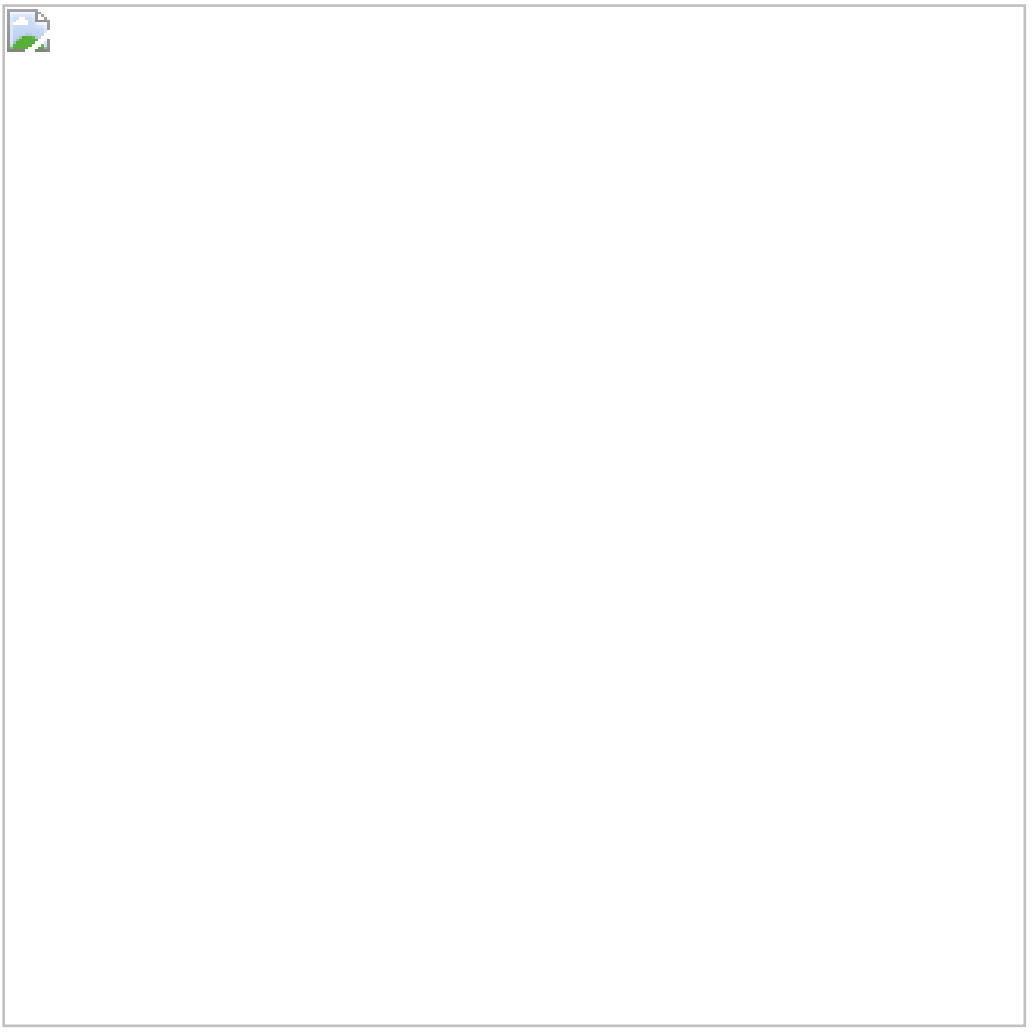

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Cryptic species diversity in the genus *Allactaga* (Rodentia: Dipodidae) at the edge of its distribution range

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Abstract. Present study aimed to address molecular diversities of the small five-toed jerboa *Allactaga elater* Lichtenstein, 1825, and the Toussi jerboa *Allactaga toussi* Darvish et al., 2008, in marginal geographic distribution of the genus in Iran. The study involved 35 individuals of *A. elater* and *A. toussi*, from the east and central parts of Iranian plateau. The two probabilistic phylogenetic algorithms, Maximum Likelihood (ML) and Bayesian Inference (BI), applied on 68 sequences of the two mitochondrial genes (34 cytochrome *b* and 34 cytochrome *c* oxidase subunit 1), retrieved reciprocal monophyly of the two species. Independent species status of *A. elater* and *A. toussi* is further evident from their sympatry in eastern Iran. Each of these species was further subdivided into two deeply divergent phylogeographic lineages within Iranian plateau, showing high level of genetic divergence ranging from 7-10.7 % for cyt*b* and 7.8-12.4 % for *cox1* genes. Such values exceed the intraspecific level of variation in rodents.

