Iranian Journal of Animal Biosystematics(IJAB)
Vol.5, No.1, 47-58, 2009
ISSN: 1735-434X

Biosystematics study of Steppe Field Mouse *Apodemus witherbyi* (Rodentia: Muridae) from North-West Iran

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Apodemus witherbyi is a species with wide distribution and geographic variation in the Iranian plateau. This species exists in syntopic, sympatric or parapatric with other four reported *Sylvaemus* species from Iran, i.e. *A. hyrcanicus*, *A. uralensis*, *A. flavicolis* and *A. avicennicus*. In this study 33 specimens from different localities in NW Iran were examined to study the taxonomic status and the intra-specific variation of the populations. The study was carried out based on the RFLP analysis of cytochrome b (mtDNA), as well as the morphological and morphometric analyses external, cranial and dental characters. The results reveal that all the specimens studied belong to a same species, *A. witherbyi*. Variation range of the morphometric characters and the frequency of the morphological traits are provided.

Key words: wood mouse, *Apodemus witherbyi,* RFLP, morphology, morphometry

INTRODUCTION

Wood mice of the genus *Apodemus* Kaup, 1829 have been the subject of many systematic and evolutionary studies in the last decades (e.g. Mezhzherin 1997; Mezhzherin et al 1989 and 1992; Fillipucci et al. 1996, 2002; Vorontsov et al. 1992, Musser et al. 1996; Zagorodnyuk et al. 1997; Michaux et al 2002, 2004; Javidkar et al. 2005, 2007; Darvish et al. 2006; Krystufek 2002; Krystufek and Hutterer 2006; Siahsarvie and Darvish 2008). Despite these vast studies, the taxonomic status and especially biogeography of the *Apodemus* taxa are not yet clear in the Asia Minor and Iranian plateau.

Steppe field mouse *A. witherbyi* (Thomas 1902) was originally recorded as a subspecies of *Mus sylvaticus* and subsequently assigned as subspecies of *A. sylvaticus* (Ellerman and Morrison-Scott, 1951). It was next considered as synonym of *A. sylvaticus arianus* (Ellerman and Morrison-Scott, 1951), or simply *A. sylvaticus* (Corbet, 1978). Some authors listed it as synonym of *A. arianus* (Musser and Carleton 1993, Pavlinov et. al. 1995, Zagorodnyuk et al. 1997). Krystufek (2002), on examining the holotype of the species *A. witherbyi*, noted that it might be identical to *A. hermonensis*. He also asserted that the type specimens of *A. arianus* are most likely the junior synonym of *A. flavicolis*. Musser and Carleton (2005) considered *A. witherbyi* as the oldest name for *A. hermonensis*.

A. witherbyi is the most widespread wood mouse in Iran with high morphological plasticity (Macholan et al 2001, Darvish et al 2006, Siahsarvie and Darvish 2008). This species occurs

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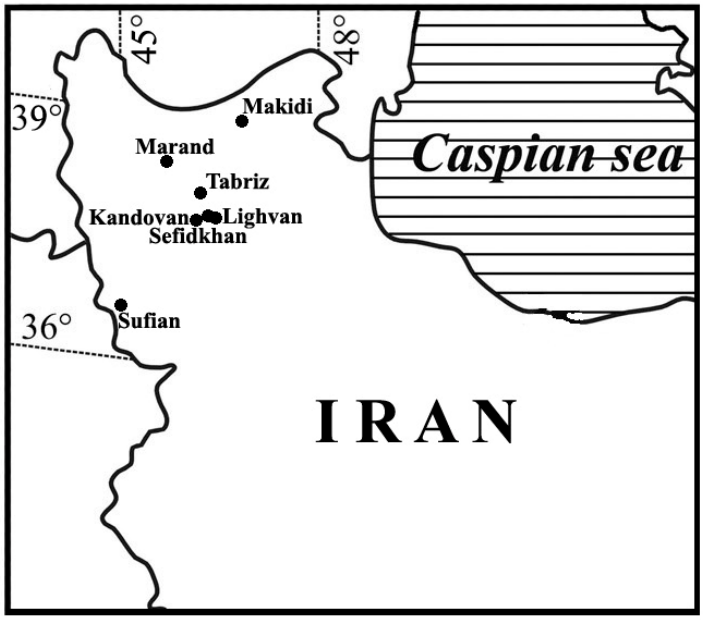


