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A preliminary molecular study of the Iranian species of *Calomyscus* (Rodentia-Calomyscidae) using RFLP

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The Calomyscidae is a small family of Rodentia that has recently been classified into 8 species in a single genus, *Calomyscus*. Currently 5 species are reported in Iran: *C. elburzensis*, *C. bailwardi*, *C. grandis*, *C. urartensis* and *C. hotsoni*. Species discrimination based on morphological characteristics is difficult, so this study re-evaluated the phylogenetic relationships and taxonomic status of the morphotypes within *Calomyscus* using PCR-RFLP as a molecular marker. Forty-seven samples of *Calomyscus* spp from 9 geographically separated regions of Iran were analyzed. The cytochrome *b* gene was amplified by PCR, digested using 3 restriction enzymes, *Hin*fI, *Hin*cII and *Hae*III, and the patterns separated electrophoretically. Species of long tailed hamster were discriminated from one another. A phylogenetic analysis of the dataset produced well distinguished clades.

Key words: *Calomyscus*, Cytochrome b, RFLP.

INTRODUCTION

The Calomyscidae is a small family of Rodentia classified into eight species within the genus *Calomyscus* (Wilson and Reeder, 2005). *Calomyscus* was first described by Thomas in 1905, and was long considered monotypic and represented by a single species, *C. bailwardi*, distributed in Iran, Afghanistan, Pakistan, Turkmenistan, and southern Azerbaijan-Naxcivan (Ellerman and Morrison-Scott, 1951).

Ellerman (1940) further classified *Calomyscus* into the superfamily Muroidea, family Muridae and subfamily Cricetinae. Later Chaline et al. (1977) re-assigned the genus to the family Cricetidae and subfamily Cricetinae. Recent classifications of *Calomyscus* place it in the subfamily Calomyscinae of the family Cricetidae. Molecular data suggest that *Calomyscus* is an isolated clade of the muroid rodent tree. Lack of morphological similarity between *Calomyscus* and any other muroid group has persuaded these authors to place it into a distinct family (Musser & Carleton, 2005).

Five species have been reported in Iran, *C. elburzensis, C. bailwardi*, *C. grandis*, *C. urartensis,* and *C. hotsoni*. All occupy well drained, barren, rocky habitats in the foothills and mountains (Malikov et al., 1999; Morshed and Patton, 2002) at elevations of 400-3500 m (Nowak, 1999; Tofts, 2003). Population distributions appear to be patchy, and some are geographically isolated (Graphodatsky et al., 2000).

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Mitochondrial DNA (mtDNA) is an appropriate marker for re-evaluation of the taxonomic status of closely related species or populations of a variety of species (Wilson et al., 1985). Mammalian mtDNA is inherited as a haploid from the mother (Hauswith and Laipis 1986). The evolutionary rate of mtDNA is 5 to 10 times faster than that of the nuclear genome (Brown et al., 1979), mainly because mitochondria do not have repair enzymes for errors in replication, or for damage to DNA (Clayton, 1982). Hence, mtDNA has a high level of transitions and transversions, as well as a high incidence of short length mutations (Cann and Wilson, 1983). Thus mitochondrial genes are important tools in a variety of fields related to the study of animal evolution, such as phylogeography, population genetics, and phylogenetics (Rokas et al., 2003). Cytochrome *b* has been considered one of the most useful genes for phylogenetic work, and has been shown to be a very effective DNA-region for species determination (Holand et al., 1993; Meyer et al., 1995).