Key words: *Allactaga elater*, *Allactaga toussi*, *cox1*, cyt*b*, molecular phylogeny

Introduction

Five-toad jerboas are typical inhabitants of arid areas in Asia and north-eastern Africa. Twelve species were recognized in Wilson & Reeder (2005) and classified as a genus *Allactaga*. The diploid number of chromosomes is stable across the genus and molecular data are available only for few regions and species groups (Arslan et al. 2012, Dianat et al. 2013, Kryštufek et al. 2013). Taxonomy has therefore been entirely based on morphology although it is known that characters used in species delimitation (e.g. size, colour, and shape of glance penis) are subjected to the intraspecific variations (Shenbrot 2009). Recent phylogenetic reconstructions, based on molecular markers, questioned the validity of established taxonomy at the level of species and of genera. Lebedev et al. (2013) proposed split into up to five genera and other analyses (Dianat et al. 2013, Kryštufek et al. 2013) showed that number of species may be higher than that of conservative 12 species in Wilson & Reeder (2005).

We address in this paper the small five-toed jerboa *A. elater* (Lichtenstein, 1825) which was recently split in Iran into two species. The new species *A. toussi* Darvish et al., 2008 was recognized on morphological ground but its distinctness from *A. elater* received support in molecular reconstruction (Dianat et al. 2013). Molecular tools have the potential to facilitate both the identification of known species and the discovery of unrecognized cryptic diversity (Hebert et al. 2003, Jaarola et al. 2004). Herewith we provide evidence on deep divergence within *A. elater* and *A. toussi*, which may be indicative of further cryptic species in this group.

Material and Methods

Our study utilized 35 specimens of small five-toed jerboas from the eastern and central regions of Iran (Fig. 1 and Table 1). Specimens were classified either as *A. elater* or as *A. toussi* on the basis of morphological characteristics provided by Darvish et al. (2008); body mass, external (HBL, TL, EL and

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Table 1. Details of the sample localities for *Allactaga* were included in phylogenetic analysis. Museum vouchers are deposited in Zoological Museum of Ferdowsi University of Mashhad (ZMFUM).

Species	Locality	Museum voucher No.	Cyt*b* accession no.	*Cox1* accession no.	Reference
A. elater 1	Golestan	2749	KX219811	KX219845	Present study
		2674	JQ954928	JQ954893	Dianat et al. 2013
	Gonabad	3524	KX219808	KX219842	Present study
	Gonbad	1905	KX219809	KX219843	Present study
	Bojnord	2842	KX219812	KX219846	Present study
		2862	KX219813	KX219847	Present study
		2897	KX219815	KX219849	Present study
	Kashmar	1412	KX219805	KX219839	Present study
		1377	KX219804	KX219838	Present study
		1374	KX219814	KX219848	Present study
		2084	KX219806	KX219840	Present study
		2128	JQ954931	JQ954902	Dianat et al. 2013
		1429	JQ954932	JQ954900	Dianat et al. 2013
		2704	KX219810	KX219844	Present study
	Torbate Heydarieh	1740	KX219807	KX219841	Present study
A. elater 2	Golestan	2738	KX219817	KX219851	Present study
		2751	KX219818	KX219852	Present study
		2732	KX219816	KX219850	Present study
		2675	JQ954927	JQ954894	Dianat et al. 2013
		2676	JQ954929	JQ954895	Dianat et al. 2013
		2677	JQ954930	JQ954896	Dianat et al. 2013
	Sarakhs	3547	KX219819	KX219853	Present study
		3548	KX219820	KX219854	Present study
		3554	KX219821	KX219855	Present study
	Torbate Jam	3601	KX219822	KX219856	Present study
		3602	KX219823	KX219857	Present study
		3603	KX219824	KX219858	Present study
		3604	KX219825	KX219859	Present study
		3609	KX219826	KX219860	Present study
		3613	KX219827	KX219861	Present study
A. toussi West	Tehran	2745	-	KX219871	Present study
		2680	KX219836	KX219870	Present study
		2679	JQ954935	JQ954898	Dianat et al. 2013
		2680	JQ954936	JQ954899	Dianat et al. 2013
	Esfahan	2678	JQ954934	JQ954897	Dianat et al. 2013
	Hamedan	4503	KX219837	-	Present study
A. toussi East	Sarakhs	3558	KX219830	KX219864	Present study
		3553	KX219831	KX219865	Present study
		3557	KX219829	KX219863	Present study
	Torbate Jam	3614	KX219835	KX219869	Present study
	Mashhad	T1045	AJ389534	-	Dianat et al. 2013
		2694	JQ954954	JQ954918	Dianat et al. 2013
		2695	JQ954955	JQ954919	Dianat et al. 2013
		2696	JQ954956	JQ954920	Dianat et al. 2013
		1415	JQ954957	JQ954921	Dianat et al. 2013
		1416	JQ954938	-	Dianat et al. 2013
		1418	JQ954958	JQ954922	Dianat et al. 2013
	Sarakhs	2130	JQ954959	JQ954923	Dianat et al. 2013
		1431	JQ954933	JQ954901	Dianat et al. 2013
	Sabzevar	1438	KX219834	KX219868	Present study
	Tabas	1434	KX219833	KX219867	Present study
	Bojnord	2864	KX219828	KX219862	Present study
		2875	KX219832	KX219866	Present study