FIG 1. Localities in northwest Iran from which *Apodemus* were examined (see table 1 for more detailes).

syntopically with *A. flavicollis* in Zagros Mountain, and is altitudinally parapatric with *A. hyrcanicus* (Macholan et al 2001, Musser and Carleton 2005). It is distributed in plains, mountain and plateau steppes and highland semi-deserts of Caucasus, Anatolian Turkish steppe. It probably occurs in Afghanistan, northeast of Iraq and through most of central and north Iranian Plateau in Zagros and Elbrouz steppic provinces including Azarbayejan, kordistan, Lorestaan, Isfahan, Fars, Semnan, Tehran, center and esat mazandaran, north and east of Khorasan and Kopet-Dagh mountains (Musser and Carleton 2005). *A. witherbyi* probably is the species represented by the sample from Qazvin in the NW Iran that Darviche et al. (1979) electrophoretically separated from *A. sylvaticus* and *A. flavicolis*, but still considered closer to the latter. Recently, Krystufek and Hutterer (2006), by reporting the *A. uralensis* for the first time from Iran, represented that this species in syntopic with *A. witherbyi* in NW Iran.

In this study, we aim to appraise the taxonomic status of the wood mice of NW Iran and to evaluate the range of intraspecific variation of this species in the area.

MATERIAL AND METHODS

Thirty six specimens were captured from seven localities in NW-Iran. The map of the collection sites and the number of specimens examined for each population are presented in Fig.1 and table 1 respectively. The morphological, morphometric and molecular characters were studied and the standard voucher specimens are deposited in the Zoology Museum of Ferdowsi University of Mashhad (ZMFUM), Iran.

MOLECULAR STUDIES

Genomic DNA of 21 specimens was extracted from 0.01-0.02 g of liver or muscle tissues preserved in 96% ethanol using Genetbio kit. Complete cytochrome b genes (around 1200 bps) was amplified using modified universal primers L7 (5'-ACTAA TGACATGAAAAATCATCGTT-3') and H6 (5'-TCTTCATTTTTG GTT TACAAGAC-3') (Montgelard et al., 2002). Amplifications were carried out in a Primus 96 thermal cycler with an initial denaturation step at 94°c for 2 min. followed by 35 cycles (45s at 94°C, 45s at 50°C and 90s at 68°C) for cytochrome b with a final

TABLE 1.- Sampling localities and the number of each sample used in this study

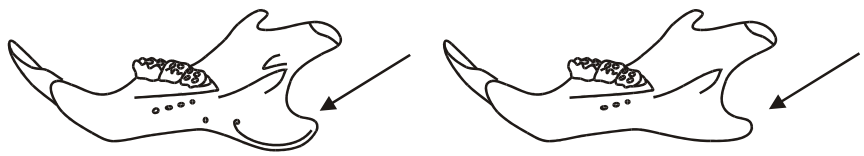
Locality	Geographic coordinates	N° of specimens
Lighvan	37° 50' N, 46° 26' E	11
Abshar	° ' N, ° ' E	4
Marand	38° 25' N, 45° 46' E	5
Kandovan	37° 48' N, 46° 14' E	2
Soufian	36° 54' N, 45° 09' E	2
Tabriz	38° 05' N, 46° 17' E	5
Sefidkhan	37° 50' N, 46° 23' E	2
Mayamay	° ' N, ° ' E	1
Makidi	38° 49' N, 46° 55' E	1
Total		33

extension time of 10 min. at 68°C (Chevret et al., 2005). The PCR products were digested with two restriction enzymes, *Alu*I and *Hin*fI. They were incubated in 37° C for 3-4 hours to digest completely. Different patterns of digested fragments were compared using electrophoresis on 1% agarose gel.

