytochrome *b* gene can be regarded as a useful tool for the differentiation of some *Apodemus* species.

KEY WORDS: Forest mouse, *Apodemus sylvaticus*, Cytochrome *b* gene, Turkey

Rodentia is the member of richest order of the classis Mammalia (Mammals), with 2277 species. Rodentia is represented by five suborders (Sciuromorpha, Castorimorpha, Myomorpha, Anomaluromorpha and Hystricomorpha). The superfamily Muroidea belonging to Myomorpha includes six families (Platacanthomyidae, Spalacidae, Calomyscidae, Nesomyidae, Cricetidae, Muridae) and has a total of 310 genera and 1518 species. The family Muridae contains 6 subfamilies, 150 genera and 730 species. The genus *Apodemus* belonging to the subfamily Murinae contains 20 species. These species are *Apodemus agrarius*, *A. alpicola*, *A. argenteus*, *A. chevrieri*, *A. draco*, *A. epimelas*, *A. flavicollis*, *A. gurkha*, *A. hyrcanicus*, *A. latronum*, *A. mystacinus*, *A. pallipes*, *A. peninsulae*, *A. ponticus*, *A. rusiges*, *A. semotus*, *A. speciosus*, *A. sylvaticus*, *A. uralensis* and *A. witherbyi* (Wilson & Reeder, 2005).

There are 21 species belonging to 9 genera of the Muridae family in Turkey. The genus *Apodemus* was represented by six species, *Apodemus agrarius* (Palas, 1771), *A. flavicollis* (Melchior, 1834), *A. mystacinus* (Danford and Alston, 1877), *A. witherbyi* (Thomas, 1902), *A. uralensis* (Pallas, 1811) and *A. sylvaticus* (Linnaeus, 1758) (Kryštufek & Vohralík, 2009).

In the late 1980s, taxonomy of Western European forest mice of the genus *Apodemus*, was re-evaluated with the application of electrophoretic analysis of all-round allozymes (Filippucci et al., 1989; Mezhzherin & Zagorodnyuk, 1989). Also the evolutionary process of the genus *Apodemus* was investigated in detail by studies of paleontology (Michaux & Pasquier, 1974; Michaux et al., 1997) and molecular phylogenetic (Serizawa et al., 2000; Michaux et al., 2002).

Filippucci et al. (1996), Frynta et al. (2001) and Macholan et al. (2001) stated that *A. sylvaticus* had a limited distribution area within the Zonguldak Province in the Western Black Sea based on the morphometric and karyological analyses. Then Yorulmaz & Albayrak (2008) recorded also *A. sylvaticus* in Kırıkkale province in Central Anatolia.

The aim of this study is to investigate the taxonomic status of *A. sylvaticus* with Cytochrome *b* gene and to contribute to the systematic of the species.

MATERIAL AND METHOD

Tissue Samples: The tissue samples of *Apodemus sylvaticus* used in the study were obtained from the collection in the Department of Biology, Faculty of Arts and Sciences of Kırıkkale University. Sample taken from ear tissues by using a 3 mm biopsy apparatus, were studied.

DNA extraction and reverse transcriptase polymerase chain reaction: DNA was extracted using a commercial kit (QIAamp DNA extraction kit, Qiagen, Germany) according to the manufacturer's instruction. Primers were selected to recognise regions of Cytochrome *b* gene of the animal as described by Martin and Gerlach (2000). Amplification was performed with the primers L14115 (5 '- AAT GAC ATG AAA CAT CGT TG -3'), H15288 (5'-ACA AGA CCA GAG TAA TGT TTA TAC TAT C-3 ', L14648 (5'-TGA ATY TGA GGR GGC TTC TCA GTA-3), H14742 (5'-GGG TTG TTD GAT CCW GTT TC-3'). All reactions were performed using SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA according to the manufacturer's instruction (Invitrogen, USA). The primer, distilled water, polymerase enzyme, target DNA and buffer solution were prepared into the eppendorf tube and put into PCR Thermalcycler (PTC100 MJ Research, USA). In PCR steps, the temperature, time and cycle numbers specified by Martin & Gerlach (2000) (1 minute at 94 ° C, 1 minute at 52 ° C, 2 minutes at 72 ° C; 40 cycles) were recorded and run by adjusting the thermal cycler. The PCR amplicons were visualised by Ethidium bromide staining after electrophoresis through 2% agarose gel (Sigma-Aldrich, Germany). The PCR products were separated in an agarose gel by electrophoresis and purified using a commercial agarose gel using the commercial kit (QIAquick® Gel Extraction Kit, Qiagen GmbH, Hilden, Germany).

RESULTS

Apodemus sylvaticus (Linnaeus, 1758) Greater Forest Mouse

1758. *Mus sylvaticus* Linnaeus, Syst. Nat. I., 10th ed., p. 62.

Type locality: Upsala, Sweden

1915. *Apodemus sylvaticus* Miller, Ann. Mag. Nat. Hist., 8th sr. VI. P.460.