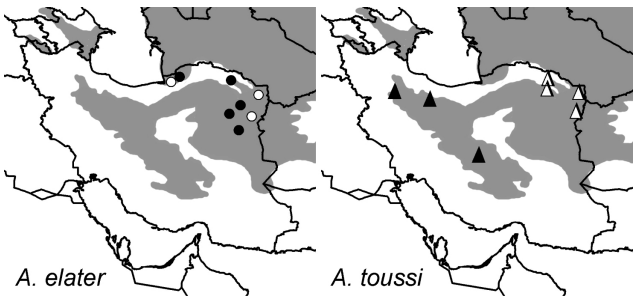

Fig. 1. Map showing collection sites of the specimens used in this study. Different symbols refer to phylogenetic sub-lineages of *A. elater*, and *A. toussi*. (The approximate distribution of small five-toed jerboa (grey shadow) follows IUCN database, Shenbrot et al. 2008b; http://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T853A13083932.en).

FL) and cranial measurements are larger in *A. toussi* than *A. elater*. Moreover, external characteristics are obviously discernable in these two species; inner surface of ear is dark in *A. toussi*, while it is light in *A. elater*, hind sole margin is naked in *A. toussi*, while it covers with black dense setae in *A. elater* (for more details see Darvish et al. 2008). Museum vouchers are deposited in the Zoological Museum of Ferdowsi University of Mashhad (ZMFUM), Iran. Whole genomic DNA of *Allactaga* was extracted from 96 % ethanol-preserved tissues using salt standard extraction method (Bruford et al. 1992). Polymerase Chain Reactions were performed using L7 and H6 primers for cyt*b* gene (Montgelard et al. 2002), and VF1d and VR1d for *cox1* gene (Ivanova et al. 2006). Purified PCR products were sequenced commercially by Macrogen Company, Republic of South Korea. 34 specimens were successfully sequenced. Sequences were edited manually using CodonCode aligner software (CodonCode Corporation) and aligned with Clustal W (Thompson et al. 1997) algorithm, using

