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Molecular, chromosomal and morphometric variation in *Calomyscus hotsoni* and *C. elburzensis* (Calomyscidae, Rodentia) in the east of Iran

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Abstract. Two species of brush-tailed mice, genus *Calomyscus*, are known from eastern Iran: *Calomyscus hotsoni* has been reported from southwestern Pakistan and southeastern Iran, and *C. elburzensis* ranges through northeastern and central Iran. Based on molecular studies of two mitochondrial genes, all the specimens from eastern Iran examined herein belong to either of these two species. Furthermore, our data expand the northern distribution limits of *C. hotsoni* to just south of Birjand and the southern limits of *C. elburzensis* to east of Birjand. Morphometric analyses conducted on three geographic groups of *C. hotsoni* within Iran, contained specimens from Birjand (group 1), Zahedan and Khash (group 2) and Saravan (as group 3) revealed a north-to-south cline of decreasing body and cranial size, such that the most significant differences were between the northern and southern most groups. Karyological studies also showed differences in autosomal arms between the two geographical groups in Iran. Although the phylogenetic analyses separated these two groups into distinct clades, along with a third clade containing most of the *C. hotsoni* from Pakistan. The morphometric and molecular partitioning of geographic populations of *C. hotsoni* were not concordant. We consider the north and south groups of *C. hotsoni* as distinct Evolutionary Significant Units. There is evidence of introgression between the two forms across a broad geographic area presented by individuals of group 2 resulting in a clinal pattern of variation.

Key words: cranial measurements, mitochondrial DNA, south Khorasan Province

Introduction

The family Calomyscidae contains a single genus, *Calomyscus*, and eight extant species distributed in southwestern Asia including areas of Syria, Iran, Turkmenistan, Azerbaijan, Afghanistan and Pakistan (Musser & Carleton 2005). Five species of *Calomycus* are recorded within Iran: *C. bailwardi* range through the Zagros Mountains from Kurdistan Province to south and southeast successively through Ilam, Luristan, Isfahan, Khuzestan, Fars, and Kerman Provinces; *C. grandis* in northern Iran and south of Caspian Sea; *C. urartensis* in Azerbaijan Province in northwestern Iran; *C. hotsoni* and *C. elburzensis* (Musser & Carleton 2005).

Thomas (1920) described Hotson's brush-tailed mouse, *C. hotsoni*, from specimens collected by Colonel Hotson at Gwambuk Kaul, 50 km SW Panjgur, Baluchistan, Pakistan (Thomas 1920). Ellerman (1940, 1961), Ellerman & Morrison-Scott (1951) and Peshev (1991) considered *hotsoni* as a subspecies of a widely-distributed *C. bailwardi*. Vorontsov et al. (1979) elevated most subspecies of *C. bailwardi*, including *hotsoni*, to species status, and this treatment was followed by Musser & Carleton (2005), Karami et al. (2008) and Norris et al. (2008). Musser & Carleton (2005) recorded *C. hotsoni* from Baluchistan region of southwestern Pakistan and Sistan-o-Baluchistan Province in southeastern Iran. Norris et al. (2008) reported this species from the Baluchistan and Sindh Provinces of southern Pakistan and Sistan-o-Baluchistan Province of southeastern Iran. Shahabi et al. (2010) reported

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two karyotypes, one with 2N = 50, FNa = 48 and the other with 2N = 48, FNa = 48 from Saravan in Sistan-o-Baluchistan. *C. elburzensis* Goodwin, 1938 is distributed in northeastern and central (Yazd Province) Iran, southeastern Turkmenistan and northwestern Afghanistan (Graphodatsky et al. 2000, Norris et al. 2003, Norris et al. 2008, Shahabi et al. 2013); its type locality is in the Kurkhud Mountains of north Khorasan Province (Goodwin 1938). The southernmost range limit reported for *C. elburzensis* in northeastern Iran is Torbate Jam in Khorasan-e-Razavi Province (Shahabi et al. 2013). Six karyotypes have been described from *Calomyscus* collected within the geographic range of *C. elburzensis*.

Graphodatsky et al. (2000) reported two diploid numbers of chromosomes, 2N = 30, FNa = 44 and 2N = 44, FNa = 58, from the Kopetdagh Mts. Meyer & Malikov (2000) recognized these two cytotypes as *C. mystax zykovi* and *C. firiusaensis* respectively, but Musser & Carleton (2005) considered both as *C. elburzensis*. Norris et al. (2008) followed Musser & Carleton's (2005) inclusion of these two cytotypes in *C. elburzensis* but tentatively recognized *zykovi* as a subspecies based on its phenotypic distinctiveness (Lebedev et al. 1998) and partial reproductive isolation (Meyer & Malikov 2000). Four other cytotypes each with 2N = 44 but with FNa ranging from 60 to 72 have been described from *C. elburzensis* by Graphodatsky et al. (2000), Somayeh et al. (2008) and Shahabi et al. (2010).

The aim of this study is to revise the distributional range of these two *Calomyscus* species, *C. elburzensis* and *C. hotsoni* in eastern Iran and, in addition, analyze geographic variation in *C. hotsoni* throughout its range in Iran using a combination of morphometrics, mitochondrial DNA sequences, and karyology.

Material and Methods

A total of 88 *Calomyscus* samples were examined, including 53 which were captured using live traps representing *C. elburzensis* from seven localities, *C. hotsoni* from four localities, *C. grandis* and *C. urartensis* each from a single locality. Thirty-three specimens from the ZMFUM (Zoology Museum of Ferdowsi University of Mashhad) were added to our morphometric analysis (Table 1, Fig. 1). All samples are deposited at the ZMFUM and voucher numbers are presented in the supplementary material.

