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IDENTIFICATION OF THREE IRANIAN SPECIES OF THE GENUS *RATTUS* (RODENTIA, MURIDAE) USING A PCR-RFLP TECHNIQUE ON MITOCHONDRIAL DNA

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ABSTRACT - Three species of the genus *Rattus* Fisher, 1803 have been reported from Iran: the brown rat (*R. norvegicus*), the black rat (*R. rattus*) and the Himalayan rat (*R. pyctoris*). The first two were introduced, whilst *R. pyctoris* is native and lives in mountainous regions from Pakistan to north-eastern Iran. In this study, the mitochondrial DNA from twenty six rats were analysed using a PCR-RFLP (Polymerase Chain Reaction - Restriction Fragments Length Polymorphism) method to investigate inter-specific variation. Part of the 16S rRNA and cytochrome *b* genes were amplified and digested with three restriction enzymes: *Alu*I, *Mbo*I and *Hin*fI. Restriction fragments resulted in four different haplotypes and allowed to distinguish the three *Rattus* species. Our results suggest that the Himalayan rats are more closely related to *R. rattus* than to *R. norvegicus* and provide the basics for further phylogenetic studies.

Key words: *Rattus norvegicus*, *R. rattus*, *R. pyctoris*, taxonomy, PCR-RFLP

RIASSUNTO - ***Identificazione di tre specie iraniane del genere* Rattus *(Rodentia, Muridae) tramite PCR-RFLP su DNA mitocondriale***. Tre specie del genere *Rattus* risultano diffuse in Iran: il surmolotto (*R. norvegicus*), il ratto nero (*R. rattus*) e il ratto himalayano (*R. pyctoris*). Le prime due specie sono state introdotte, mentre *R. pyctoris* è presente nelle aree montane che si sviluppano dal Pakistan all'Iran nordorientale. In questo studio, il DNA mitocondriale di 26 individui è stato analizzato tramite PCR-RFLP per evidenziare variazioni inter-specifiche. Parte dei geni del rRNA 16S e del citocromo *b* è stata amplificata e quindi sottoposta a digestione tramite tre diversi enzimi: *Alu*I, *Mbo*I e *Hin*fI. I frammenti di restrizione hanno permesso di individuare quattro aplotipi mitocondriali e di distinguere le tre specie. I risultati ottenuti suggeriscono che il ratto himalayano sia più vicino a *R. rattus* che non a *R. norvegicus* e pongono le basi per ulteriori studi filogenetici.

Parole chiave: *Rattus norvegicus*, *R. rattus*, *R. pyctoris*, tassonomia, PCR-RFLP

INTRODUCTION

Three species of the genus *Rattus* (Fisher, 1803) have been reported from Iran: the brown rat (*R. norvegicus*), the black rat (*R. rattus*) and the Himalayan rat (*R. pyctoris*; Fig. 1). The first two have a worldwide distribution, inhabiting also urban areas as pest species. Although *R. rattus* remains have been reported from Pleistocene deposits in western Iran (Hashemi *et al.,* 2006), the black rat has only recently been transported by ship from south-east Asia to the Persian Gulf and has successively spread in this region, especially on the Iranian border and in Mangrove woods near Bandar Abbas and Gheshm. During the 19th century, human activities also favoured its expansion into Shiraz, Esphahan and Tehran, and more recently the species has also been reported from the coasts of the Caspian Sea (Misonne, 1959). The brown rat has also moved from central Asia to the border of the Caspian Sea and to Gorgan and Rasht cities in the north. It has also been unintentionally introduced to Tehran and Tabriz in the north-west, while recently, it has been transported by train to Mashhad, in the north-east of Iran (Etemad, 1978; Panteleyev, 1998; Darvish *et al*., 2006). The Himalayan rat lives in mountainous regions, from Pakistan and Himalaya to Afghanistan and the north-east of Iran (Etemad, 1978; Ziaee, 1996; Seyed Mousavi *et al*., 2001). It has never been found in urban regions of Iran. It has been recently reported from the north-eastern part of Kerman province, and included in the same group of *R. norvegicus* (Musser and Carleton, 2005).

