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Morphologic, Morphometric and Molecular Comparison of Two Sister Species of Rodents as Potential Reservoir Hosts of Zoonotic Cutaneous Leishmaniasis in the Southwest of Iran

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ABSTRACT

Introduction: Rodents are reservoir hosts of various infectious diseases. Many species and subspecies of genus *Rattus* play a significant role as potential reservoir hosts of different emerging and re-emerging diseases, including leishmaniasis. **Methods:** Rodents were captured using live wooden traps from different localities of Khuzestan Province, southwest of Iran. To precise identification of two sister species of rats, including *Rattus rattus* and *Rattus norvegicus*, morphological, molecular, and biosystematics characters were examined using amplification of mitochondrial Cytochrome *b* (Cyt*b*) gene fragment. **Results:** Out of 119 captured rodents, 44 were *R. rattus*, 12 were *R. norvegicus,* and 63 belonged to other species (*Tatera indica*, *Nesokia indica*, *Mus musculus*). Partial Cyt *b* gene (≤624 bp) was amplified to characterize *R. rattus* and *R. norvegicus,* accurately. Three haplotypes of *R. rattus* (six samples) and a unique haplotype of *R. norvegicus* (three samples) were identified with some nucleotide variations. **Conclusion:** Mitochondrial results confirmed morphological disparity between the two *Rattus* species in Khuzestan Province. Therefore, we recommend applying an integrative approach to identify host reservoirs for infectious diseases, especially those suspected as reservoirs of cutaneous Leishmaniasis.

INTRODUCTION

Rodents are reservoir hosts of at least 60 zoonotic diseases [1-2]. Asian rodents of the genus *Rattus* are reservoir hosts of several significant infectious diseases such as plague, murine typhus, scrub typhus, leptospirosis, and Hantavirus hemorrhagic fever [2-3]. Leishmaniasis is an emerging infectious disease caused by the protozoan parasites of the genus *Leishmania.* This disease is endemic in 98 countries in the world; about 90% of new cases occurs in 13 countries, including Iran and different rodent species serve as reservoir hosts of the pathogenic agents [4-5]. Leishmaniasis presents in three primary forms: visceral, cutaneous, and mucocutaneous, with the last mostly confined to the new world.

Some rodents, as proven or potential reservoir hosts of diseases, have similar or close morphologic characters and are indistinguishable from each other. Accurate morphologic, morphometric, and molecular characterization of different rodent species is essential in the strategic planning for disease control [6].

Many reports on detection, isolation, and molecular identification of *Leishmania* parasites from different rodent species are available [7-11], but there is not much data on molecular identity and systematics of the two closely related rodent species [12] *Rattus rattus,* and *Rattus norvegicus* (also called sister species).

Recently, following the detection of *Leishmania major*, the causative agent of zoonotic cutaneous leishmaniasis, in *R. norvegicus*, the species was considered as a potential reservoir of ZCL in Fars Province of Iran [13]. Genus *Rattus* from the subfamily Murinae is a taxonomically mixed group, comprising many species and subspecies worldwide [14]. The brown rat, *R. norvegicus*, and the black rat, *R. rattus*, share some similar morphological features and may co-occur in some geographical areas. In the present, to examine the morphological disparity between these two sympatric species in Khuzestan Province, we examined the morphological characteristics, along with molecular characterization based on the mitochondrial cyt*b* marker.

MATERIAL AND METHODS

Ethics Statement. The animals were treated in accordance with the guidelines of the ethics committee of the Pasteur Institute of Iran (approval reference: 91/0201/4558).

Rodent sampling. Regarding the prevalence of cutaneous leishmaniasis disease in the area, the rodents were collected from 16 localities in North, South, East, West, and center of Khuzestan Province. The area is about 18 m above mean sea level (MAMSL) and is within the geographical coordinates 30˚19′40″ N to 32˚ 22′59″ N and 47˚ 55′ 59″E to 50˚ 13′ 8″E (Fig. 1).

Fig. 1. Map of Khuzestan Province, Iran. Sixteen localities in ten cities in which the rodents were collected

First, the active colonies of rodents were identified, and then 50 live traps for each location were put near the rodent burrows [15]. The specimens were captured using live wooden traps baited with cucumber, butter, cheese, or bread from June 12th to July12th, 2014.

Morphological identification. Specimens were identified first based on morphological characters using external criteria according to the standard reference for the rodents in Iran [16]. In all animals, the head and body length, tail length, ear length, hindfoot length were measured (Table 1). After preparing the skulls, 15 cranial variables were measured as described by others [14, 17] using a digital caliper with a 0.1 mm precision [12] (Table 2).