In this study, the inter- and intra-specific variability of mitochondrial DNA of 47 samples of the five species of *Calomyscus* were analyzed by, PCR-RFLP. The cytochrome *b* gene was amplified by PCR amplification. RFLP analyses involved cutting DNA with one or more nucleases, separating the resulting fragments by molecular weight with gel electrophoresis, and visualizing the size-sorted fragments. Differences in digestion profiles among individuals may result from base substitutions within cleavage sites, insertions or deletions of DNA, or sequence rearrangements, with each source of variation producing characteristic banding changes. Restriction analyses therefore encompass a wide diversity of technical approaches. This PCR-based RFLP method offered some advantages: it required a small amount of template DNA, of a defined length, so size differences underlying RFLPs could be readily distinguished from restriction site differences; PCR amplifies DNA in an unmethylated condition, so natural DNA methylation is not a potential confounding source of variation in restriction digests; and the method does not require radioactive isotopes or autoradiography (Avise, 2004).

MATERIAL AND METHODS

Forty seven specimens of long tailed hamsters were collected from nine localities in Iran (Fig. 1). Sampling sites are listed in Table 1. A 0.01-0.02 g sample of liver or muscle was excised and stored in 98% ethanol. Total genomic DNA was extracted with an extraction kit (Genetbio) according to manufacturer's instructions.

TABLE 1. Sources and number of samples of *Calomyscus* spp.

Number of specimens	Sample localities	Province
8	Khajemorad	Khorasan Razavi
9	Aghdarband	Khorasan Razavi
3	Bojnord Field	Khorasan Shomali
1	Tandureh	Khorasan Razavi
6	Fasham	Tehran
5	Eslamiyeh, Fakhrabad	Yazd
3	Anjerk	Kerman
8	Zagros Mountain	Fars
4	Saravan	Sistan & Baluchestan

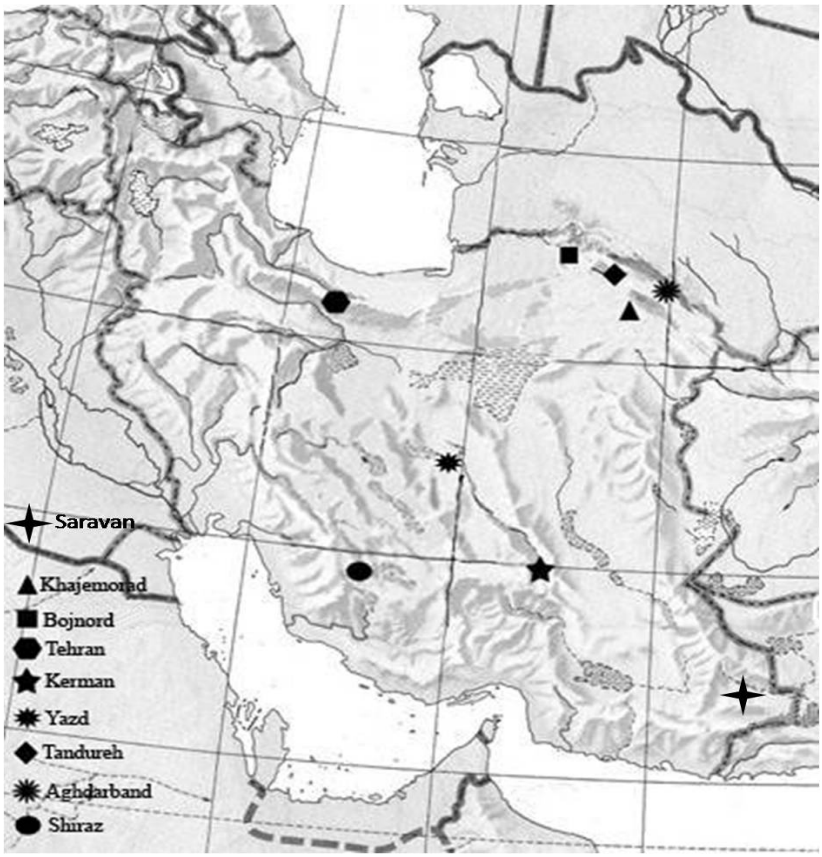

FIGURE 1. Map of sampling localities for *Calomyscus* spp. in Iran.