R. norvegicus differs from the two other species in having the tail shorter than body-length and short ears (Yigit *et al*., 1998); also its skull is distinctly different from those of *R. rattus* and *R. pyctoris* (Kayvanfar, in press). *R. norvegicus* has brown dorsal hair, while the dorsal fur of black rats from Shiraz and Mangrove forests is lighter than that of *R. norvegicus* (Kayvanfar, in press).

R. rattus and *R. pyctoris* are, however,

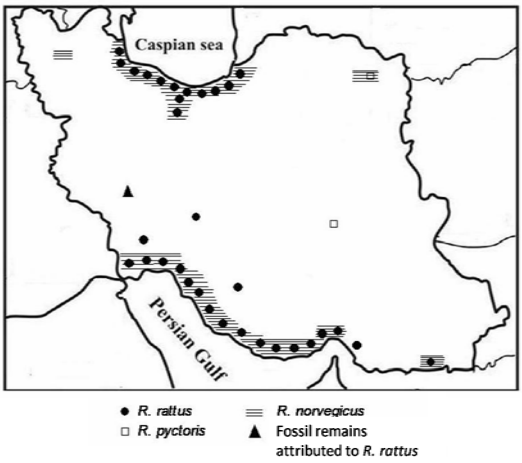

Figure 1 - Distribution of the genus *Rattus* in Iran.

very similar in both external morphology and skull. *R. pyctoris* has a shaggy, dense fur, six pairs of teats and a reduced antrolabial cusp (t3) relative to two adjacent cusps forming the anterior lamina (Musser and Carleton, 2005).
Karyological studies have shown that brown and Himalayan rats have 42 chromosomes, whilst in the black rat 2n = 38 (Kayvanfar, in press).
While morphologic, morphometric and karyologic studies have been carried out on Iranian rats by different investigators (Etemad, 1978; Kayvanfar, in press), until now no molecular studies have been attempted to answer the questions concerning the origin and radiation of Iranian rats and the taxonomic status of both the Mangrove rat populations of the Persian Gulf and those of the mountain rat in north-eastern Iran. Mitochondrial genes are important tools for the investigation of taxonomic status and phylogeography of different populations, especially for those with similar morphological phenons (Mayr and Ashlock, 2001; Rokas *et al*., 2003). In this study, mitochondrial DNA was analysed to determine the interspecific variability of Iranian *Rattus* species.

METHODS

The mtDNA of one laboratory rat (*R. norvegicus* strain wistar) and 25 wild rats from seven localities in Iran and Armenia (Table 1) was analysed.
From each rat, both liver and femur muscle were isolated and preserved in 98% ethanol. Genomic DNA was then extracted from 0.01-0.02 g of the tissues (Genetbio, Korea) and used as template for the amplification of both 16S rRNA (590 bps) and cytochrome *b* genes (1242 bps). A region of the DNA encoding 16S rRNA was amplified using two slightly modified primers, L2510 and H5080 (Palumbi *et al*., 1991; see also Klossa-Kilia *et al*., 2001), while the complete cytochrome *b* was amplified by two modified primers, L7 and H6 (Montgelard *et al*., 2002) (Table 2).
Amplification of cytochrome *b* was carried out in a Primus 96 thermal cycler with an initial denaturation step at 94°C for 2 min., followed by 35 cycles of 45s at 94°C, 45s at 50°C and 90s at 68°C, with a final extension time of 10 min. at 68°C (Chevret *et al*., 2005). For the 16S rRNA mtDNA segment, amplification conditions were as fol-

Table 1 - Number of analysed samples (N) and locality of collection for each *Rattus* species.