Molecular study

Of the 88 captured and deposited *Calomyscus* specimens from the ZMFUM, sequenced data consist of 44 individuals belonging to four species (22 *C. elburzensis*, 4 *C. grandis*, 16 *C. hotsoni* and 2 *C. urartensis*; see Table 1 and supplementary Table S1). Additional cyt*b* sequences of *C. hotsoni* and *C. baluchi* were retrieved from GenBank and three *COI* sequences of *C. baluchi* were obtained from ZTNH (Zadock Thompson Natural History, University of Vermont) (Table 1). For our phylogenetic analyses, we used sequences from the Spalacidae (Muroidea) as the appropriate outgroup; these were also obtained from GenBank (Table 1).

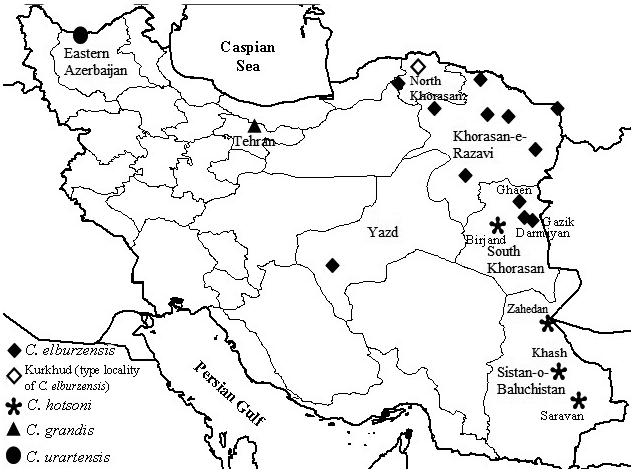

Fig. 1. Collection sites of four species of *Calomyscus* from Iran. The samples from all of the localities of *C. hotsoni* and *C. elburzensis* were utilized in molecular and morphometric analyses; while some individuals from Kurkhud, Gazik, Birajnd and Zahedan which were named on the map were used in karyological analysis (see also Table 1).

The genomic DNA from samples of four species was isolated from fresh muscle or kidney tissues, according to standard salt method extraction of Bruford et al. (1992). Amplifications of two mitochondrial genes were performed with modified universal L7 (5′-ACT AAT GAC ATG AAA AAT CAT CGT T-3′) and H6 (5′-TCT TCA TTT TTG GTT TAC AAG AC-3′) primers in thermal cycling conditions from Montgelard et al. (2002) for the cytochrome *b* gene, and with VF1d (5′-TTC TCA ACC AAC CAC AAR GAY ATY GG-3′) and VR1d (5′-TAG ACT TCT GGG TGG CCR AAR AAY CA-3′) primers using PCR conditions were given from Ivanova et al. (2006) for the *cytochrome oxidase* I (*COI*) gene. Single strands of PCR products were sequenced by the Macrogen Company, Republic of South Korea.

Sequences of both genes were edited and aligned separately with BioEdit 7.0.5 (Hall 1999) and were checked for stop codons. Cyt*b* and *COI* sequences were analyzed separately and as a combined, concatenate dataset, under Bayesian inference and

Table 1. Information on taxa, number of specimens (N) and sequenced samples in parenthesis, sampling localities in Iran and GenBank accession numbers. ZMFUM – localities which their samples were obtained from Zoology Museum of Ferdowsi University of Mashhad.

Taxon	N	Locality	Coordinates	cyt*b* Accession number	*COI* Accession number
C. elburzensis	11 (2)	Khajemorad Mts., Mashhad, Khorasan-e-Razavi, Iran (ZMFUM)	36.25 N, 59.5667 E	KT878581 KT884547	KT878542 KT884576
C. elburzensis	4 (3)	Kopetdagh Mts., Aghdarband, Khorasan -e-Razavi, Iran (ZMFUM)	36.5 N, 61.1167 E	KT878585 KT884549 KT878586	KT878546 KT878547
C. elburzensis	2 (2)	Kopetdagh Mts., Daregaz, Khorasan-e-Razavi, Iran (ZMFUM)		KT884548	KT884577 KT884578
C. elburzensis	5 (1)	Shahneshin Mts., Torbat-e-Jam, Iran Khorasan-e-Razavi, Iran (ZMFUM)	35.15 N, 60.4 E	KT878587	KT878548
C. elburzensis	27 (2)	Binalud Mts., Neyshabur, Khorasan-e-Razavi, Iran	36.3167 N, 58.8831 E	KT884550 KT884551	KT884580 KT884581
C. elburzensis	1 (1)	Siahkuh Mts., Bajestan, Khorasan-e-Razavi, Iran	34.4081 N, 58.1722 E	KT884552	KT884582
C. elburzensis	2 (2)	Shaskuh Mts., Ghaen, South Khorasan, Iran	33.6267 N, 60.0372 E	KT884553 KT884554	KT884583
C. elburzensis	4 (2)	Kurkhud Mts., North Khorasan, Iran	37.8167 N, 56.6833 E	KT878590 KT884555	KT878550 KT884584
C. elburzensis	1 (1)	Elburz Mts., Dasht, North Khorasan, Iran (ZMFUM)		-	KT884579
C. elburzensis	1 (1)	Elburz Mts., Sbazevar, Khorasan-e-Razavi, Iran	36.5258 N, 57.1861 E	KT884556	KT884585
C. elburzensis	2 (1)	Gazik, South Khorasan	33 N, 60.2261 E	KT884557	KT884586
C. elburzensis	1 (1)	Darmiyan, South Khorasan, Iran	33.0406 N, 60.1183 E	KT884558	-
C. elburzensis	3 (3)	FakhrAbad, Yazd, Iran (ZMFUM)	31.6667 N, 54.3167 E	KT878582 KT878584 KT878583	KT878582 KT878584 KT878583
C. hotsoni	8 (7)	Birk Mts., Saravan, Sistan-o-Baluchistan, Iran (ZMFUM)	27.3 N, 61.7667 E	KT884560 KT884561 KT884562 KT884563 KT884564 KT884565 KT884566	KT878577 KT878578 KT884588 KT884589 KT884590
C. hotsoni	4 (4)	MalekSiahkuh Mts., Zahedan, Sistan-o-Baluchistan, Iran	29.7433 N, 60.7883 E	KT884568 KT884569 KT884570 KT884571	KT884592 KT884593
C. hotsoni	1 (1)	Abkhan Mts., Khash, Sistan-o-Baluchistan, Iran	28.1572 N, 61.1778 E	KT884567	KT884591
C. hotsoni	4 (4)	Bagheran Mts., Birjand, South Khorasan, Iran	32.8180 N, 59.2148 E	KT884572 KT884573 KT884574 KT884575	KT884594 KT884595
C. hotsoni	1	Panjgur Dist., Mitha Singh, Pakistan	26.7647 N, 64.1569 E	EU135579	-
C. hotsoni	1	Panjgur Dist., Mitha Singh, Pakistan	26.7647 N, 64.1569 E	EU135580	-
C. hotsoni	1	Panjgur Dist., Mitha Singh, Pakistan	26.7647 N, 64.1569 E	EU135581	-
C. hotsoni	1	Sindh, Dadu Dist., Rani Kot near Shergart, Pakistan	26.3819 N, 66.8875 E	EU135582	-
C. hotsoni	1	Khuzder Dist., Dancer village, Pakistan	28.0211 N, 65.8428 E	EU135583	-
C. baluchi	1	Kalat Dist., Khan's palace Bungalow, Pakistan	29.2647 N, 66.7144 E	EU135586	-
C. baluchi	1	Kalat Dist., Kargaz, Pakistan	29.5344 N, 66.7558 E	EU135587	-
C. baluchi	1	Datta Khel, SW Miran Shaw, Pakistan	33.0806 N, 69.7317 E	EU135591	-