Morphological studies

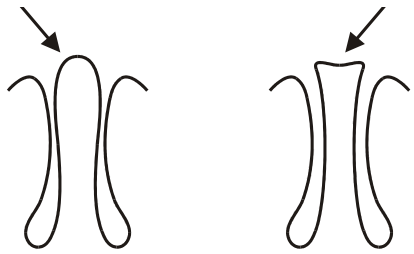
For this purpose, only adult specimens with similar age group (according to tooth wear) were studied. This procedure enables us to rule out the effect of age from evolutionary effects (Frynta and Zizkova, 1992). Thirteen dental and cranial characters were studied and the frequencies of their different states were figured out. These characters (see Javidkar et al. 2007 for details) include:

1. Angular process of mandible. a: Well developed and wide; b: Tender and blade shaped



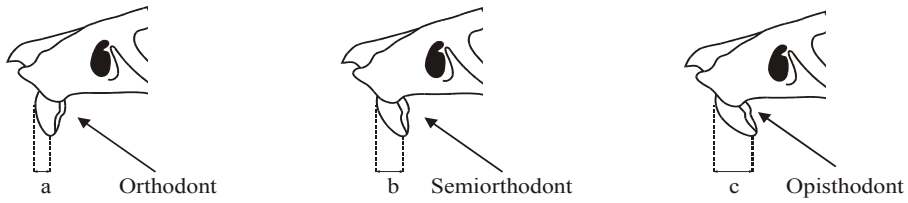
a b

2. Fronto-parietal suture.
a: V shaped and is angled
b: U shaped and is Curved
3. Posterior edge of palatine.
a: Curved
b: Rather Straight



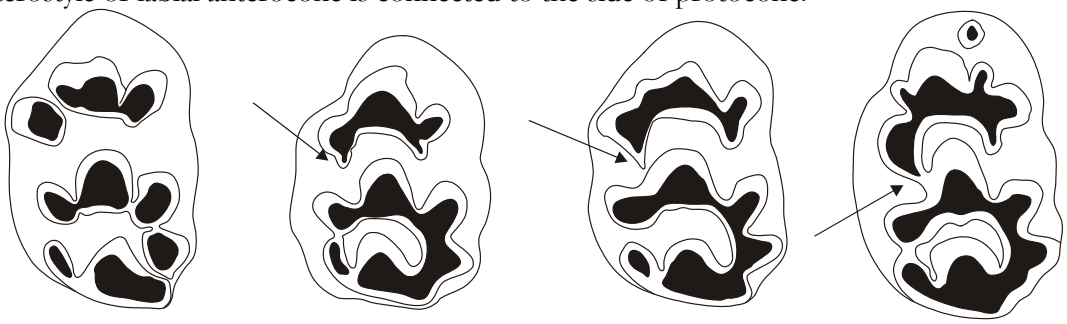
a b

4. Position of Incisor.
a: Orthodont
b: Semiorthodont
c: Opisthodont



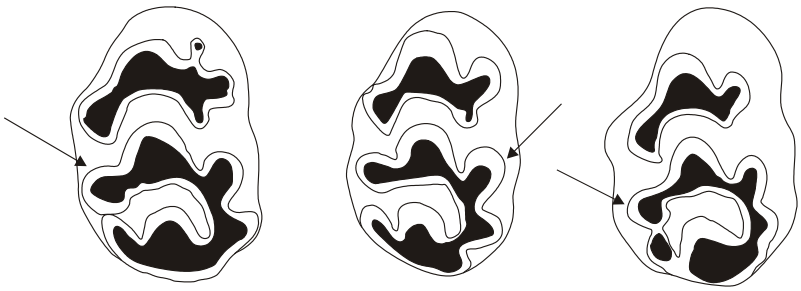
a<b<c

5. Connective plan of labial anterocone (t3) - anterostyle (t1) to protocone (t5) in upper M1/.
a: Anterostyle or labial anterocone without any connection to protocone.
b: Anterostyle or labial anterocone with a short enamel horn towards protocone.
c: Anterostyle or labial anterocone with a long enamel horn towards protocone.
d: Anterostyle or labial anterocone is connected to the side of protocone.



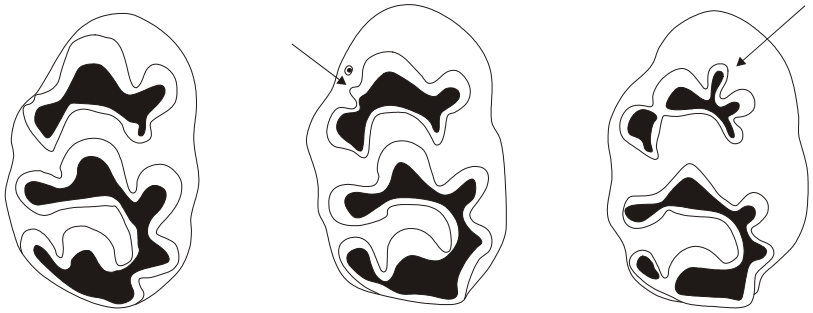
a b c d

6. Position of enterostyle (t4) to paracone (t6) in upper M1/.
a: Enterostyle and paracone locate in a row.
b: Enterostyle is upper than paracone.
c: Enterostyle is lower than paracone.