Species	N	Sampling station	Geographic coordinates	Elevation (m a.s.l.)
R. norvegicus	9	Railway of Mashhad	36°17′N 59°36′E	970
R. norvegicus	6	Shahre rey, Tehran	35°34′N 51°26′E	1191
R. norvegicus	1	Armenia	40°N 45°E	-
R. pyctoris	3	Noghondar	36°22′23″N 59°17′35″E	1400
R. rattus	1	Shiraz	29°36′N 52°32′E	1600
R. rattus	4	Minab, mangrove forest	27°07′04″N 56°53′41″E	5
R. norvegicus	1	Train Semnan to Mashhad	-	-

Table 2 - Sequences of the primers used for PCRs.

Primer	Sequence	References
L2510	5′-CGCCTGTTTACCAAAAACAT-3′	Palumbi *et al*., 1991
H5080	5′-CCGGTCTGAACTCAGATCACGT-3′	
L7	5′-ACTAATGACATGAAAAATCATCGTT-3′	Montgelard *et al.,* 2002
H6	5′-TCTTCATTTTTGGTTTACAAGAC-3′	

lows: one preliminary denaturation step at 94°C for 5 min., followed by 35 cycles of 60s at 94°C, 60s at 50°C and 90s at 72°C, and final extension at 72°C for 5 min. (Klossa-Kilia *et al*., 2001).

Each PCR product was then digested by three restriction enzymes; *Mbo*I, *Alu*I and *Hin*fI (Fermentas). The reactions were incubated at 37°C for 3-4 h for complete digestion. The results were analysed by electrophoresis on either 1% or 1.5% agarose gels or 12% polyacrylamide gels, and the resulting patterns were compared among different species.

The rate of differentiation of the amplified and digested fragments from different samples was assessed by the software Popgen (Population Genetics Analysis, 32-bit version). A dendrogram based on Nei's genetic distance (1978) was constructed and organized with the aid of Treeview.

RESULTS AND DISCUSSION

The mammalian mitochondrial DNA is a 16-17 kb double-stranded circular DNA molecule, characterized by its low-molecular mass, simple structure, high evolution rate and maternal inheritance (Schlick *et al*., 2006). On average, each somatic cell has 100-500 mitochondria, and each mitochondrion has 1-15 mtDNA molecules. Because of its high sensitivity to damage and its low ability to repair lesions, the rate of sequence evolution in mtDNA is 10-20 times higher than that in the nuclear ge-

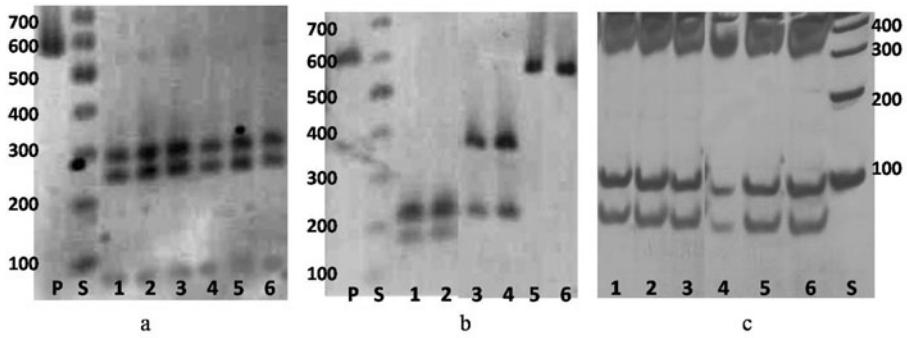

Figure 2. - Restriction patterns of 16S rRNA on agarose and polyacrylamide gels. (a: *Alu*I cleavage pattern on 1% agarose gel; b: *Hin*fI cleavage pattern on 1.5% agarose gel; c: *Mbo*I cleavage pattern on 12% polyacrylamide gel. 1: *R. norvegicus* from Armenia; 2: *R. norvegicus* from Iran; 3, 4: *R. pyctoris*; 5, 6: *R. rattus*; P: undigested PCR product; S: 100 bps size marker).