C. baluchi	1	Ziarat Dist., Ziarat, Pakistan	30.385 N, 67.7278 E	-	KT878579
C. baluchi	1	Sibi Dist., Pakistan	29.5472 N, 67.8714 E	-	KT884596
C. baluchi	1	Datta Khel, Tore Shore, Pakistan	33.7169 N, 71.3878 E	-	KT878580
C. grandis	5 (4)	Fasham, Tehran, Iran (ZMFUM)	35.9344 N, 51.5242 E	KT878591 KT878592 KT878593 KT884559	KT878551 KT878552 KT878553 KT884587
C. urartensis	2 (2)	Kordasht, Eastern Azerbaijan, Iran	46.01 N, 38.8669 E	KT878594 KT878595	KT878554 KT878555
Spalax ehrenbergi	1	-	-	AJ389537	AJ416891
Rhizomys pruinosus	1	-	-	KC789518	JQ601434

maximum likelihood criteria. The model GTR + I + G was determined for cyt*b* and GTR + I for *COI* data by Modeltest 3.7 (Posada & Crandall 1998). However, the phylogenetic reconstruction for the combined data of the two genes using Bayesian Inference (BI) was conducted with a GTR + I + G model which was determined to be the appropriate model for combined data set. This same evolution model was used to determine bootstrap values in a maximum likelihood (ML) analysis and both ML bootstrap and posterior probability (BI) supported for nodes were provided. A maximum likelihood (ML) tree was generated in PAUP 4.0b10 (Swofford 2002) with the heuristic search algorithm. The robustness of nodes in the ML analysis was assessed by 100 bootstrap replicates. Bayesian inference analysis (BI) was conducted in MrBayes 3.1.1 (Ronquist & Huelsenbeck 2003) with 10×10^6 generations with four Markov Chains Monte Carlo and the first 10000 trees were discarded as "burn-in". The mean of Kimura 2-parameter distances within and among species were calculated using MEGA 5 (Tamura et al. 2011). Due to the lack of data in *COI* sequences for some specimens of *C. hotsoni* those from Pakistan, the Bayesian and maximum likelihood analyses were performed with two data sets, the combined data set where samples for which *COI* sequences were not available were coded as missing data (?), and a second data set that included only the cyt*b* sequences.

Regarding the lacking of individuals from Pakistan in the resulted tree of *COI* sequences, we compared the alternative phylogenetic hypothesis of these specimens in tree generated by combined data set and by cyt*b*-only data using Shimodaria-Hasegawa test (Shimodaria & Hasegawa 1999), as implemented in RA × ML v.7.0.3 (Stamatakis 2006). The tested topologies were obtained enforcing the monophyly of clade 3 versus all of the specimens and haplotypes of *C. hotsoni* from Pakistan in a single clade in the maximum likelihood searches in RA × ML.

Morphometric study

Four external (BL: body length, TL: tail length, FL: hind foot length, EL: ear length) and 27 cranial and dental characters (Fig. 2) were measured in 88 individuals by means of a hand-held ruler, digital caliper, and measuring-scope, to the nearest 0.1, 0.01, and 0.001 mm, respectively. All specimens were adult with their three molar teeth fully erupted. The data were checked for normality with the Shapiro-Wilk test. All the individuals of *C. hotsoni* were assorted in three groups based on the geographical localities from which they were collected, from north to south in south Khorasan and Sistan-o-Baluchistan Provinces (Fig. 1). Univariate ANOVA's and Tukey's tests were performed to determine which morphometric characters differed significantly between groups and species. Mean and standard errors were calculated for all variables. Canonical variate analysis was used for the ordination of specimens along each canonical axis (CV). Statistical analyses were conducted using the SPSS Base 20 package (IBM Corp. Released 2011) and PAST v2.08 (Hammer et al. 2001).