DNA extraction and PCR. Whole genomic DNA was extracted from a piece of an ear of animals using ISH-Horovize and DynaBio kit. A partial sequence of mitochondrial cytochrome *b* gene was amplified using a pair primers UNFOR403-(5'-TGAGGACAAATATCATT-CTGAGG-3') and UNREV1025- (5'-GGTTGTCCT-CCAATTCATGTTA-3') [18] using *Taq* polymerase enzyme (TakapooZist, Iran) and 50 ng DNA.

The amplification was performed in a thermocycler (Eppendorf, Hamburg, Germany) programmed for an initial denaturation of 5 min at 95˚C, followed by 35 cycles, each consisting 95˚C for 1 min, 58˚C for 1 min, and 72˚C for 1 min and a final extension at 72˚C for 7 min [18]. PCR products were visualized on 2% agarose gel along with a premix DNA ladder (Parstous Biotechnology, Iran). The PCR products were sequenced in both directions by a commercial company (Bioneer company, South Korea) using the Sanger method.

Alignment and phylogenetic analysis. DNA sequences were edited and aligned using SequencherTm v.4.1.4 software (Gene Codes Corporation). The final alignment was checked for unexpected stop codons using MEGA6, and a 601 bp was used to construct a phylogenic tree with Maximum Likelihood (ML) procedure using parsimony criteria in MEGA6 software [19].

RESULTS

Morphology and morphometry. We captured 44 *R. rattus* and 12 *R. norvegicus.* The external morphological features and cranial criteria were used to identify the two species (Tables 1 and 2, Figs. 2 and 3). Unlike *R. rattus*, the length of the tail in *R. norvegicus* was less than the head-body. In *R. norvegicus*, the ear was shorter and, when laid forward, did not reach the eye, while in *R. rattus* specimens, the ears reached the eyes when laid forward (Fig. 2). Some morphological characters are summarized in Tables 1 and 2.

Molecular characterization and DNA analysis of *R. rattus* and *R. norvegicus.* From 56 examined *Rattus* specimens, only nine were used for molecular characterization. Our study revealed three unique haplotypes within six *R. rattus* sequences (Accession Nos. MH311782, MH345733, MH345734) and one haplotype within three *R. norvegicus* sequences (Accession No. MH281952).

A phylogenetic tree was constructed using the sequences obtained in this study and those of other species available in the GenBank database (AB033702, *R. rattus* from Japan; AF295545, *R. norvegicus* from China; KP001566, *Tatera indica* from Khuzestan, Iran; KF783119 and KF783118, *Erinaceus europaeus* from Russia), (Fig. 4)

Table 1. Morphological and morphometric characters of *R. rattus* (R.r) and *R. norvegicus* (R.n) collected in Khuzestan province.

		Head and body length						Tail length						Ear length					Hindfoot length							
		10.5-23						**11-22**						**1-2.3**					**3-4.5**					**Gender**		**Total**
Location	**Species**	10.5-12.5	13-15	16-18	18.5-20	20.5-23	*P**	11-13	13.5-15	16-18.5	19-20	20.5-22	*P**	1-1.5	1.7-2.3	*P*§	*r*£	*P*¥	3	3.5	4	4.5	*P**	M	F	N (%)
Shadegan	R.r	7	17	11	3	0		4	9	9	9	7		22	16				21	17	0	0		23	15	38 (67.9)
Abadan	R.r	0	0	0	0	0		0	0	0	0	0		0	0				0	0	0	0		0	0	0 (0)
Khoramshahr	R.r	0	0	0	0	0	0.51	0	0	0	0	0	0.64	0	0	0.48	-0.43	0.11	0	0	0	0	0.48	0	0	0 (0)
Shushtar	R.r	1	2	0	0	0		0	1	2	0	0		1	2				1	2	0	0		2	1	3 (5.4)
Dezful	R.r	0	3	0	0	0		0	1	2	0	0		3	0				0	2	0	1		2	1	3 (5.4)
Shadegan	R.n	0	0	0	0	0		0	0	0	0	0		0	0				0	0	0	0		0	0	0 (0)
Abadan	R.n	2	2	0	0	2		4	0	1	0	1		3	3				0	5	1	0		0	6	6 (10.7)
Khoramshahr	R.n	0	0	0	4	0	0.55	0	0	3	1	0	0.34	0	4				0	0	1	3	0.41	3	1	4 (7.1)
Shushtar	R.n	0	0	0	0	0		0	0	0	0	0		0	0				0	0	0	0		0	0	0 (0)
Dezful	R.n	2	0	0	0	0		2	0	0	0	0		2	0				0	2	0	0		0	2	2 (3.6)
Total No. of R.r Species		8	22	11	3	0		4	11	13	9	7		26	18				22	21	0	1		27	17	44 (78.5)
Total No. of R.n Species		4	2	0	4	2		6	0	4	1	1		5	7				0	7	2	3		3	9	12 (21.4)
Total		12	24	11	7	2		10	11	17	10	8		31	25				22	28	2	4		30	26	56 (100)