Morphological Characters

The Foramen incisiva is long and exceeds the line joining anterior edges of the the alveoli of the first upper molar (M1). In most of the examples, there is a dirty yellow stain of 2-4 mm wide and 6-14 mm in height starting from the line where the front legs are attached to the shoulder under the chin. The back of the palate makes a relatively rounded tip. A small t12 peak on M^1 is connected to the slightly evident t3 peak on M^2. M^1 has four roots (Yorulmaz & Albayrak, 2009).

Interpretation of Results of PCR

PCR amplification was carried out on 2% agarose gel. The results were evaluated on UV transilluminator. For the expected positivity as a result of PCR amplification, it was found that specific bands were formed in the 100 bp regions for the primer pair L14648 and H14742 of about 100 bp, for the primer pair L14115 and H15288 of about 1000 bp. The results were recorded by taking a polaroid photograph (Fig. 1). The obtained products were used in DNA sequencing test.

Phylogenetic Analysis

Mitochondrial DNA of *Apodemus sylvaticus* was isolated and sequence analysis of Cytochrome *b* gene was performed. Sequences were determined with the ABI PRISM® 310 Genetic Analyzer. The base sequences obtained by BioEdit software were compared.

DNA Sequencing

The PCR products were sequenced. Sequences were examined using BioEdit software and alignment procedures were performed with ClustalX1.83 software (Thompson et al., 1997). BLAST program was used to compare the indexes obtained.

A 139-b-length index of the Cytochrome *b* gene was obtained from the ear tissue of *Apodemus sylvaticus*. Base sequence for the resulting index T TGA ATC TGA GGA GTC GTC TTC TTA ATC TTC GTC GTC TTC ATC TTC TTC GTC TTC ATC TTC ATC CTT ATC CTT ATC CTT GTC GTA TTC GTC GTC GTA TTC TTC ATC CTC CTA CTG ACA ACC CAA A". In the total 139 bp length sequence analysis, Adenine bases were 25.89%, Thymine 31.65%, Guanine 13.66% and Cytosine 28.05%.

DISCUSSION

In this study, the specimens identified as *Apodemus sylvaticus* after morphological examination, showed similarity to the sequences of other *Apodemus* species obtained from the gene bank. Our result was most similar to the sequences of *Apodemus hermonensis* with 96%. Since *Apoemus hermonensis* is synonymous to *Apodemus witherbyi*, these results should be attributed to *Apodemus witherbyi*. Thus, Cytochrome *b* gene appear to be a suitable alternative, if it is desired to be used for the delimitation of *Apodemus* species. The results are consistent with the findings obtained by Martin & Gerlach (2000) in terms of Cytochrome *b* gene.

The DNA sequence consisting of 139 base pairs obtained in the study was significantly shorter than the 1140 bp DNA sequence obtained by Martin & Gerlach (2000). This is due to the use of only first pair of primers used by Martin & Gerlach (2000) to detect the Cytochrome *b* gene in this study. Therefore, it is insufficient to make species comparison. In later studies, also the use of second pair of primers used by Martin & Gerlach (2000) will give much better results for having a complete assessment.

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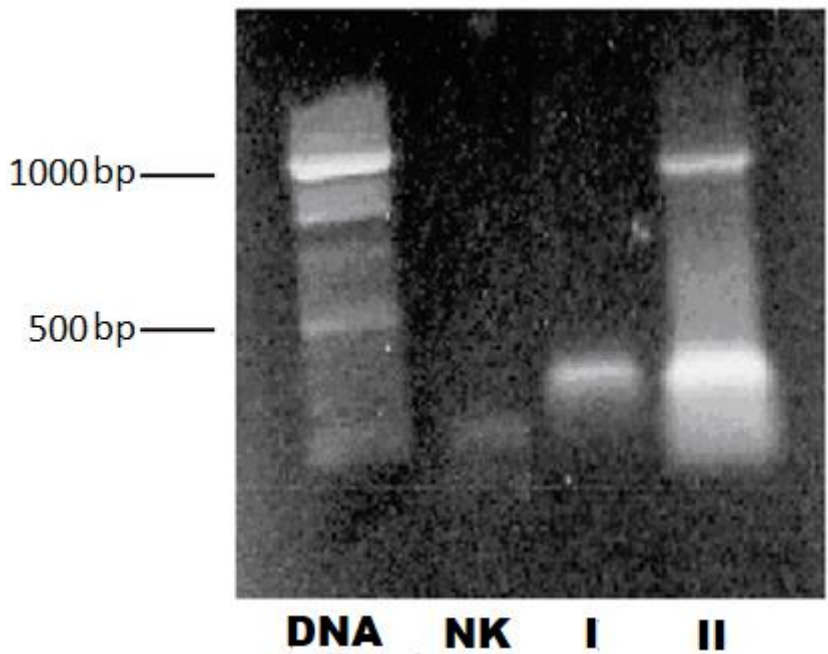

Figure 1. Reaction image (DNA ladder: Molecular marker, NK: Negative control, I: reaction image with primer L, II: reaction image obtained with Primer H) on agarose gel with primer L and Primer H sets.