Karyological study

Karyotypes were obtained from two specimens of *C. elburzensis* from two localities (Kurkhud Mts. and Gazik) and three *C. hotsoni* samples from two localities (Zahedan and Birjand) (Table 1). Animals were injected with a 10 % vinblastin solution, 1 ml/100 g of body weight, 45 minutes before euthanizing and harvesting femoral and tibial bone marrow cells (Dutrilaux et al. 1982). The diploid number (2N) and autosomal fundamental number (FNa) were determined from counts of approximately 10 lysed nuclei where the chromosomes were not overlapping

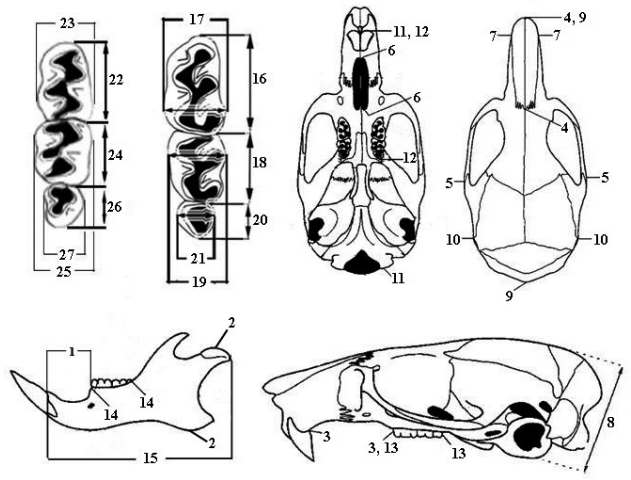

Fig. 2. Description of cranial, mandibular and dental measurements. 1-LLD (lower length of diastema), 2-MH (mandibular height), 3-UDL (upper diastema length), 4-NL (nasal length), 5-ZW (zygomatic width), 6-ForI (length of anterior palatine foramen), 7-NW (nasal width), 8-SH (skull height), 9-Occl (occipitonasal length), 10-CW (cranium width), 11-CBL (condylobasal length), 12-PatI (palatal length), 13-MxI (maxillary tooth row), 14-MnI (mandibular tooth row), 15-MndI (mandible length), 16-M.1L (length of M1), 17-M.1W (width of M1), 18-M.2L (length of M2), 19-M.2W (width of M2), 20-M.3L (length of M3), 21-M.3W (width of M3), 22-m1.L (length of m1), 23-m1.W (width of m1), 24-m2.L (length of m2), 25-m2.W (width of m2), 26-m3.L (length of m3), 27-m3.W (width of m3).

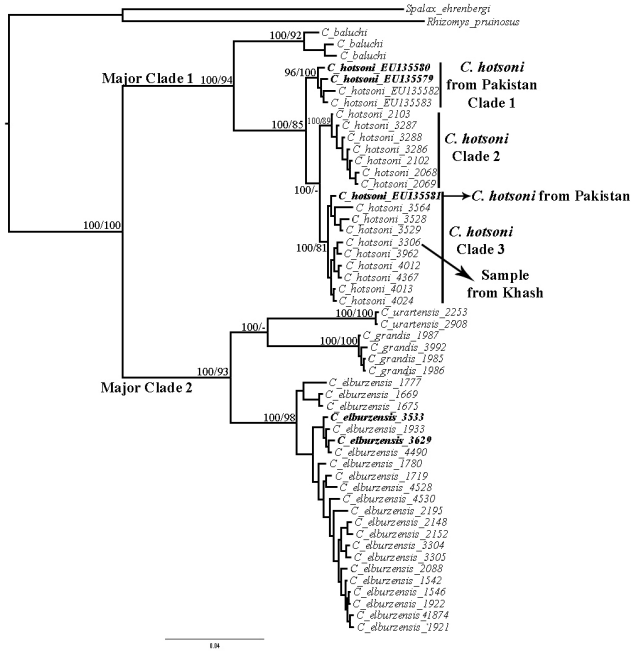

Fig. 3. Combined data tree resulting from Bayesian inference (BI) and maximum likelihood (ML) analyses of cyt*b* and *COI* gene sequences. The type locality samples for *C. hotsoni* and *C. elburzensis* are indicated by bold. Numbers on branches refer to Bayesian posterior probabilities (first values) and bootstrap values derived from the maximum likelihood (second values) analysis (give only if > 95 % for posterior probabilities with high support in > 99 % and good in > 95 %, following Aliabadian et al. 2007; and if > 70 % for ML bootstraps with robust support in > 70, moderate in < 70 and > 50, and weak support in < 50, Zander 2004).

Table 2. The mean of Kimura-2-parameters distance matrix on cyt*b* and *COI* sequences of three clades of *C. hotsoni* (lower half of interclade distances belong to cyt*b* and upper half of distances to *COI*; bold intraclade distances: cyt*b*, italic intraclade distances: *COI*).

Taxa	Clade 1	Clade 2	Clade 3
Clade 1	**0.5**, -	-	-
Clade 2	2.1	**0.3**, *0.3*	1.2
Clade 3	1.9	1.5	**0.5**, *0.6*

using the Chromosome Processing software (CIP) developed in the Rodentology Research Department of Ferdowsi University of Mashhad.

Results

Molecular analyses

After editing sequences and checking for the presence of stop codons, a total 1616 bps of the two mitochondrial genes were available for phylogenetic analyses, 988 bps for cyt*b* and 628 bps for *COI*. The *Calomyscus* samples from eastern Iran were separated into two distinct major clades in both BI and ML trees (Fig. 3), each of which also contained samples from either the type locality (*C. elburzensis* from Kurkhud Mts.) or near the type locality (*C. hotsoni*, from GenBank). These two taxa were quite divergent molecularly with the mean of genetic distances (K2P) between them of 17.7 % for cyt*b* and 15.3 % for *COI* sequences. The individuals of each taxon were well supported in Bayesian inference of 100, maximum likelihood support for *C. elburzensis* is 100 % and for *C. hotsoni* is 85 %. Furthermore, both species were found in close geographic proximity in south Khorasan Province, Iran, with *C. hotsoni* occurring in the Bagheran Mountains in southern Birjand and *C. elburzensis* occurring in eastern Birjand (Fig. 1).