* *P*-value is generated using the ANOVA test. § *P*-value is generated using Tajima's D index. £ Correlation coefficient. ¥ *P*-value is generated using Tajima's D index. M, Male; F, Female; R.r, *Rattus rattus*; R.n, *Rattus norvegicus*

Table 2. Morphological characters of *R. rattus* and *R. norvegicus* based on the skulls in the study areas (Student's paired t-test: one-tailed p-values was derived from the two-tailed p-values)

Morphological Characters	**Rodent species**										**Correlation coefficient (r)**	**T test (*P* value) (*P* <0.05)**
	R. rattus					*R. norvegicus*						
	Rodent code numbers											
	88	90	91	95	97	120	121	122	123	125		
Width of rostrum	0.64	0.61	0.59	0.62	0.60	0.73	0.54	0.54	0.54	0.54	0.82	0.0419*
Occipitonasal length	4.45	4.22	4.24	4.12	4.01	4.84	3.55	3.52	3.38	3.60	0.80	0.0490*
Condylbasal length	4.24	4.08	4.11	3.98	3.89	4.66	3.45	3.37	3.30	3.50	0.72	0.0842
Zygomatic width	2.07	1.95	2.00	1.99	1.91	2.21	1.77	1.76	1.70	1.78	0.73	0.0775
Least interorbital width	0.60	0.59	0.60	0.58	0.58	0.67	0.58	0.57	0.62	0.57	0.28	0.3186
Cranial width	1.40	1.30	1.33	1.35	1.30	1.44	1.28	1.26	1.34	1.26	0.93	0.0100*
Length of nasal	1.70	1.52	1.55	1.45	1.48	1.90	1.26	1.25	1.14	1.28	0.94	0.0070*
Length of diastema	1.21	1.11	1.17	1.09	1.01	1.32	0.94	0.93	0.89	0.93	0.67	0.1075
Length of anterior palatine foramina	0.81	0.73	0.78	0.70	0.71	0.82	0.64	0.63	0.60	0.62	0.82	0.0412*
Length of tympanic bullae	0.77	0.70	0.65	0.70	0.63	0.79	0.66	0.66	0.68	0.63	0.92	0.0129*
Width of tympanic bullae	0.65	0.64	0.47	0.67	0.51	0.67	0.49	0.45	0.47	0.50	0.41	0.2417
Upper cheekteeth	0.73	0.71	0.69	0.68	0.69	0.74	0.68	0.69	0.60	0.72	0.67	0.1054
Lower cheekteeth	0.66	0.65	0.61	0.61	0.60	0.72	0.68	0.59	0.69	0.59	0.76	0.0660
Height of skull	1.45	1.38	1.31	1.30	1.26	1.51	1.15	1.22	1.07	1.26	0.63	0.1235
Length of mandible	2.44	2.29	2.20	2.29	2.16	2.62	1.90	0.87	1.87	0.97	0.96	0.0036*