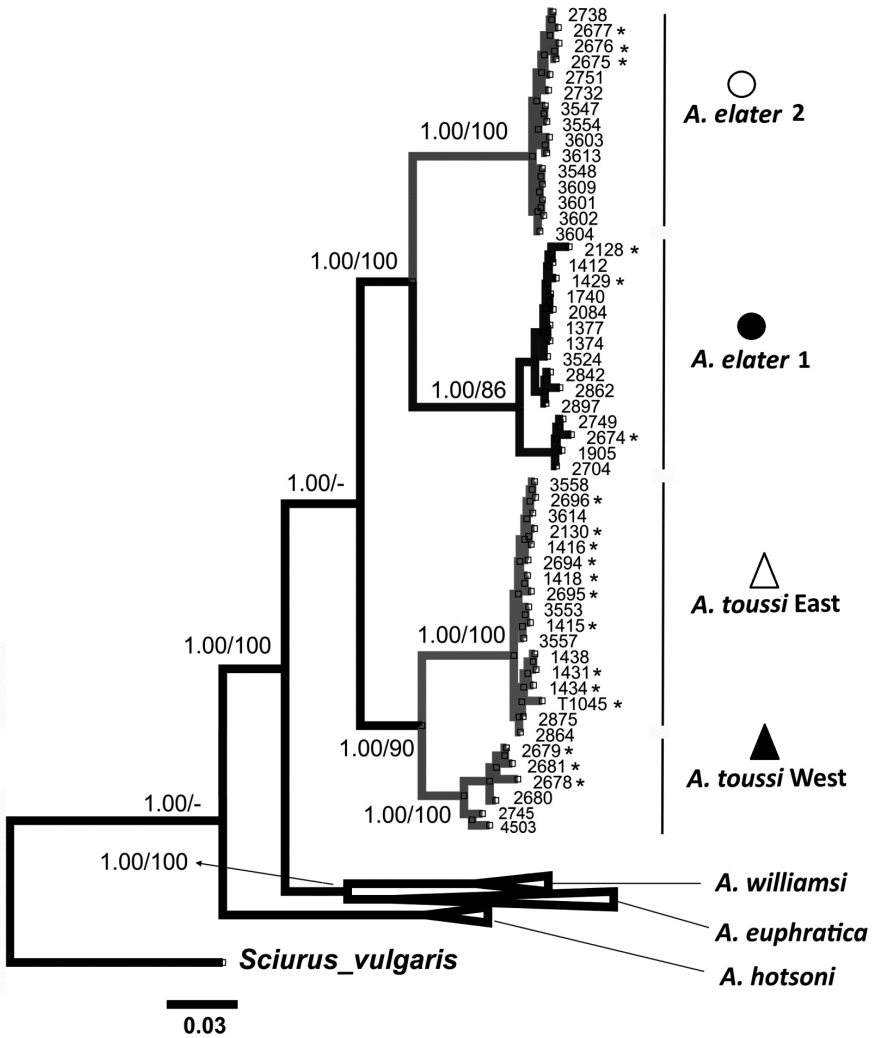

Fig. 2. Phylogenetic relationships among five species of *Allactaga* obtained from concatenated dataset (cyt*b* and *cox1*). Bayesian Inference and Maximum Likelihood analyses pose an identical topology. The first and second numbers on the branches correspond to posterior probability (BPP) and bootstrap (BP) values in the BI and ML tree analyses, respectively. (*Sequences were downloaded from GenBank; published by Dianat et al. 2013). The symbols correspond to those in Figure 1.

Table 2. The K2P (mean ± SD) estimates of intraspecific and interspecific cyt*b* and *cox1* divergences in five species of *Allactaga*.

Description of node	Cyt*b*: K2P (± SD)	*Cox1*: K2P (± SD)
A. elater /A. toussi	12.6 ± 0.9	13.5 ± 1.3
A. elater /A. hotsoni	15.9 ± 1.2	18.1 ± 1.4
A. elater /A. euphratica	16.2 ± 1.0	20.2 ± 1.8
A. elater /A. williamsi	15.4 ± 1.1	18.4 ± 1.6
A. elater /A. sibirica	18.6 ± 1.4	18.9 ± 1.7
A. toussi /A. hotsoni	16.5 ± 1.2	13.4 ± 1.2
A. toussi /A. euphratica	16.4 ± 1.1	18.5 ± 1.8
A. toussi /A. williamsi	15.5 ± 1.3	17.1 ± 1.5
A. toussi /A. sibirica	17.3 ± 1.3	19.1 ± 1.9
A. hotsoni /A. euphratica	18.3 ± 1.2	20.9 ± 1.8
A. hotsoni /A. williamsi	17.9 ± 1.4	18.3 ± 1.5
A. hotsoni /A. sibirica	18.4 ± 1.5	19.9 ± 1.7
A. euphratica /A. williamsi	15.0 ± 1.0	14.6 ± 1.6
A. euphratica /A. sibirica	20.7 ± 1.4	19.6 ± 1.9
A. williamsi /A. sibirica	18.7 ± 1.5	19.5 ± 1.9
A. toussi: toussi the east/*toussi* the west	7.0 ± 0.8	7.8 ± 1.1
A. elater: elater 1/*elater* 2	10.7 ± 1.1	12.4 ± 1.4

BioEdit 7.0.5 (Hall 1999). Genetic distances were analyzed assuming Kimura 2 parameter (K2P) model with 10000 bootstraps in Mega v6 (Tamura et al. 2013).

Phylogenetic analyses were performed on a combined multiple sequence alignment including 68 sequences obtained in this study (34 *cox1*, and 34 cyt*b*), and 75 sequences (34 *cox1*, and 41 cyt*b*) belonging to five species of *Allactaga* which were retrieved from GenBank (Dianat et al. 2013, Kryštufek et al. 2013). The best fitting model of nucleotide substitution was estimated, using jModeltest 0.1.1 (Posada 2008). Bayesian Inference (BI), was performed under the General Time Reversible model (GTR) with a proportion of invariable sites (I) and a gamma distribution (G) using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Four Monte Carlo Markov chains were run simultaneously for 4000000 generations. The trees were sampled every 100th generation after removing the first 5000 trees as the burn-in stage. Branch support was assessed as Bayesian Posterior Probability (BPP). Maximum Likelihood analysis was performed based on the Akaike Information Criterion (AIC), with the assumed model. The branch support of the ML tree was assessed as bootstrap value (BP) with 200 replicates. We considered a BPP ≥ 0.95 as "good" and BPP = 0.90-0.95 as "moderate" supports, while BP > 90 % as "good" support, and BP = 80-90 % as "moderate" support, in line with other authors (e.g. Kryštufek et al. 2009). The phylogenetic trees were rooted with red squirrel, *Sciurus vulgaris* (AJ238588) as an out-group (Reyes et al. 2000).