a b c

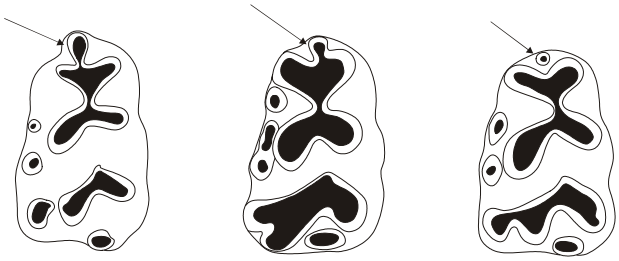
7. Position of anteroconule (t1bis-t3bis) in upper M1/.
a: Anteroconule is absent.
b: Anteroconule is present.
c: Anteroconule is well developed and similar to a real cusp.



a b c

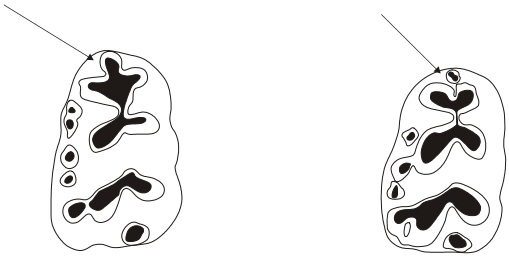
8. Position of metacone (t9) in upper M1/.
a: Massive, large and comparable with paracone.
b: Relatively small and tender that seems paracone (t6) is connected to hypocone (t8) straight.

9. Position of median anteroconid (tma) in lower M/1.
a: Well developed and comparable with lower cusps.
b: Medium size.
c: Tiny.



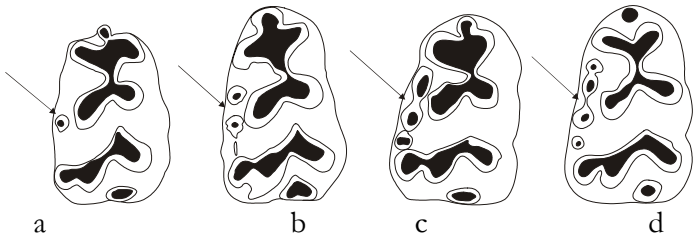
a b c

10. Position of median anteroconid (tma) to paired anteroconid cusps.
a: Mediam Anteroconid is connected to paired labial and lingual Anteroconid.
b: Isolated from the paired Anteroconid cusps.

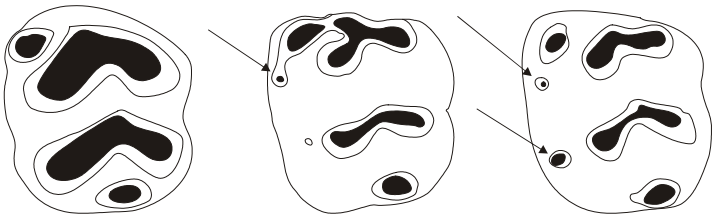


a b

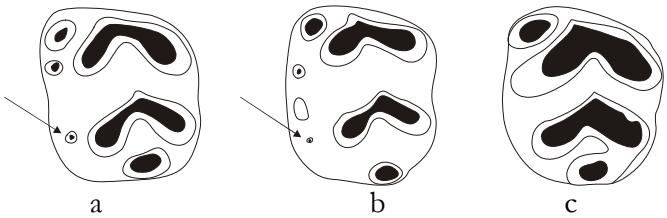
11. Number of labial cingulum cusps in lower M/1.
a: 1 Cingulum.
b: 2 Cingulums.
c: 3 Cingulums.
d: 4 Cingulums.



12. Number of labial cingulums in lower M/2.
a: Absent.
b: 1 Cingulum.
c: 2 Cingulums.



13. Size of first labial cingulum in lower M/2.
a: Large / medium.
b: Small / tiny.
c: Absent.



Besides, the presence of pectoral spot and its shape and size were defined in all specimens.