Phylogenetic trees from combined data set and only the cyt*b* sequences (Fig. 3 and supplementary Fig. S1) both identified three clades of *C. hotsoni* in Iran and southwest of Pakistan. Clade 1 contained 4 cyt*b* haplotypes (A, B, D and E), the two of which occurred in the vicinity of the type locality and others in localities west of the type locality in southern Pakistan. Unfortunately, *COI* data were not available for these specimens. Clade 2 contained samples with both cyt*b* and *COI* sequences from Saravan, the southernmost locality for *C. hotsoni* from Iran. Clade 3 contained samples with both cyt*b* and *COI* sequences from Birjand, Zahedan, and Khash from Iran along with the cyt*b* haplotype C from the site in Pakistan near the type locality. All three clades were supported in the tree generated from both the combined and cyt*b*

Table 3. Comparison of alternative phylogenetic hypotheses using Shimodaria-Hasegawa test performed with RA × ML. △-lnL – difference in tree likelihood compared to the best tree. NS – not significantly worse than the best topology; significant, $p < 0.05$.

Topology tested	Tree likelihood	△-lnL	SH test
Best tree of cytb data	–4568.517122		best
Monophyly of all samples from Pakistan (Calde 1 + sample 81 in clade 3)	–4587.130249	–18.613127	significant
Monophyly of clade 3	–4571.996658	–3.479536	NS
Beast tree of combined data	–7213.362066		best
Monophyly of all samples from Pakistan (clade 1 + sample 81 in clade 3)	–7222.923718	–9.561652	significant
Monophyly of clade 3	–7204.380463	–8.981603	NS

data (Fig. 3), but, in contrast to the ML analysis, clade 1 did not have any acceptable support in the cyt*b*-only Bayesian analyses (supplementary Fig. S1). These three molecular clades exhibited low genetic divergence or K2P distances (Table 2) with the mean of distance among the three clades of 1.83 % for cyt*b* and the mean of distance between clades 2 and 3: 1.2 % for *COI*.

The results of Shimodaria-Hasegawa test are presented in Table 3 which rejects the hypothesis of the monophyly of enforcing all of the specimens and haplotypes of *C. hotsoni* from Pakistan in a single clade in combined and cyt*b* trees. The clade 3 consist of specimens from Birjand, Khash, Zahedan and haplotype C was not significantly different from our best topology of both trees.

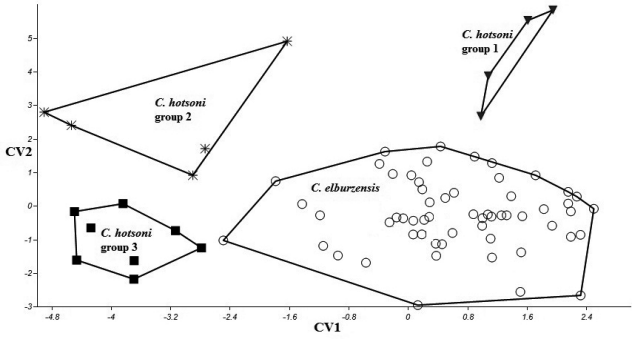

Fig. 4. Plot of the first and second canonical variate axes in analyses of three geographic groups of *C. hotsoni* and *C. elburzensis*.

Morphometric analyses

We pooled male and female specimens as no sexual dimorphism was found ($p > 0.05$) and all of the variables were normally distributed. For more accurate statistical analysis on *C. hotsoni*, these samples were sorted into three groups according to their geographic places from north to south. These three geographic groups of *C. hotsoni* were analysed using both uni- and multivariate approaches. Group 1 samples included specimens from Birjand in south Khorasan Province, those of group 2 included individuals from Zahedan

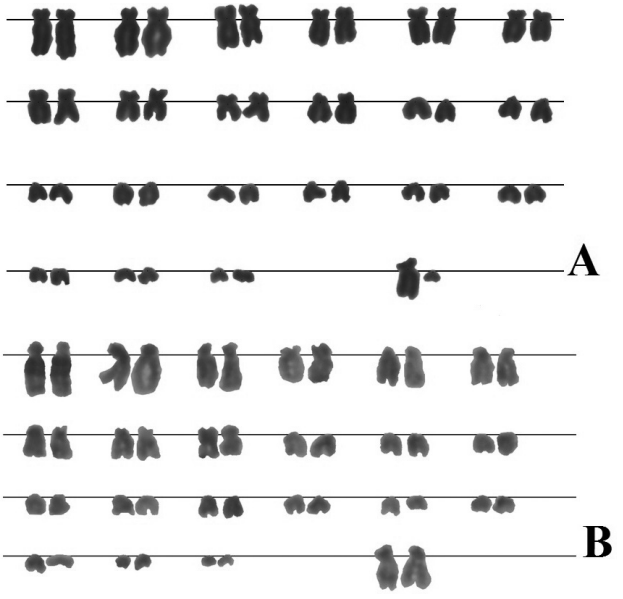

Fig. 5. Diploid chromosomes of two samples of *C. elburzensis*, (A) a male from Kurkhud Mts., voucher number: 3630 ZMFUM and (B) a female sample from Gazik (4529 ZMFUM).

in the northern half of Sistan-o-Baluchistan Province; samples of these two groups are part of molecular clade 3 (Fig. 3). Finally, group 3 specimens, representing molecular clade 2, are from Saravan, which situated in southern half of Sistan-o-Baluchistan Province (Fig. 1). Box plots of morphometric variables of these groups showed clinal variation in thirteen variables (BL, EL, CBL, Forl, NL, Patl, UDL, SH, NW, Mxl, M.2W, m1.W and m2.W) with the highest values in samples of the northern group 1 and the lowest values in specimens of group 3. Brush-tailed mice from group 2 exhibited intermediate values. Means and standard errors of these measurements are presented in Table 4. These values in the only sample from Khash were more close to group 2 than to group 3 (except BL and EL, which were less than the means of both groups). Hence, this sample was placed in a same group with specimens from Zahedan for multivariate analysis.

