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Phylogeography and taxonomic status of the greater mouse-tailed bat *Rhinopoma microphyllum* (Chiroptera: Rhinopomatidae) in Iran

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The taxonomic status of the greater mouse-tailed bat (*Rhinopoma microphyllum*) in Iran is not clear and researchers have reported conflicting results. The initial suggestion of the presence of two subspecies *R. m. microphyllum* and *R. m. harrisoni* has been questioned on the basis of small differences between the populations. These differing inferences are based on analysis of morphological characteristics. Here we present a study of the phylogeography of this species using 567 bp of the mitochondrial control region in several localities in Iran and from across its distribution to infer the taxonomic status of this species. Based on the control region sequences, we found high genetic diversity in the Iranian population but variation between populations was not statistically significant. The phylogenetic trees and statistical parsimony network showed all Iranian samples were grouped in the same clade and Levant samples belonged to another clade. These results support the hypothesis that all Iranian specimens belong to one subspecies. We therefore recommend that *R. m. microphyllum* and *R. m. harrisoni* can be synonymized as the same subspecies with the name *R. m. harrisoni*, because molecular results indicate that Iranian samples differ from Levant and Moroccan samples (*R. m. microphyllum*).

Key words: *Rhinopoma microphyllum*, phylogeography, control region, Iran

INTRODUCTION

The greater mouse-tailed bat (*Rhinopoma microphyllum*) is a medium-sized bat with a free tail, inhabiting arid and semi-arid regions of the Old World. Records of *R. microphyllum* in Iran are restricted to the northern shores of the Oman Sea and the Persian Gulf and adjacent arid lands in the northern Mesopotamian plain where the climate is characteristically hot and prolonged in summer with no freezing period in winter (DeBlase, 1980). Although some reports indicate that *R. microphyllum* enters the Iranian plateau and stays in marginal areas of the Zagros range to the Mesopotamian plain for a short period in summer (Hemmati, 2001), this bat occurs mainly in dry lands of southern parts of Iran.

Rhinopoma microphyllum (Brünnich, 1782) is one of six species (*R. microphyllum*, *R. muscatellum*, *R. hardwickei*, *R. macinnesi*, *R. cystops* and *R. hadramauticum* of the mouse-tailed bats (Rhinopomatidae) that are found in the arid and semi-arid regions of the Old World (Van Cakenberghe and De Vree, 1994; Hulva *et al.*, 2007; Benda *et al.*, 2009), covering about 12,000 km, from Sumatra and India in the east, the Middle East, and southern Asia, throughout Arabia, to north-western Africa (e.g., Hill, 1977; DeBlase, 1980; Van Cakenberghe and De Vree, 1994; Altringham, 1996; Schlitter and Qumsiyeh 1996; Simmons 2005; Hulva *et al.*, 2007). *Rhinopoma microphyllum* is considered to be polytypic and four to six subspecies (*R. m. microphyllum*, *R. m. sumatrae*, *R. m. kinneari*, *R. m. tropicalis*, *R. m. harrisoni* and *R. m. asirensis*) have been reported (Hill, 1977; Koopman, 1994; Van Cakenberghe and De Vree, 1994; Simmons, 2005; Levin *et al.*, 2008).

The taxonomic status of *R. microphyllum* subspecies in Iran is unclear, as different researchers have reported either one or two subspecies. Schlitter and DeBlase (1974) recognized *R. m. harrisoni* in southern Iran. According to these authors, *R. m. harrisoni* is smaller than *R. m. microphyllum*. In a more detailed survey of this genus, Hill (1977) confirmed the presence of two subspecies from Iran.

Nevertheless, in the most recent study, Van Cakenberghe and De Vree (1994) showed that differences between the two subspecies are very small. These authors suggested that the validity of *R. m. harrisoni* may be questioned and *R. m. harrisoni* should be considered a synonym of the nominal form.

In the first study on the intraspecific genetic variability of *R. microphyllum*, Levin *et al.* (2008) used two mitochondrial markers (the cytochrome *b* gene and the control region) from Israeli colonies and as well as from across the species range to discover three clades: an Oriental one comprising the Indian bats, an intermediate clade comprising the Iranian bats and Palaearctic clade including the Moroccan and Levant populations. Their results show that the Israeli and Moroccan populations belong to the subspecies *R. m. microphyllum*, while the Indian group belongs to the subspecies *R. m. kinneari.* Here, based on only two samples collected from migrating populations of *R. microphyllum* in the western Zagros Mountains, they suggested that Iranian *R. microphyllum* can be divided into two subspecies: *R. m. harrisoni* and *R. m. microphyllum*, and both can be found in sympatry at the edge of their distributional range.