MORPHOMETRIC STUDIES

In total, four external, 12 cranial and 12 dental characters were measured accurate to the nearest millimeter, 0.05mm and 0.01mm respectively. Abbreviations used are as follows: BL, body length; TL, tail length; HFL, hind foot length; EL, ear length; BCH, braincase height; RH, rostral height; ZYGW, zygomatic width; RW, rostral width (maximum distance); IOW, interorbital width; BCW, braincase width; FL, facial length; PAL, palatal length; CBL, condylobasal length; IBW,interbullar width; BULL, bulla length; FI, length of foramen incisivum; M1/L, first upper molar length; M2/L, second upper molar length; M3/L, third upper molar length; M1/W, first upper molar width; M2/W, second upper molar width; M3/W, third upper molar length; M/1L, first lower molar length; M/2L, second lower molar length; M/3L, third lower molar length; M/1W, first lower molar width; M/2W, second lower molar width; M/3W, third lower molar width (Fig. 2).

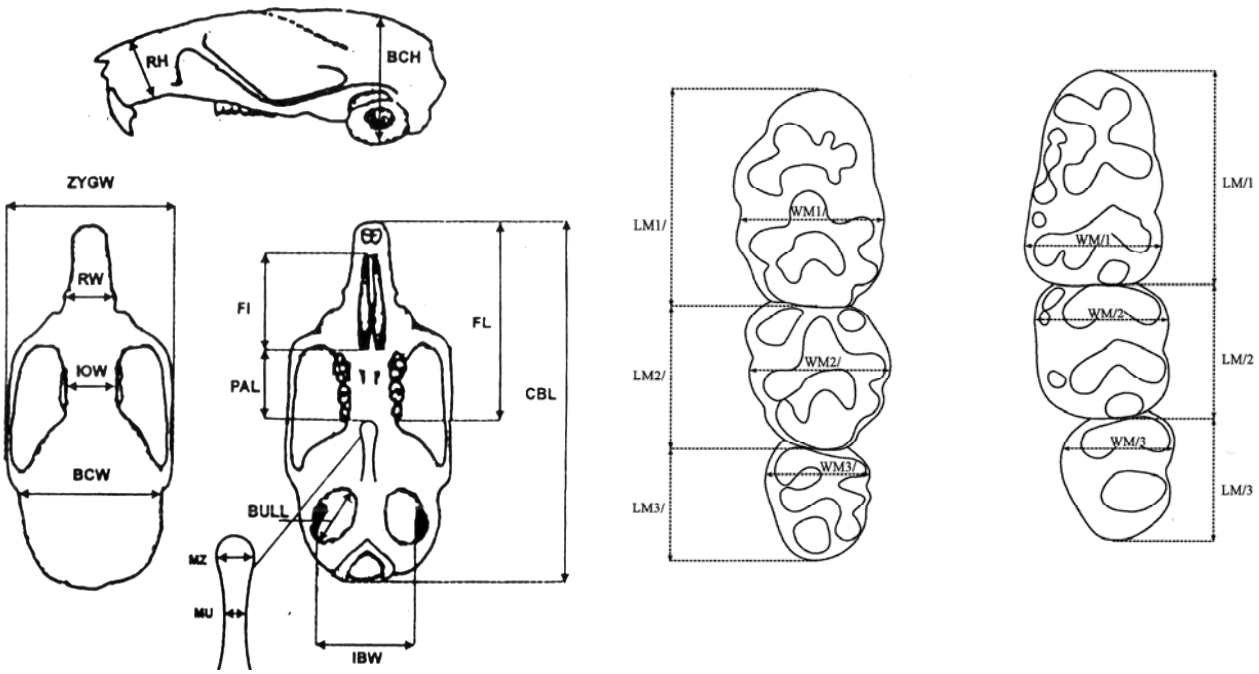


FIG2. The characters measured on skull (left image after Ferynta and Žižkova .) and molars (right). See text for abbreviations.

Analysis of Variance (ANOVA) and Multivariate Analysis of Variance (MANOVA) were used to determine significant differences among the populations. For this purpose we excluded the samples with less than three specimens as their standard deviations are not reliable. Mean and standard deviation of the characters were given for each. SPSS version 11.5 was used for all statistical procedures.

RESULTS

Molecular results

All specimens have a same haplotype for each enzyme indicating that these specimens belong to the same taxon, *A. witherbyi* (Fig. 3).