No significant geographic variation or trend in morphometric characters was observed among our

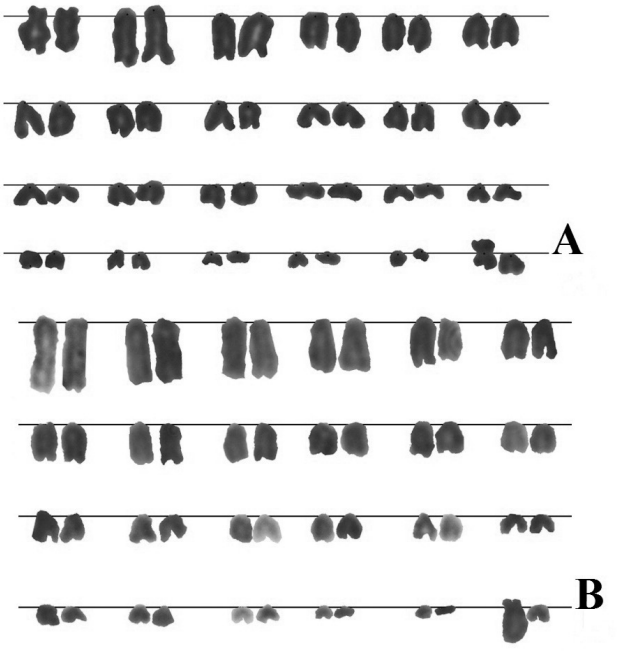

Fig. 6. Diploid chromosomes of two specimens of *C. hotsoni*, (A) a male from Zahedan (2N = 48, FNa = 48) with voucher number 3529ZMFUM and (B) a male from Bagheran Mts. in Birjand (2N = 48, FNa = 46), 4012 ZMFUM.

samples of *C. elburzensis*, from northern localities in the Kurkhud Mts. to the southern localities in Darmiyan (Fig. 1).

ANOVA's and Tukey's tests showed that some external, cranial and dental characters differed significantly ($p < 0.05$) among three groups of *C. hotsoni* and differentiated each of these groups in comparison to *C. elburzensis* (Table 5). Group 1 was different in several traits from groups 2 and 3 but groups 2 and 3 were not significantly different for any trait. Specimens belong to group 1 had longer ears and nasals than those of group 2; also this group had a longer body, ear, upper diastema, nasal, palatine and anterior palatine foramen and wider nasal and the first lower molar than *C. hotsoni* from group 3.

Besides, *C. elburzensis* had the greatest number of significant differences of traits with *C. hotsoni* group 3, the geographically most distant from *C. elburzensis*. *C. elburzensis* in variables NL, SH, M.2W, m1.W, and m2.W were significantly smaller than individuals from group 1 of *C. hotsoni*, the closest in geographic proximity, and ZW, M.2W, M.3L and m1.W were significantly smaller than group 2 members of *C. hotsoni* (Table 5). The width of the first lower molar (m1.W) is a good character to differentiate two species of *C. elburzensis* and *C. hotsoni*, the former having narrower m1.W.

The plot of canonical variate analysis (CVA) of three groups of *C. hotsoni* and *C. elburzensis* is shown in Fig. 4. The first and second axes contained 57.4 % and 36.0 % of total variance, respectively. The first axis (CV1) separates *C. elburzensis* and group 1 of *C. hotsoni* from both groups 2 and 3 of the latter species, while CV2 separated *C. hotsoni* group 2 from group 3, and *C. hotsoni* group 1 from *C. elburzensis* (Fig. 4). However, the pairwise group comparison indicated that the separation between *C. hotsoni* groups 2 and 3 was not significant ($p > 0.05$) (Table 6).

Karyological analyses

Examination of *C. elburzensis* and *C. hotsoni* indicated the diploid number of 44 for the former and 48 for the latter species. However, the number of autosomal arms was different in individuals of the same species. The single male of *C. elburzensis* from the Kurkhud Mts. had FNa = 62, including 10 pairs subtelocentric autosomes, 11 pairs acrocentric autosomes, subtelocentric X and acrocentric Y chromosome; (Fig. 5A) whereas the female from Gazik in South Khorasan province showed 60 autosomal arms with 9 pairs subtelocentric, 12 pairs acrocentric autosomes and two subtelocentric X chromosomes (Fig. 5B). Male of *C. hotsoni* from Zahedan in Sistan-o-Baluchistan Province had 48 arms in its autosomal complement, which contained one pair subtelocentric and 22 pairs acrocentric autosomes as well as the X and Y chromosomes which were subtelocentric and acrocentric, respectively (Fig. 6A). A male and female individuals from Birjand in south Khorasan Province had 46 autosomal arms including 23 pairs acrocentric autosomes with subtelocentric X and acrocentric Y chromosomes (Fig. 6B).

Discussion

C. hotsoni is one of the most poorly known species in the genus *Calomyscus*, with its recognized range covering southwestern Pakistan and southeastern Iran in Sistan-o-Baluchistan Province (Karami et al. 2008, Norris et al. 2008) but its full distribution is incompletely known (Musser & Carleton 2005). The examined samples studied by molecular and morphometric analyses were assigned to two species of *Calomyscus*, *C. hotsoni* which this study expands its known range within Iran substantially to the south of Birjand and near to the southern-most localities of the other species, *C. elburzensis* (east of Birjand).

According to Norris et al. (2008), individuals from northern localities of the *C. hotsoni* (Zahedan and Khash) had a significantly longer hind foot, condylobasal and mandibular tooth row length and wider interorbital breadth than southern populations

Table 4. Comparison of means ± standard errors of 27 external and cranial measurements between three groups of *C. hotsoni* and *C. elburzensis*.