TABLE 2. Primers and sequences.

Primer	Sequence	Designer
L7	5'-ACTAATGACATGAAAAATCATCGTT-3'	Montgelard *et al.,* 2002
H6	5'-TCTTCATTTTTGGTTTACAAGAC-3'	Montgelard *et al.,* 2002

The complete cytochrome *b* gene was amplified according to the following program with modified L7 and H6 primers (Montgelard *et al.,* 2002) (Table 2).

For cytochrome *b*, amplifications consisted of an initial 2 min denaturation at 94°C followed by 35 cycles: 45s at 94°C, 45s at 50°C 90s at 68°C with a final extension cycle of 10 min. at 68°C, in a Primus 96 thermal cycler (Chevret *et al*., 2005).

Each PCR product was digested by three restriction enzymes, *Hae*III (GG/CC), *Hin*cII (GTY/RAC), and *Hin*fI (G/ANTC). A restriction enzyme (or restriction endonuclease) cuts double-stranded or single stranded DNA at specific recognition nucleotide sequences known as restriction sites (Roberts, 1967; Kessler and Manta, 1990; Pingoud *et a*l., 1993). Recognition of sequences in DNA differ for each restriction enzyme, producing differences in the length, sequence and strand orientation (5' end or the 3' end) of a sticky-end "overhang" of an enzyme restriction (Goodsell, 2002). When restriction enzymes cut at their prescribed nucleotide sequences, the fragments may

migrate differently (shorter or longer distances) on electrophoretic gels due to differing fragment lengths (Linebaugh, 2008). PCR products were incubated at 37°C for 3-4 h to be completely digested. The products were then separated by electrophoresis on a 1% agarose gel, according to the different patterns of band distribution on gels. The computer program PAUP*4 beta10 were used for analyzing the results.

RESULTS

The PCR products of cytochrome *b* gene were approximately 1200 bps in length. Each PCR product was digested by three restriction enzymes. The fragments produced by digestion of cyt *b* [using different restriction enzymes] represented usually different patterns on agarose gels among different populations (Figs 2-4 and Table 3).

A dendrogram of *Calomyscus* spp., using the polymorphic fragments, reconstructed by Phylip essence relatively resolved relationships among populations. As the cladogram of Figure 5 shows, the *C. elburzensis* populations represent one distinct clades contains the samples of Khajemorad, Yazd and Aghdarband. One clade contains samples from Aghdarband and Tandureh that do not belong to the other four species. The populations of Kerman and Shiraz produced a distinct clade which also contained *C. bailwardi*. The samples of the Tehran grouping with the C. *grandis* specimens formed the other distinct clade and the latter clade is the long tailed hamsters from Saravan that belong to *C. hatsoni* (Fig. 5).

Uncorrected genetic distance among the different populations of *Calomyscus* was also calculated using the program *Mega* version 4 (Tamura et al., 2007) (Table 4). The highest interpopulation genetic distance was 7.02%. Calculated between the Saravan and Tehran as well as Saravan and Tandoreh populations (Table 3).