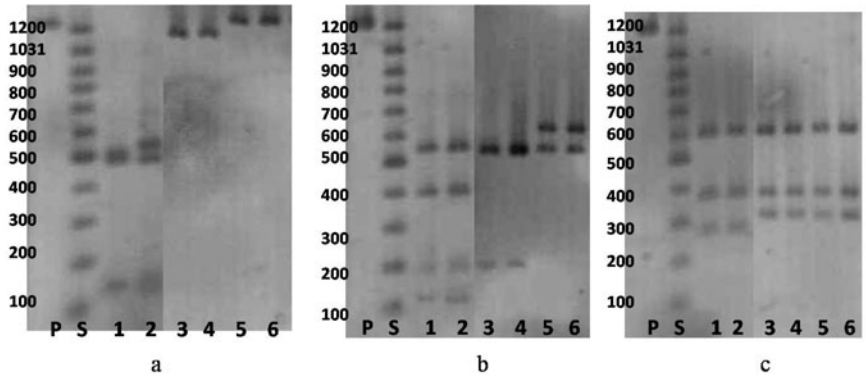

Figure 3 - Restriction patterns of cytochrome *b* on 1% agarose gel (a: *Alu*I cleavage pattern; b: *Hin*fI; c: *Mbo*I; 1: *R. norvegicus* from Armenia; 2: *R. norvegicus* from Iran; 3, 4: *R. pyctoris*; 5, 6: *R. rattus*; P: PCR product; S: 100 bps size marker).

nome and, consequently, any two mtDNA genomes may differ in 10-66 nucleotides (Dai *et al*., 2005). In this study, restriction morphs (Fig. 2 and 3) were analysed for all 26 samples. Tables 3 and 4 show the four haplotypes - H01, H02, H03 and H04 -, that were generated by the three restriction enzymes.

Studies on mtDNA using RFLP techniques revealed the presence of 4 groups and 4 subgroups for brown rats and 5 groups and 3 subgroups for black rats (Brown and Simpson, 1981). Hilsdorf and Krieger (1999) also studied the mtDNA of laboratory rats and found 4 different groups using the same method.

The digestion of amplified regions of 16S rRNA gene with *Hin*fI, and cytochrome *b* with *Hin*fI and *Alu*I, differentiated the three species. Cytochrome *b* treated with *Alu*I distinguished between *R. norvegicus* from Iran and Armenia, while the use of *Mbo*I allowed to separate *R. norvegicus* from *R. rattus* and *R. pyctoris* (Table 3).

No difference emerged between the restriction patterns for brown rats from Tehran and Mashhad (Fig. 2 and 3). Also the laboratory rat did not show any difference with commensally wild brown rats, except for the only sample from Armenia (H02), which differed from the Iranian *R. norvegicus* (H01).

The genetic distance between these two haplotypes was 0.18, while both resulted in distinct separation from the other two species (1.098). The general lack of intraspecific variability might be explained by the low mutation rate of the markers (about 0.02 mutations per site per one million years for cytochrome b and rRNA genes of laboratory mice; Goios *et al*., 2007), which probably is not able to highlight the intraspecific variability of recently diverged populations. The application of the RFLP method on a more variable marker, such as the mitochondrial control region, could provide more reliable information (Robins *et al*., 2008).

The genetic distance between *R. pyctoris* and *R. rattus* (H03 and H04)

Table 3 - Restriction patterns and haplotypes produced by *Alu*I, *Hin*fI and *Mbo*I (*brown rat from Armenia; ***R. norvegicus* strain wistar).