Variables	*C. hotsoni* group 1	*C. hotsoni* group 2	*C. hotsoni*from Khash	*C. hotsoni* group 3	*C. elburzensis*
BL*	82.7 ± 3.2	75.7 ± 4.0	69.0	71.4 ± 9.7	80.07 ± 5.2
TL	85.0 ± 4.2	89.3 ± 6.3	90.0	94.3 ± 25.3	90.0 ± 5.1
FL	20.0 ± 1.0	19.0 ± 1.0	18.0	19.4 ± 1.4	20.1 ± 1.1
EL*	19.7 ± 0.5	16.7 ± 1.1	13.0	15.9 ± 3.2	18.7 ± 1.7
LLD	3.46 ± 0.15	3.37 ± 0.11	3.24	3.24 ± 0.29	3.42 ± 0.22
MH	5.99 ± 0.28	6.06 ± 0.29	5.84	5.97 ± 0.39	5.77 ± 0.24
UDL*	6.67 ± 0.15	6.43 ± 0.26	6.41	6.16 ± 0.27	6.53 ± 0.25
NL*	10.25 ± 0.11	9.47 ± 0.27	9.48	9.28 ± 0.43	9.76 ± 0.34
ZW	12.39 ± 0.17	12.07 ± 0.30	-	12.44 ± 0.42	12.66 ± 0.34
Forl*	4.96 ± 0.23	4.67 ± 0.37	4.65	4.35 ± 0.25	4.62 ± 0.27
NW*	3.01 ± 0.18	2.74 ± 0.28	2.71	2.65 ± 0.13	2.83 ± 0.16
SH*	8.52 ± 0.18	8.33 ± 0.32	8.30	8.24 ± 0.09	8.23 ± 0.19
Occl	25.88 ± 0.18	25.14 ± 0.86	25.07	25.00 ± 0.41	25.09 ± 0.48
CW	11.19 ± 0.26	11.15 ± 0.32	11.39	11.28 ± 0.40	11.29 ± 0.26
CBL*	22.55 ± 0.17	22.12 ± 0.22	21.96	21.87 ± 0.38	22.07 ± 0.48
Patl*	11.76 ± 0.15	11.38 ± 0.45	11.12	10.99 ± 0.37	11.49 ± 0.31
Mxl*	3.62 ± 0.11	3.55 ± 0.12	3.53	3.48 ± 0.09	3.49 ± 0.12
Mnl	3.45 ± 0.11	3.39 ± 0.13	3.44	3.34 ± 0.11	3.38 ± 0.12
Mndl	13.09 ± 0.35	12.83 ± 0.26	12.99	12.75 ± 0.42	12.97 ± 0.36
M.1L	1.706 ± 0.015	1.702 ± 0.055	1.671	1.652 ± 0.05	1.646 ± 0.066
M.1W	1.147 ± 0.018	1.134 ± 0.027	1.155	1.09 ± 0.036	1.091 ± 0.44
M.2L	1.168 ± 0.038	1.179 ± 0.035	1.166	1.172 ± 0.048	1.176 ± 0.048
M.2W*	1.121 ± 0.034	1.101 ± 0.050	1.126	1.074 ± 0.032	1.064 ± 0.032
M.3L	0.617 ± 0.035	0.650 ± 0.034	0.614	0.608 ± 0.036	0.579 ± 0.039
M.3W	0.752 ± 0.056	0.683 ± 0.056	0.746	0.712 ± 0.053	0.720 ± 0.035
m1.L	1.531 ± 0.037	1.467 ± 0.094	1.514	1.443 ± 0.052	1.435 ± 0.154
m1.W*	1.039 ± 0.014	1.020 ± 0.044	1.052	0.972 ± 0.028	0.963 ± 0.035
m2.L	1.238 ± 0.046	1.242 ± 0.051	1.252	1.218 ± 0.062	1.214 ± 0.039
m2.W*	1.087 ± 0.012	1.060 ± 0.037	1.087	1.031 ± 0.029	1.034 ± 0.031
m3.L	0.742 ± 0.031	0.755 ± 0.047	0.739	0.740 ± 0.037	0.726 ± 0.045
m3.W	0.700 ± 0.018	0.700 ± 0.019	0.709	0.679 ± 0.023	0.699 ± 0.034

* variables which showed clinal variation in three geographic groups of *C. hotsoni*.

from Chah-Bahar and Nik-Shahr in southeastern Iran; these northern populations also had a significantly deeper braincase and longer diastema and interorbital breadth than specimens from the type locality in Pakistan and southern populations in Iran. Hence, based on this geographic variation in morphometric features, we separated our samples of *C. hotsoni* into three geographic groups from north to south in eastern and southeastern Iran for morphometric analyses which were in accordance with two of three phylogenetic clades. Samples from Birjand (group 1), the northern-most locality of the species, had the longest means of body and ear length and some cranial measurements (Fig. 4). Samples from Saravan, in the extreme south (group 3), were uniformly the smallest in the means of the same variables and concordant with molecular clade 2 in molecular analysis. Finally, those individuals from the geographically intermediate localities of Zahedan and Khash (group 2) displayed intermediate variable means which constitute molecular clade 3 within group 1 (Fig. 4). *C. hotsoni* thus exhibits a general north to south cline in body and some cranial dimensions from large to small from north to south. Morphometric results showed that groups 2 and 3 were not significantly different for any variable in univariate analysis and did not separate in

Table 5. Significantly different variables between three groups of *C. hotsoni* and *C. elburzensis*; pairwise Tukey' test, $p < 0.05$.

Pairs of taxa	Different variables
C. elburzensis-*C. hotsoni*, group 1	NL, SH, M.2W, m1.W, m2.W
C. elburzensis-*C. hotsoni*, group 2	ZW, M.2W, M.3L, m1.W
C. elburzensis-*C. hotsoni*, group 3	BL, EL, UDL, NL, Forl, NW, Patl, m1.W
C. hotsoni, group 1-*C. hotsoni*, group 2	EL, NL
C. hotsoni, group 1-*C. hotsoni*, group 3	BL, EL, UDL, NL, Forl, NW, Patl, m1.W
C. hotsoni, group 2-*C. hotsoni*, group 3	-

Table 6. Significance of differences between pairwise of three groups of *C. hotsoni* and *C. elburzensis* in canonical variate analysis (CVA), $p = 0.05$.