Morphological results

The pectoral spot is present in 100% of the specimens but it is polymorphic in size and shape. This character is narrow limited diffuse in 45% and spread wide diffuse in 50% of the specimens. The rest have a big dark spot.

In skull, angular process is tender in 65% and developed in 35% of the specimens (n=23); Fronto-parietal suture is U-shaped in 96% and V-shaped in 4% of the specimens (n=25); Posterior edge of palatine is rather straight in 95.7% (74% with a notch, 21.7% without notch) and curved in 4.3% of the specimens (n=23) and the incisors are semiorthodont in 59% and orthodont in 41% of the specimens (n=22).

In molars, connective plan of labial antrocone (t3)- antrostyle (t1) to protocone (t5) in upper M1/ is with long enamel horn in 45%, with short enamel horn in 25%, connected to the sides of protocone in 30% and with no connection in 0% of the specimens (n=20); position of entrostyle (t4) to paracone (t6) in the first upper molar is located in a row in 88%, entrostyle upper than the paracone in 6% and lower than the paracone in 6% of the specimens (n=17); position of entroconul (t1 bis-t3

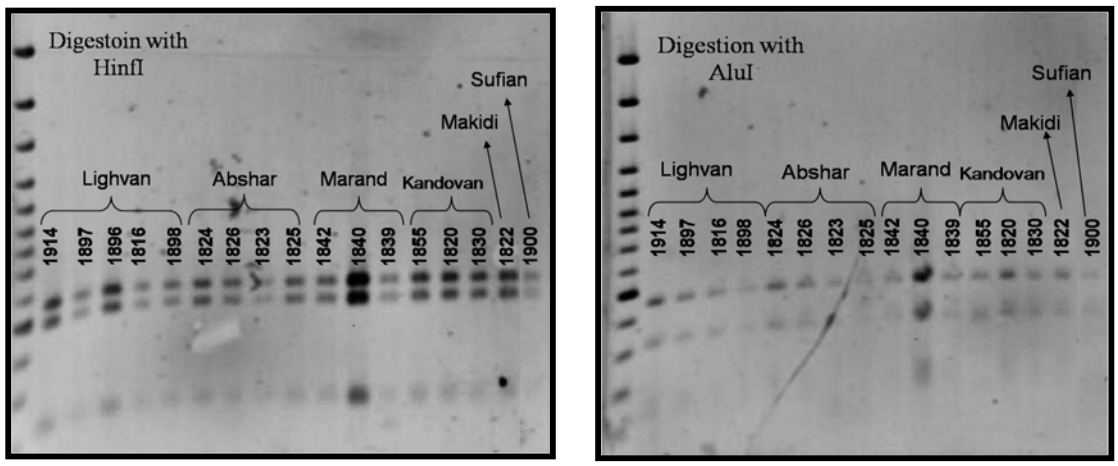


FIG. 3. Restriction patterns of cytochrome b PCR product by *Hin*fI (left) and *Alu*I (right) on 1% agarose gel

bis) in upper M1/ is absent in 50%, t1 bis in 50% and t3 bis in 0% of the specimens (n=20); position of metacone (t9) in upper M1/ is relatively small in 75% (13/16) and massive in 25% of the specimens (n=16); position of median antroconid (tma) in lower M/1 is well developed and comparable with lower cusp in0%, medium sized in 52.4%, tiny in 42.9% and absent in 4.7% of the specimens (n=21); position of median antroconid (tma) in lower M/1 is isolated from the paired entroconid cusp in 94% and connected to paired labial and lingual antroconid in 6% of the specimens; number of labial cingulums cusps in lower M/1 is 1 or 2 in 0%, 3 in 55%, 4 in 20% and 5 in 25% of the specimens (n=20); number of labial cingulums in lower M/2 is absent in 5.9%, 1 in 64.7% and 2 in 29.4% of the specimens (n=20); size of labial cingulum in lower M/2 is medium in 13.3%, small in 80% and absent in 6.7% of the specimens.

Morphometric results

The mean of standard external, cranial and dental characters are given in tables 2, 3 and 4 respectively. Tail length is always longer than head and body (Mean of tail to head and body length = 1.11, SD=0.12). Multivariate analysis of variance on cranial characters showed no significant differences among populations (Wilk's Lambda, P=0.641). The same result was obtained for dental characters (Wilk's Lambda, P=0.178). Analyses of Variance (ANOVA) revealed that the populations studied have no significant differences in most of their cranial and dental characters except in upper M1/ and M2/ length and lower M/2 width in which the specimens of Marand are a bit larger. It should be considered that in these analyses, specimens of Makidi, Mayamay, Sefidkhan, Soufian and Kandovan were not included because only one or two specimens from these localities were present, consequently their standard deviations were not reliable. These results indicate that the specimens studied from NW Iran belong to a unique species.