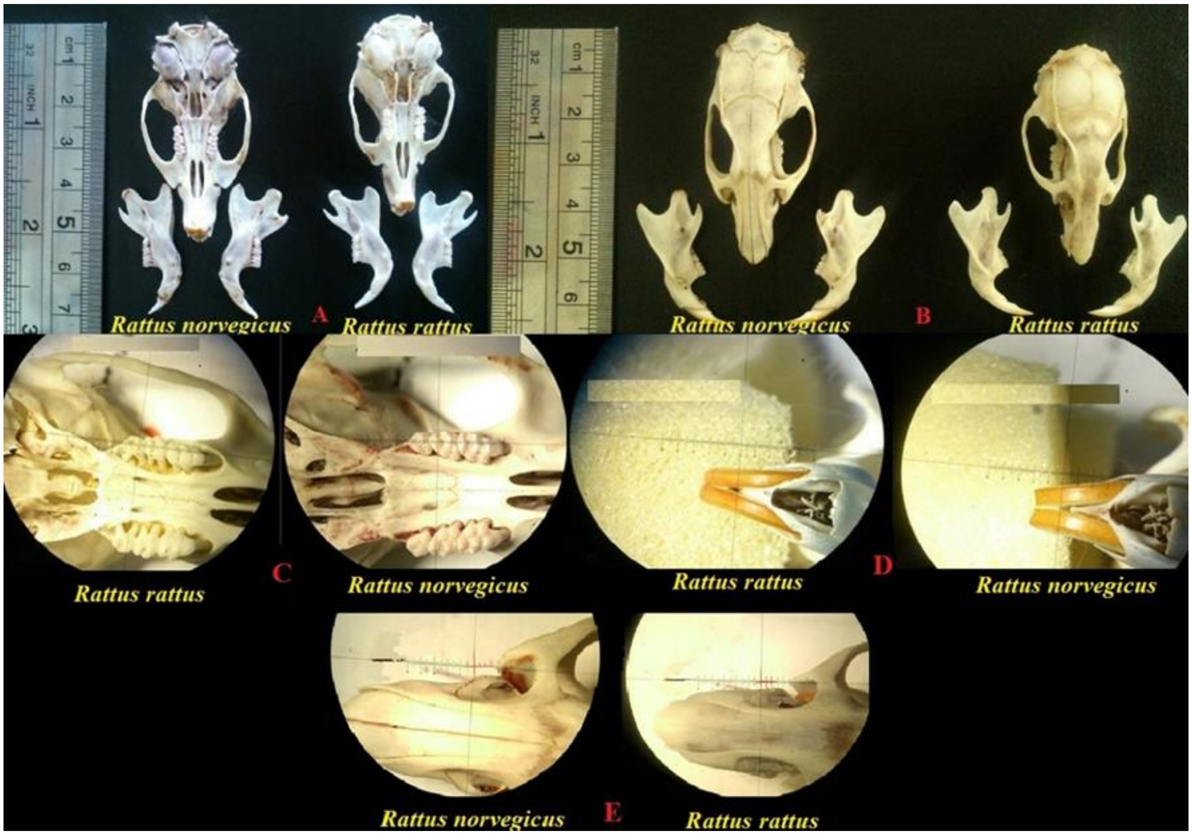

Fig. 2. The skulls of *R. rattus* and *R. norvegicus*. The ventral (A) and dorsal (B) surface of the skull of the two species with morphologic differences reflected in the zygomatic plate (C) upper molars (D) upper incisors (E).

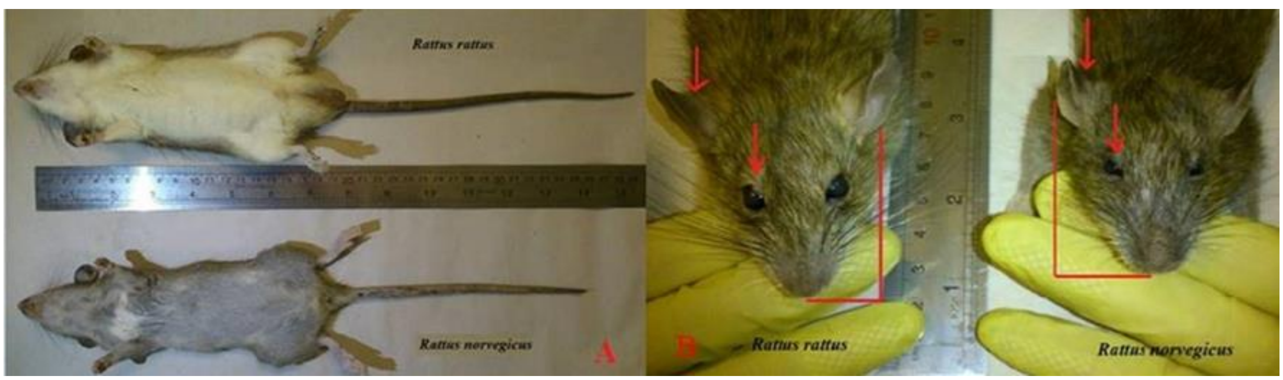

Fig. 3. *Rattus rattus* and *R. norvegicus* external view, including morphologic differences in ventral surface (A) and skulls (B).

DISCUSSION

Rodents are known as reservoir hosts for many infectious diseases [20-23]. These diseases can be transmitted through bites or direct contact with contaminated food, feces, and urine of the rodents or infective bites of arthropod vectors. Classification of rodents based on morphological criteria is the subject of numerous publications in the world [24]. However, the relationships among rodent families are confounded by the current morphological characters [25, 26]. The *R. norvegicus* and *R. rattus* are known as reservoir hosts of ZCL in Khuzestan Province and have a significant role in maintaining *Leishmania* parasites in this area, which highlights the importance of current research on these two species. Since these species vary in preference habitat, behavior, and biology, accurate identification of host species is of high importance and is critical in adopting strategies for controlling programs.