Pairs of groups	*C. elburzensis*	*C. hotsoni*, group 1	*C. hotsoni*, group 2	*C. hotsoni*, group 3
C. elburzensis	-			
C. hotsoni, group 1	0.001	-		
C. hotsoni, group 2	0.000	0.032	-	
C. hotsoni, group 3	0.001	0.019	0.130	-

multivariate analysis, while groups 1 and 2 have two and groups 2 and 3 have eight significant differences of variables and group 1 was separated from other two groups also in multivariate analysis.

The karyotype of the samples of *C. hotsoni* from Zahedan (group 2) and Birjand (group 1) were characterized both with 2N = 48 but FNa = 48 and 46, respectively (Fig. 6). Two females from the southern-most locality of Saravan (group 3), were reported to have two different karyotypes, both with the same number of autosomal arms (FNa = 48) but different diploid numbers (2N = 48 and 2N = 50) which showed chromosomal polymorphism (Shahabi et al. 2010). So individuals from morphological groups 2 and 3 share a karyotype (2N = 48, FNa = 48) with a similar diploid number to group 1, but the latter has a different autosomal arm number. This pattern is concordant with the lack of significant morphometric variables between individuals belonging to groups 2 and 3 and greater differences between those groups and specimens of group 1. However three phylogenetic clades of *C. hotsoni* are best considered as conspecific based on high nodal support from Bayesian inference and moderate nodal support from maximum likelihood (Fig. 3) with shallow molecular distances and a constant diploid number 2N = 48 (Fig. 6 and Shahabi et al. 2010) for most specimens of *C. hotsoni* with only one record of 2N = 50 by Shahabi et al. (2010).

Nevertheless, the placement in different molecular clades, significant separation in morphometric analyses and different number of autosomal arms between individuals of groups 1 and 3 in northern and southern distribution area of *C. hotsoni* provide support that these groups are two Evolutionary Significant Units, or even possibly subspecies in the initial stages of speciation. However, *C. hotsoni* samples from group 2 were similar in morphometric measurements and karyotypic characteristics with group 3, whereas they were placed in clade 3 with group 1 individuals in the phylogenetic analyses. This discordance in three datasets reveal a broad area of introgression between Birjand (group 1) and Saravan (group 3) within a single species, resulting in a clinal morphometric variation within a north to south distribution of *C. hotsoni*. In addition, clade 1 could constitute another Evolutionary Significant Unit in *C. hotsoni* based on the phylogenetic analyses with *cytb* sequences, and with owing smaller cranium measurements than northern populations from Zahedan and Khash was morphometrically more similar to southern populations in Chah-bahar and Nik-Shahr (Norris et al. 2008).

The north-to-south cline of decreasing general size within *C. hotsoni* generated a pattern wherein the samples of this species from Zahedan and Khash (group 2) are similar in several cranial dimensions and body length to specimens of *C. elburzensis*. This observation suggests the possibility of character displacement in northern population of *C. hotsoni* in group 1 (Bagheran Mts.), which occur in proximity to the southern-most known localities of *C. elburzensis*, and thus contribute to their larger size of some external and cranial variables than sample from group 2. However, the observed clinal variation in *C. hotsoni* may result from evolutionary responses to climatic or ecological variables.

C. elburzensis, which did not exhibit any geographic variation in cranial and dental variables, is strongly

separable from each three groups of *C. hotsoni* by some of morphometric variables, but the width of first lower molar is a good character to differentiate *C. elburzensis* from all samples of three groups of *C. hotsoni*. Shahabi et al. (2011) also found that *C. hotsoni* samples from Saravan (group 3 in the present study) were significantly smallest in almost all the cranial and dental measurements. The two karyotypes reported in the present study for *C. elburzensis*, one with 2N = 44, FNa = 60 from south Khorasan was recorded from Khorasan-e-Razavi by Somayeh et al. (2008) and the other with 2N = 44, FNa = 62 that is similar to karyotypes from Khorasan-e-Razavi and North Khorasan was described by Shahabi et al. (2010). Overall, *C. elburzensis* appears to have a uniform 2N = 44 but extensive diversity in number of autosomal arms, which range from 58 to 72 (Malikov et al. 1999, Graphodatsky et al. 2000, Shahabi et al. 2010). A karyotype with 2N = 30 and FNa = 44 from central and western Kopetdagh reported by Malikov et al. (1999) had been associated with *C. elburzensis* (Musser & Carleton 2005), however Norris et al. (2008) recognized this cytotype as the subspecies *C. elburzensis zykovi* based on laboratory hybridization studies. Phylogenetic analysis indicated that the mean genetic distances comparing all samples of this species in the present study showed very low intraspecific values.

Despite the apparent phenotypic similarity of these two species, the molecular phylogenetic analyses not only support the monophyly of both *C. elburzensis* and *C. hotsoni*, but also place each of them within a different major clades in the phylogenetic analysis of the genus *Calomyscus* (Fig. 3). The different karyotypic characteristics of 2N = 44 for *C. elburzensis* and 2N = 48 or 50 for *C. hotsoni* also serve to diagnose the two as separate species.

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Supplementary online materials

Table S1. Localitites and voucher numbers of taxa included in our analyses with Genbank accession numbers of the cyt*b* and *COI* sequences used in the present study (URL: http://www.ivb.cz/folia/download/akbarirad_et_al._table_s1_supplementary_material.pdf).

Fig. S1. Phylogenetic tree derived from Bayesian inference (BI) and maximum likelihood (ML) analyses of the cyt*b* gene sequences. Numbers on branches indicate the Bayesian posterior probabilities (first values) and bootstrap values derived from the maximum likelihood (second values) analysis (give only if > 95 % for posterior probabilities and if > 70 % for ML bootstraps) (URL: http://www.ivb.cz/folia/download/akbarirad_et_al._fig_s1_supplementary_material.jpg).