DISCUSSION

The results of RFLP on mitochondrial cytochrome b indicate that all the studied specimens from northwest Iran belong to a same species. This result is supported with morphmetric data seeing almost no significant differences among populations. Krystufek and Hutterer (2006) reported both *A. witherbyi* and *A. uralensis* from Makidi near Aras river in the NW Iran. They reported the maxillary tooth row of 3.65-4.00 mm and Bulla length of 4.00-4.7 mm for the latter species. Our specimens

TABLE 2. Mean and standard deviation of four external characters in different populations of *A. witherbyi* from NW Iran.

	BL	TL	HFL	EL	N
Lighvan	75.68±6.62	88.56±10.45	21.00±1.12	12.56±1.95	9
Abshar	92.01±11.49	96.00±9.54	21.75±0.50	15.00±0.82	4
Marand	89.34±3.22	96.67±6.66	21.34±0.58	14.67±0.58	3
Kandovan	101.01±4.25	97.50±0.71	20.00±1.42	15.50±0.71	2
Soufian	79.01±12.73	87.00±16.98	19.00±1.42	10.00±0.00	2
Tabriz	86.01±6.56	96.75±18.84	20.75±1.50	12.00±1.64	4
Sefidkhan	85.50±2.13	94.00±4.25	21.50±0.71	15.50±0.71	2
Mayamay	97.00	103.00	22.00	14.00	1
Makidi	98.00	106.00	22.00	14.00	1
Total	84.90±10.42	93.63±11.08	21.00±1.19	13.40±2.05	28

TABLE 3. Mean and standard deviation of 12 cranial characters in different populations of *A. witherbyi* of NW Iran. *: The populations with less than three specimens have been excluded from the ANOVA.

	BCH	RH	ZYGW	RW	IOW	BCW	FL	PAL	CBL	IBW	BULL	FI	N
Lighvan	8.26±0.33	3.82±0.24	12.70±0.37	4.38±0.31	4.15±0.10	11.55±0.50	13.51±0.70	4.66±0.33	24.07±1.15	8.79±0.33	4.88±0.36	4.76±0.31	4-11
Abshar	8.28±033	4.05±0.32	14.15	4.72±0.42	4.23±017	11.69±0.34	14.02±1.05	4.86±0.44	25.30±1.59	9.09±0.49	4.93±0.17	4.90±0.14	1-4
Marand	8.42±0.28	4.04±0.26	12.27±0.61	4.42±0.22	4.21±0.16	11.47±0.24	13.51±0.49	4.96±0.32	24.49±0.96	8.91±0.06	4.84±0.23	4.74±0.09	3-5
Kandovan	8.43±0.32	4.13±0.04		4.55±0.22	4.10±0.07	11.60±0.15	14.45±0.36	5.18±0.25	25.53±1.03	8.90	5.25±0.22	5.01±0.20	1-2
Soufian	8.90±0.14	4.20±0.50	13.05±0.22	4.50±0.00	4.28±0.18	11.80±0.15	14.11±0.73	5.19±0.97	25.25±1.35	9.06±0.20	4.60±0.29	4.80±0.12	2
Tabriz	8.10±0.45	3.92±0.17	12.15	6.48±4.29	4.19±0.09	11.59±0.14	13.85±0.60	7.32±4.73	24.00±0.15	8.60	4.74±0.44	4.59±0.29	1-5
Sefidkhan	8.15±0.50	4.08±0.39	12.50	4.15±0.07	4.23±0.04	11.55±0.07	13.55±0.64	4.65±0.15	24.78±0.53	9.28±0.53	4.65±0.36	4.83±0.11	1-2
Mayamay	8.86	4.65	13.35	4.60	4.55	11.80	14.20	5.00	25.75	9.65	5.15	5.00	1
Makidi	8.50	4.15	13.45	4.50	4.50	12.00	12.95	4.70	24.00	8.90	4.50	4.80	1
P. V.* (ANOVA)	0.689	0.305	0.075	0.230	0.645	0.881	0.596	0.131	0.401	0.492	0.879	0.409	
Total	8.35±0.36	3.99±0.29	12.81±0.64	4.76±1.71	4.20±0.14	11.61±0.34	13.73±0.69	5.14±1.73	24.64±1.13	8.97±0.37	4.85±0.32	4.78±0.25	14-33