According to Montgelard *et al.* [27], mitochondrial genes, as well as nuclear exonic and intronic sequences, can help to classify mouse-related clades. Mitochondrial DNA markers are considered useful tools in identifying potential cryptic species [28]. Cytochrome *b* is a commonly used mitochondrial gene for species identification and determination of phylogenetic relationships [29].

There are many reports on using molecular approaches for the identification of rodents in Iran, while the data on the identity of rodents that serve as reservoirs of

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leishmaniasis is not much [30, 31], and most studies focused on taxonomy, phylogeny, and phylogeography of the rodents [12, 16, 32, 33]. Recently, the Cyt*b* gene revealed intraspecific variations among *Tatera indica* specimen, a reservoir host of cutaneous leishmaniasis in southern Iran (from the area where we collected *R. rattus* and *R. norvegicus* specimen) [12].

Our phylogenetic tree exhibited separation of two species, *R. norvegicus*, and *R. rattus* into two clades and confirmed the morphological disparity between these species. Our results indicated three haplotypes for *R. rattus* (MH311782, MH345733, MH345734) and one haplotype for *R. norvegicus* (MH281952) in the studied area, southwest of Iran.

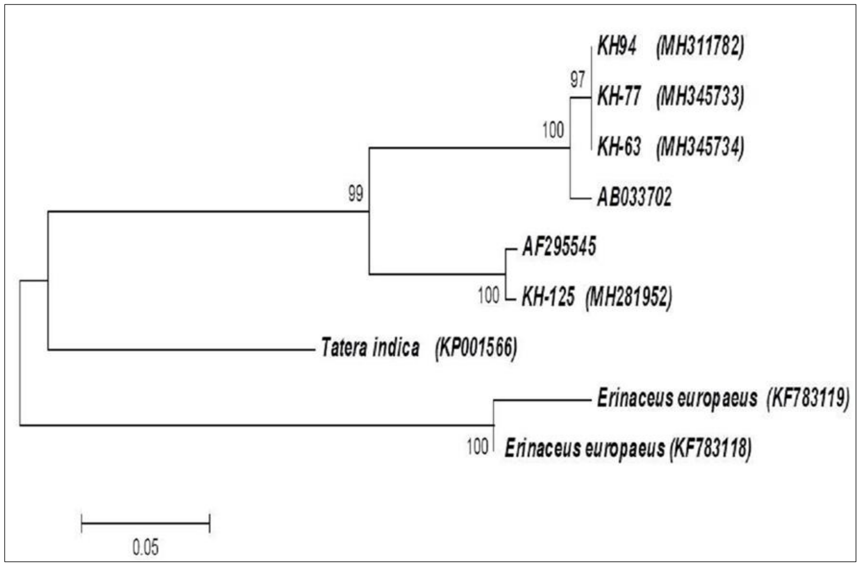

Fig. 4. The ML tree constructed based on the partial sequence of the cyt*b* gene. Only the bootstraps ≥ 90 are shown. The scale represents the number of base substitutions per site. KH-94, KH-77, and KH-63 represent *R. rattus* and KH-125 *R. norvegicus* investigated in this study. (AB033702, *R. rattus* from Japan; AF295545, *R. norvegicus* from China; KP001566, *Tatera indica* from Khuzestan, Iran; KF783119 and KF783118, *Erinaceus europaeus* from Russia).

Our study provided three haplotypes for *R. rattus*, which differed by 1 to 6 nucleotides, while the *R. norvegicus* rats, including those obtained from GenBank differed by 1–5 nucleotides. The variation between *R. rattus* and *R.norvegicus* sequences was high (14.17% or 34 among 240 bp). One-tailed and two-tailed tests revealed no significant differences among morphometric measurements and morphologic features ($P>0.05$) and confirmed the previous reports on these two species that showed similarity in morphological criteria [14]. The correlation coefficient (r) was used to measure how strong a relationship between the ear length of two species (*R. rattus* and *R. norvegicus*) was and revealed no significant linear relationship (r=-0.4291 correlation) between *R. rattus* and *R. norvegicus* in the population. In our study, molecular characterization of *R. rattus* and *R.norvegicus* species based on cyt*b* sequence corroborated morphological and morphometric findings.

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CONFLICT OF INTEREST

The authors declare that there are no issues to be perceived as a conflict of interest.

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