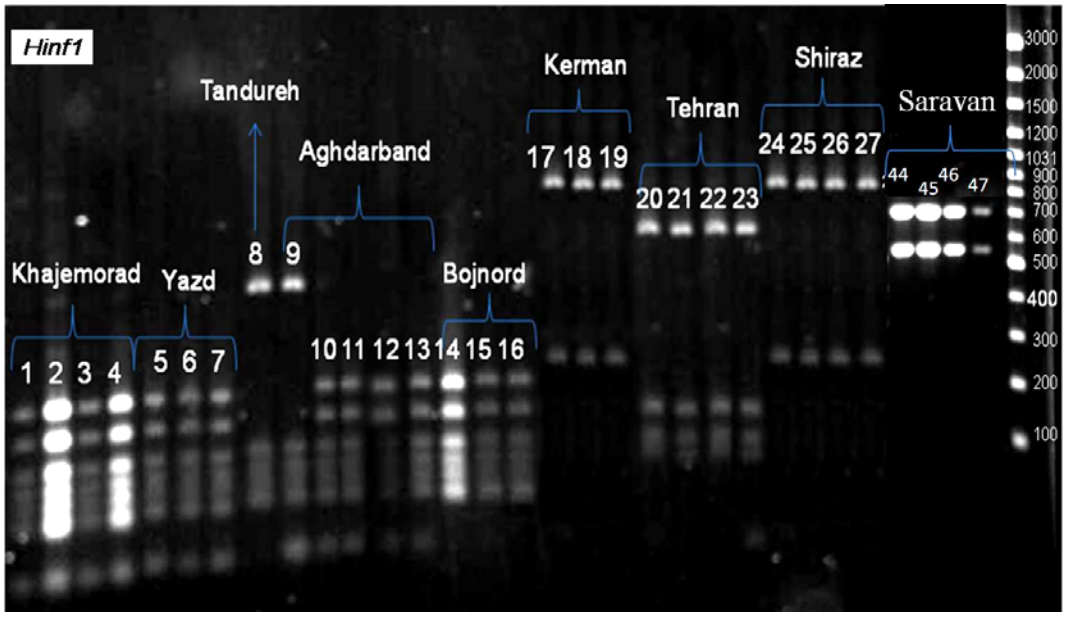

FIGURE 2. Restriction patterns of cytochrome *b* from *Calomyscus* spp on 1% agarose gel (*Hin*fI).

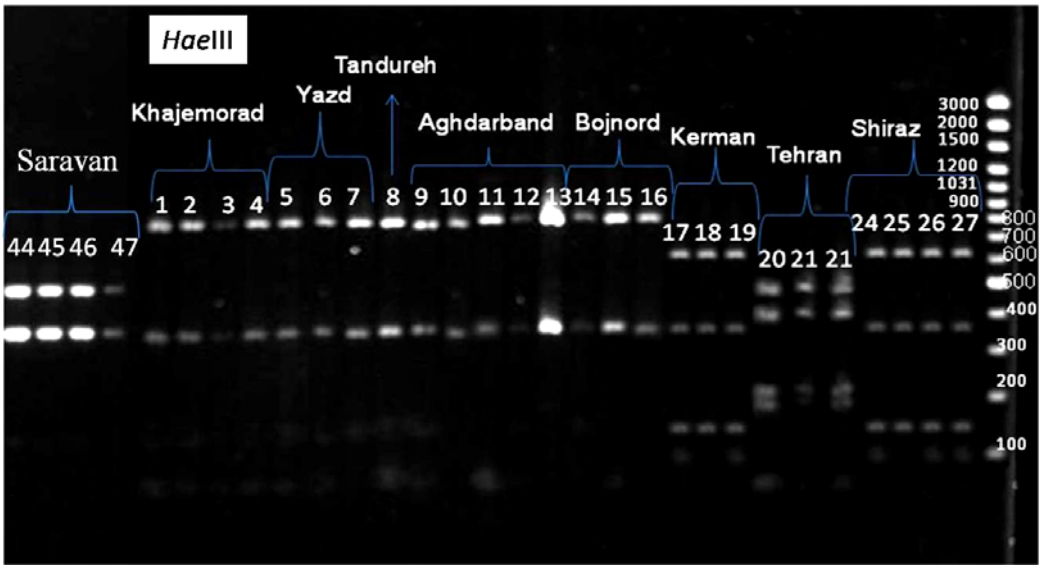

FIGURE 3. Restriction patterns of cytochrome *b* from *Calomyscus* spp. on 1% agarose gel (*Hae*III).