are in accord with the presented data for the former character (3.71-3.93 mm) but they show a larger bulla length (4.53-5.17 mm). However, the bulla length of the only specimen captured from Makidi (4.50) is in accord with what was reported by Krystufek and Huttere (2006) for the *A. witherbyi* of this area. Morphological data also support the affiliation of our specimens to *A. witherbyi*. In the studied specimens, tail length is longer than head and body (93.6 mm versus 84.9 mm).

Dorsal fur is gray and ventral body is pure white. Feet have light fur and ears are gray. Tail is obviously bicolor with lighter fur in ventral side. Dental pattern of maxilla is stephanodont, the first cusp of lower M/1 have an edge that is pulled to the fifth cusp. On the first upper molar Cusps t4 and t6 are located in a row and cusps t1 and t2 are linked to each other. Cusp t7 on the first upper M/1 is large. Posterior portion of palatine is straight and fronto-parietal suture is U-shaped.

TABLE 3. Mean and standard deviation of 12 dental characters in different populations of *A. witherbyi* of NW Iran. *: The populations with less than three specimens have been excluded from the ANOVA.

	M1/L	M2/L	M3/L	M1/W	M2/W	M3/W	M/1L	M/2L	M/3L	M/1W	M/2W	M/3W	N
Lighvan	1.78±0.07	1.13±0.05	0.88±0.05	1.15±0.05	1.12±0.04	0.83±0.06	1.66±0.05	1.14±0.05	0.93±0.06	1.02±0.03	1.05±0.03	0.88±0.05	11
Abshar	**1.88±0.05**	1.17±0.07	0.88±0.04	1.17±0.04	1.11±0.04	0.79±0.03	1.70±0.08	1.15±0.05	0.92±0.03	1.02±0.04	1.02±0.04	0.82±0.09	4
Marand	**1.88±0.05**	**1.20±0.04**	0.87±0.02	1.20±0.02	1.15±0.04	0.83±0.04	1.72±0.05	1.13±0.06	0.88±0.07	1.04±0.03	**1.08±0.02**	0.86±0.07	5
Kandovan	1.83±0.00	1.16±0.02	0.83±0.01	1.20±0.04	1.14±0.05	0.80±0.01	1.63±0.00	1.12±0.05	0.91±0.05	1.02±0.07	1.05±0.00	0.85±0.05	2
Soufian	1.72±0.03	1.13±0.02	0.92±0.03	1.17±0.04	1.10±0.02	0.88±0.13	1.77±0.10	1.15±0.05	0.92±0.07	1.14±0.15	1.12±0.06	0.88±0.05	2
Tabriz	1.82±0.08	1.14±0.03	0.81±0.08	1.17±0.04	1.13±0.06	0.83±0.05	1.65±0.04	1.11±0.06	0.91±0.07	1.03±0.03	1.05±0.02	0.89±0.02	5
Sefidkhan	1.79±0.03	1.11±0.03	0.79±0.05	1.11±0.00	1.07±0.07	0.81±0.01	1.67±0.07	1.11±0.10	0.92±0.02	0.98±0.06	0.99±0.05	0.83±0.00	2
Mayamay	1.94	1.20	0.82	1.23	1.20	0.85	1.71	1.19	0.99	1.11	1.17	0.94	1
Makidi	1.94	1.11	0.82	1.22	1.11	0.85	1.78	1.13	0.85	0.99	1.05	0.88	1
P. V.* (ANOVA)	**0.023**	**0.040**	0.113	0.266	0.444	0.542	0.052	0.524	0.381	0.544	**0.047**	0.182	
Total	1.82±0.08	1.15±0.05	0.86±0.06	1.17±0.05	1.13±0.05	0.83±0.05	1.68±0.06	1.13±0.05	0.92±0.06	1.03±0.06	1.05±0.05	0.87±0.06	33

ACKNOWLEDGEMENTS

This is the publication number 2009-113 of Institut des Science de l'Evolution of Montpellier (ISEM).

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