		16S rRNA			Cytochrome *b*		
		Enzymes					
Species	ID numbers	*Alu*I	*Hin*fI	*Mbo*I	*Alu*I	*Hin*fI	*Mbo*I
R. norvegicus	1	A	A	A	A	A	A
	2	A	A	A	A	A	A
	3	A	A	A	A	A	A
	4	A	A	A	A	A	A
	5	A	A	A	A	A	A
	6	A	A	A	A	A	A
	7	A	A	A	A	A	A
	8	A	A	A	A	A	A
	9	A	A	A	A	A	A
	10	A	A	A	A	A	A
	11	A	A	A	A	A	A
	12	A	A	A	A	A	A
	13	A	A	A	A	A	A
	14	A	A	A	A	A	A
	15	A	A	A	A	A	A
	20	A	A	A	A	A	A
	24*	A	A	A	D	A	A
	26**	A	A	A	A	A	A
R. pyctoris	16	A	B	A	B	B	B
	17	A	B	A	B	B	B
	18	A	B	A	B	B	B
R. rattus	19	A	C	A	C	C	B
	21	A	C	A	C	C	B
	22	A	C	A	C	C	B
	23	A	C	A	C	C	B
	25	A	C	A	C	C	B

Table 4 - The four mtDNA haplotypes identified from the 26 analysed samples (*brown rat from Armenia; ***R. norvegicus* strain wistar).

Code	Haplotype	ID numbers	Species
H01	AAAAAA	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 26**	*R. norvegicus*
H02	AAADAA	24*	*R. norvegicus*
H03	ABABBB	16, 17, 18	*R. pyctoris*
H04	ACACCB	19, 21, 22, 23, 25	*R. rattus*

Table 5 - Nei's genetic distance between the four haplotypes.

Code	H01	H02	H03	H04
H01	*	0.1823	1.0986	1.0986
H02	0.1823	*	1.0986	1.0986
H03	1.0986	1.0986	*	0.6931
H04	1.0986	1.0986	0.6931	*

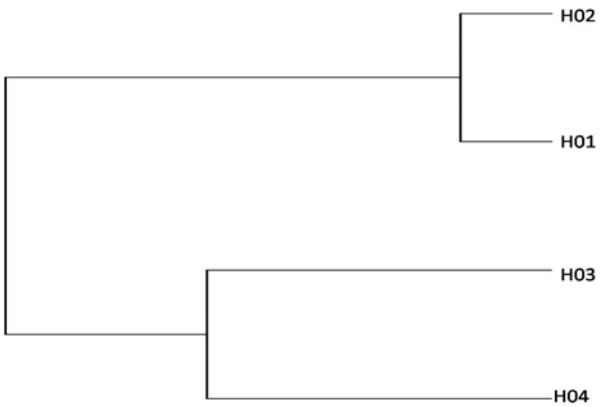

Figure 4 - Dendrogram of the genetic distances between the four haplotypes.

was 0.69 (Table 5), suggesting, contrary to the results of Musser and Carleton (2005), that *R. pyctoris* is more closely related to *R. rattus* than *R. norvegicus* (Fig. 4); the ongoing sequencing of the amplified fragments will provide further useful information. Although the analyses have to be extended to a higher number of samples, our initial results demonstrate that the species of genus *Rattus* from the Iranian plateau need further phylogenetic studies to better highlight their origin and taxonomic status.

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REFERENCES

Brown G.G. and Simpson M.V. 1981. Intra- and interspecific variation of the mitochondrial genome in *Rattus norvegicus* and *Rattus rattus*: restriction enzyme analysis of variant mitochondrial DNA molecules and their evolutionary relationship. *Genetics*, 97: 125-143.

Chevret P., Veyrunes F. and Britton-Davidian J. 2005. Molecular phylogeny of the genus *Mus* (Rodentia: Muridae) based on mitochondrial and nuclear data. *Biol. J. Linn. Soc*., 84: 417-427.

Dai J., Min J., Xiao Y., Lei X., Shen W. and Wei H. 2005. The absence of mi-

tochondrial DNA diversity among common laboratory inbred mouse strains. *J. Exp. Biol*., 208: 4445-4450.

Darvish J., Siahsarvie R., Mirshamsi O., Kayvanfar N., Hashemi N. and Sadeghie Shakib F. 2006. Diversity of the rodent of northeastern Iran. *Iranian J. of Animal Biosystematics*, 2(1): 57-76.