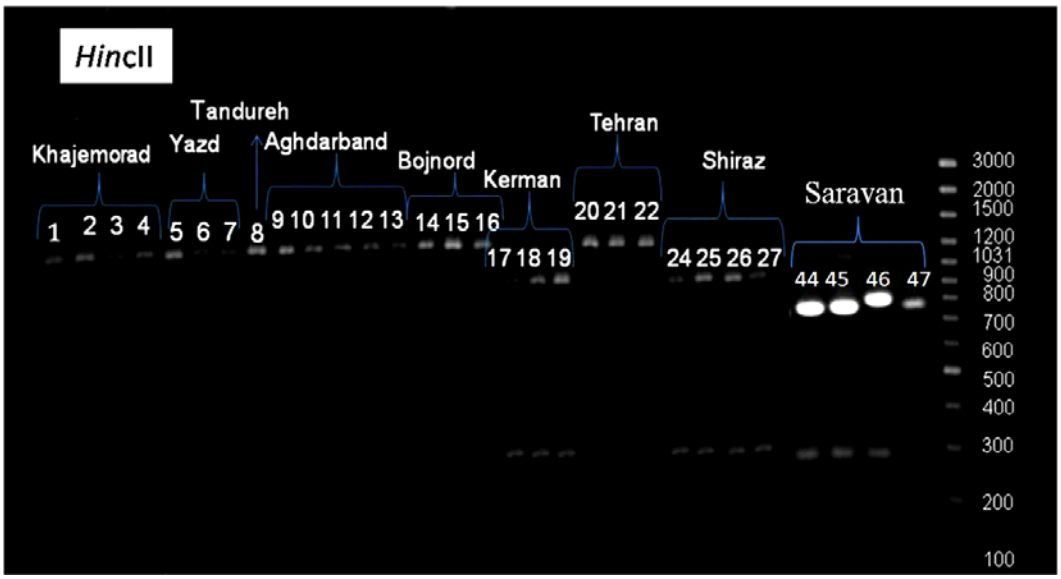

FIGURE 4. Restriction patterns of cytochrome *b* from *Calomyscus* spp. on 1% agarose gel (*Hin*cII).

TABLE 3. Different localities that were separated by three enzymes.

Enzymes	Locality				
*Hin*fI	Khajemorad Yazd Aghdarband Bojnord	Aghdarband (one sample) Tandure	Kerman Shiraz	Tehran	Saravan
*Hae*III	Khajemorad Yazd Tandure Aghdarband Bojnord		Kerman Shiraz	Tehran	Saravan
*Hin*cII	Khajemorad Yazd Tandure Aghdarband Bojnord		Kerman Shiraz	Tehran	Saravan