Results

Together with data reported in Dianat et al. (2013), 40 cyt*b* and 34 *cox1* haplotypes are known for lesser five-toed jerboas. Of the 911 bp-long cyt*b* sequence, 218 polymorphic sites (23.92 %) were found, 182 (83.48 %) of which were parsimony informative. Of the 620 bp-long *cox1* sequence, 241 (38.87 %) and 183 (75.93 %) sites were polymorphic and parsimony informative sites, respectively. No stop codons, insertions or deletions were observed in the alignments.

We constructed BI and ML trees for the two mitochondrial genes independently (cyt*b*, *cox1*) and for the concatenated sequence. Because of congruence between the outputs only the BI tree drawn for combined sequences is shown (Fig. 2). Seven highly supported (BPP = 1.00) lineages are eminent. *Allactaga hotsoni* was basal in the tree and *williamsi* + *euphratica* were in a sister position against the small five-toed jerboas. All specimens of small five-toed jerboas were in two lineages which matched their morphological classification. In north-eastern Iran ranges of these two species overlapped and in Bojnord, Torbat Jam, and Sarakhs, they were sympatric. Each of the two small five-toed jerboas' species was further structured into two sub-lineages. Sub-lineages were strictly allopatric in *A. toussi*, being confined to north-eastern Iran (*A. toussi* the east) and

to the area between the Zagros and Elbruz Mountain ranges (*A. toussi* the west). The two sub-lineages of *A. elater* were allopatric in the extreme north-eastern Iran, but were found in sympatry in Golestan. K2P genetic distances between the species of five-toed jerboas ranged between 12.6 and 20.7 % for cyt*b* and between 13.4 and 20.9 % for *cox1*. Corresponding values for the sub-lineages of *A. elater* and *A. toussi* were 10.7 and 7.0 % for cyt*b*, and 12.4 and 7.8 % for *cox1* (Table 2).

Discussion

Our results confirmed association between the morphotype and the molecular profile in small five-toed jerboas classified as *A. elater* and *A. toussi*. Cyt*b* divergence between them (12.6 %) was above the values reported for intraspecific divergences in rodents (Baker & Bradley 2006). Also importantly, these two jerboas were broadly sympatric in a large area of north-eastern Iran. It is therefore beyond doubt that *elater* and *toussi* are two distinct species.

Of no lesser interest are divergences within both, *A. toussi* and *A. elater*. In both species, the intraspecific cyt*b* distances (7.0 and 10.7 % for *toussi* and *elater*, respectively) exceeded the intraspecific divergences in rodents (≤ 4.7 %; Baker & Bradley 2006), being well within the range of K2P distances found between species. Since the two lineages of *A. elater* were sampled from the same place in Golestan, one may assume for them to be at least parapatric. Although we believe that cryptic species are most likely involved in both cases, we refrain at this stage from taxonomic conclusions. First of all, nuclear genetic markers have to be included into analyses, sampling in the zone of overlap has to be intensified, and morphological variation of glans penis is to be taken into consideration for these highly divergent sub-lineages. Following recent reports on cryptic species richness in five-toed jerboas (*Paralactaga*; Kryštufek et al. 2013), our results further emphasize the extent of undetected species richness in dipodids. It seems that the phenomenon is widespread, if not overwhelming and that cryptic species will follow to be reported along with progress of molecular screening in the family. Small five-toed jerboas seem a particularly good candidate, considering large range, high number of subspecies and categorical divergence evidenced by the morphology of glans penis. Noteworthy, significant portion of variation takes place outside Iran since only one of the two groups of subspecies discerned from glans penis was reported for the country (Shenbrot et al. 2008a).

Allactaga vinogradovi as the closest relative to *A. elater* (Lebedev et al. 2013) has not been sequenced so far. Information on nucleotide sequences in *A. vinogradovi* is essential to get an unbiased insight into the phylogenetic structuring of the entire group of small five-toed jerboas (tentatively classified as *Microalactaga*; Lebedev et al. 2013) and particularly the relationships between *A. vinogradovi* and *A. toussi*. It came as a surprise that *A. toussi* is more widespread in Iran than is *A. elater*.

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