Etemad E. 1978. Mammals of Iran; Vol. I: Rodents and key to their identification. National society of natural sources and human environmental protection Publications, Tehran.

Goios A., Pereira L., Bogue M., Macaulay V. and Amorim A. 2007. mtDNA phylogeny and evolution of laboratory mouse strains. *Genome Res.*, 17(3): 293–298.

Hashemi N., Darvish J., Mashkur M and Biglari F. 2006. Rodents and Lagomorphs remains from late Pleistocene and early Holocene caves and rochshelter sites in the Zagros region, Iran. *Iranian J. of Animal Biosystematics*, 2(1): 25-33.

Hilsdorf A. and Krieger J. 1999. Characterization of six rat strains (*Rattus norvegicus*) by mitochondrial DNA restriction fragment length polymorphism. *Braz. J. Med. Biol. Res*., 32: 267-273.

Kayvanfar N., Siahsarvie R. and Darvish J. (in press). Investigation of genus *Rattus* (Muridae, Rodentia) in Iran based on karyologic, morphometric and morphological studies. *Iranian J. of Animal Biosystematics*.

Klossa-Kilia E., Papasotiropoulos V. and Alahiotis S. 2001. Authentication of Messolongi (Greece) fish roe using PCR-RFLP analysis of 16S rRNA mtDNA segment. *Food control*, 13(3): 169-172.

Mayr E. and Ashlock P.D. 2001. Principles of systematic zoology, McGraw-Hill Book Co., Singapore.

Misonne X. 1959. Analayse zoogéographique des Mammifères de l'Iran. Inst. Royal Sciences Nat.

Montgelard C., Bentz S., Tirard C., Verneau O. and Catzeflis F. 2002. Molecular systematics of Sciurognathi (Rodentia): the mitochondrial cytochrome *b* and 12S rRNA genes support the Anomaluroidea (Pedetidae and Anomaluridae). *Mol. Phylogenet. Evol*., 22(2): 220-233.

Musser G.G. and Carleton M.D. 2005. Subfamily Murinae. In: Wilson D.E. and Reeder D.M. (eds.). Mammal species of the world. The Johns Hopkins University Press Baltimore, Vol. 2: 1247-1252, 1460- 1494.

Palumbi S., Martin A., Romano S., McMillan W.O., Stice L. and Grabowski G. 1991. The simple fools guide to PCR, version II. University of Hawaii, Honolulu.

Panteleyev P.A. 1998. The rodents of the Palearctic: composition and areas. Russian Academy of Sciences, Zoological Institute.

Robins J.H., McLenachan P.A., Phillips M.J., Craig L., Ross H.A. and Matisoo-Smith E. 2008. Dating of divergences within the *Rattus* genus phylogeny using whole mitochondrial genomes. *Mol. Phylogenet. Evol*., 49: 60-466.

Rokas A., Ladoukakis E. and Zouros E. 2003. Animal mitochondrial DNA recombination revisited. *Trends Ecol. Evol*., 18: 411-417.

Schlick N., Jensen-Seaman M., Orlebeke K., Kwitek A., Jacob H. and Lazar J. 2006. Sequence analysis of the complete mitochondrial DNA in 10 commonly used inbred rat strains. *Am. J. Physiol. Cell Physiol*., 291: C1183-C1192.

Seyed Mousavi F., Darvish J. and Aliabadian M. 2001. Biosystematics of *Rattus turkestanicus* (Rodentia) of Mashhad region. Plant Pests and Disease Research

Institute, 68: 1-2 (in Persian).

Yigit N., Colak E. and Sozen M. 1998. The taxonomy and karyology of *Rattus norvegicus* (Berknhout, 1769) and *Rattus rattus* (Linnaeus, 1758) (Rodentia: Muridae) in Turkey. *Turkish Journal of Zoology*, 22: 203- 212.

Ziaee H. 1996. A field guide to the Mammals of Iran, Department of the environment, Tehran, Iran.