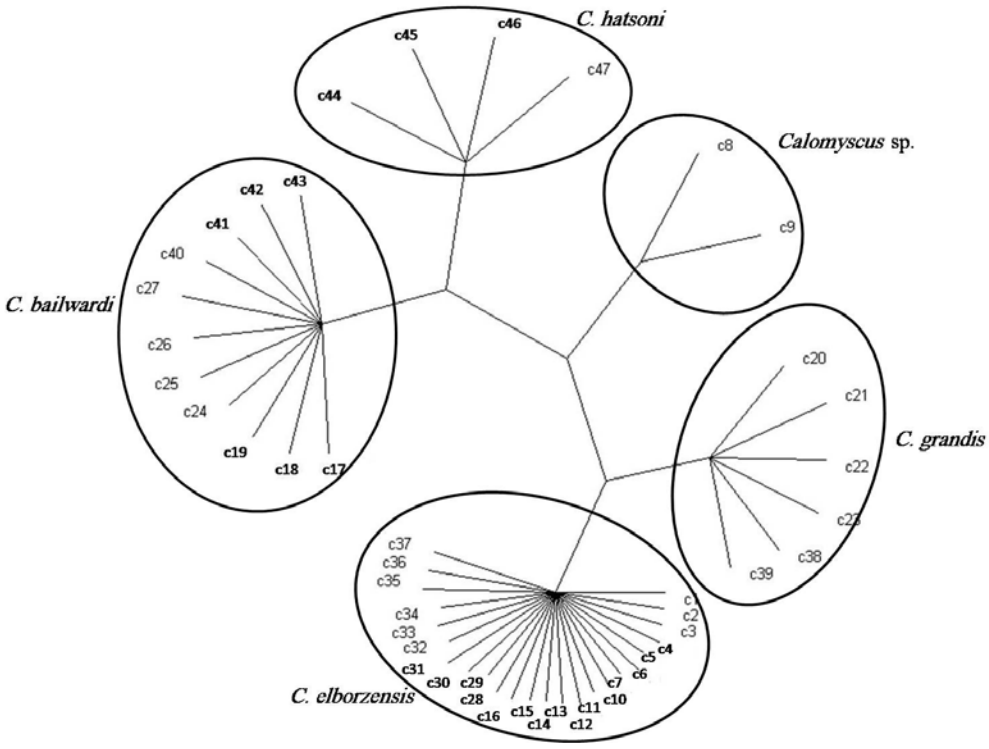

FIGURE 5. An unrooted dendrogram of *Calomyscus* spp. populations (1-4, 28-31 from Khajemorad; 5-7, 36 and 37: Yazd; 8: Tandure; 9-13, 32-35: Aghdarband; 14-16: Bojnord; 17-19: Kerman; 20-23, 38 and 39: Tehran; 24-27, 40-43: Shiraz; 44-47: Saravan).

TABLE 4. Uncorrected genetic distance among the different populations of four *Calomyscus* spp. included in this study (not unknown two samples from Tandure and Aghdarband). 1. Khajemorad, Aghdarband and Yazd, 2. Tandureh, 3. Bojnord, 4. Kerman and Shiraz, 5. Tehran, 6. Saravan.

	1	2	3	4	5	6
1						
2	0.160					
3	0.040	0.120				
4	0,600	0.520	0.560			
5	0.400	0.400	0.360	0.600		
6	0.640	0.720	0.680	0.680	0.720	

Since discrimination of species based on morphological characteristics of *Calomyscus* is difficult, the present investigation focused on using PCR-RFLP as a molecular marker to resolve the taxonomic status of the Iranian populations of *Calomyscus*. The use of mitochondrial DNA (mtDNA) to study phylogenetic relationships among natural populations of closely related species has many advantages: many mtDNA copies are present in each cell, gene content is highly conserved, increased rate of nucleotide substitution occurs within the molecule, and strict maternal inheritance prevents reduction of genetic divergence. Due to length variation of regions of mtDNA among species, the most widely used method for obtaining phylogenetic data is restriction enzyme fragment analysis. Restriction Fragment Length Polymorphisms (RFLPs) occur due to differences in lengths of the same DNA fragment among species (Linebaugh, 2008). In the present study, the cytochrome b gene was used to identify three species of *Calomyscus*. Using patterns produced after digestion, the species of *Calomyscus* were reliably discriminated from each other but the *Hin*cII enzyme could not separate thediscriminate *C. grandis* from *C. elburzemsis*. A phylogenetic analysis of the dataset produced well separated clades. The genetic distances among the populations were also in accord with the phylogeny recorded.

The smallest genetic distance was observed between the specimens of Khajemorad-Yazd-Aghdarband and Bojnoord (Table 3), subsequently as is seen in the tree the Bojnoord population is the sister taxon for the Khajemorad-Aghdarband-Yaze clade. On the other hand, the highest degree of genetic distance was observed between the Kerman-Shiraz clade and the Khajemorad-Aghdarband-Yazed populations which in turn are the most distantly related clades in the tree.

One of the outstanding consequences of the present study is that whereas the morphological data failed to clarify the taxonomic status of the Yazd population, the molecular data successfully clarified its taxonomic status as belonging to the *C. elburzensis* clade.

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