In this study, we used 567 bp partial mitochondrial DNA (mDNA) control region for evaluating the subspecific status of *R. microphyllum* in Iran and over most of the species' range. The present study is the first attempt to infer the phylogeography, genetic variability and intraspecific differentiation of *R. microphyllum* in Iran.

Materials and Methods

Sampling

To assess the phylogeography of the greater mouse-tailed bat (*R. microphyllum*) we included 71 samples from 13 localities of Iran, five samples from Levant and seven sequences were retrieved from GenBank (Table 1). In three consecutive years (2006–2008), a total of 71 adults were captured from 13 roosts (Fig. 1) in west, south, southwestern and southeastern Iran. The bats were captured using hand or mist nets and a 3-mm wing membrane biopsy punch was taken from each bat and placed in Eppendorf vials containing 96% ethanol for DNA analysis (Worthington Wilmer and Barratt, 1996). All bats were released immediately after sampling. Five Levant samples were collected from a colony in the Hula Valley (33°06'N, 35°39'E) and kindly provided by Dr. Eran Levin. In the mitochondrial control region analysis we included five sequences from Levant (GenBank [accession numbers AM886153, AM886154, AM886155, AM 886156 and AM886157]), one sequence from India (GenBank [accession number AM886158]), and one sequence from Morocco (GenBank [accession number AM886161]). *Rhinopoma hardwickei*, *R. muscatellum* and *R. cystops* were used as outgroups. *R. hardwickei* were sampled from southwestern Iran, *R. muscatellum* was sampled from Kuhe-Khaje (20 km SW Zabol) in eastern Iran and *R. cystops* was kindly provided by Dr. Eran Levin. A distribution map of collection sites was created using DIV GIS 3.1 (Hijmans *et al.*, 2002) by superimposing locality records on layers depicting political boundaries and topography.

Table 1. Name and locality from which skin samples were taken from *R. microphyllum* and three species of *Rhinopoma* used as outgroups

Species	Region	Locality	Latitude	Longitude	*n*	References
R. microphyllum	Iran	Dehloran, Ilam province	32°55'N	47°35'E	8	This study
		Bishehderaz, Ilam province	32°48'N	46°58'E	4	This study
		Darab, Fars province	28°45'N	54°34'E	5	This study
		Nojivaran, Kermanshah province	34°29'N	47°29'E	6	This study
		Biston, Kermanshah province	34°25'N	47°28'E	5	This study
		Hashilan, Kermanshah province	34°35'E	46°15'N	7	This study
		Damin, Sistan and Baluchistan province	27°22'N	60°47'E	6	This study
		Jiroft, Kerman province	28°51'N	57°27'E	4	This study
		Gotvand, Khuzestan province	32°15'N	48°50'E	7	This study
		Lar, Fars province	27°43'N	54°19'E	7	This study
		Tadovan, Fars province	28°47'N	53°21'E	5	This study
		Kazeron, Fars province	29°34'N	51°44'E	6	This study
		Bastak, Hormozgan province	27°12'N	54°15'E	1	This study
R. microphyllum	Levant	Hula valley	33°06'N	35°39'E	5	This study
		Tabaqat Fahl	32°27'N	35°37'E	1	Levin *et al.* (2008)
		Jordan valley	33°11'N	35°39'E	4	Levin *et al*. (2008)
R. microphyllum	Morocco	Anti-Atlas Mts.	29°15'N	9°11'W	1	Levin *et al*. (2008)
R. microphyllum	India	New Delhi	28°38'N	77°12'E	1	Levin *et al*. (2008)
R. hardwickei	Iran	Gotvand	32°15'N	48°50'E	1	This study
R. muscatellum	Iran	Kuhe-Khaje (Zabol), Sistan and Baluchistan province	30°56'N	61°15'E	1	This study
R. cystops	Levant	Kineret Valley	32°44'N	35°38'E	1	This study

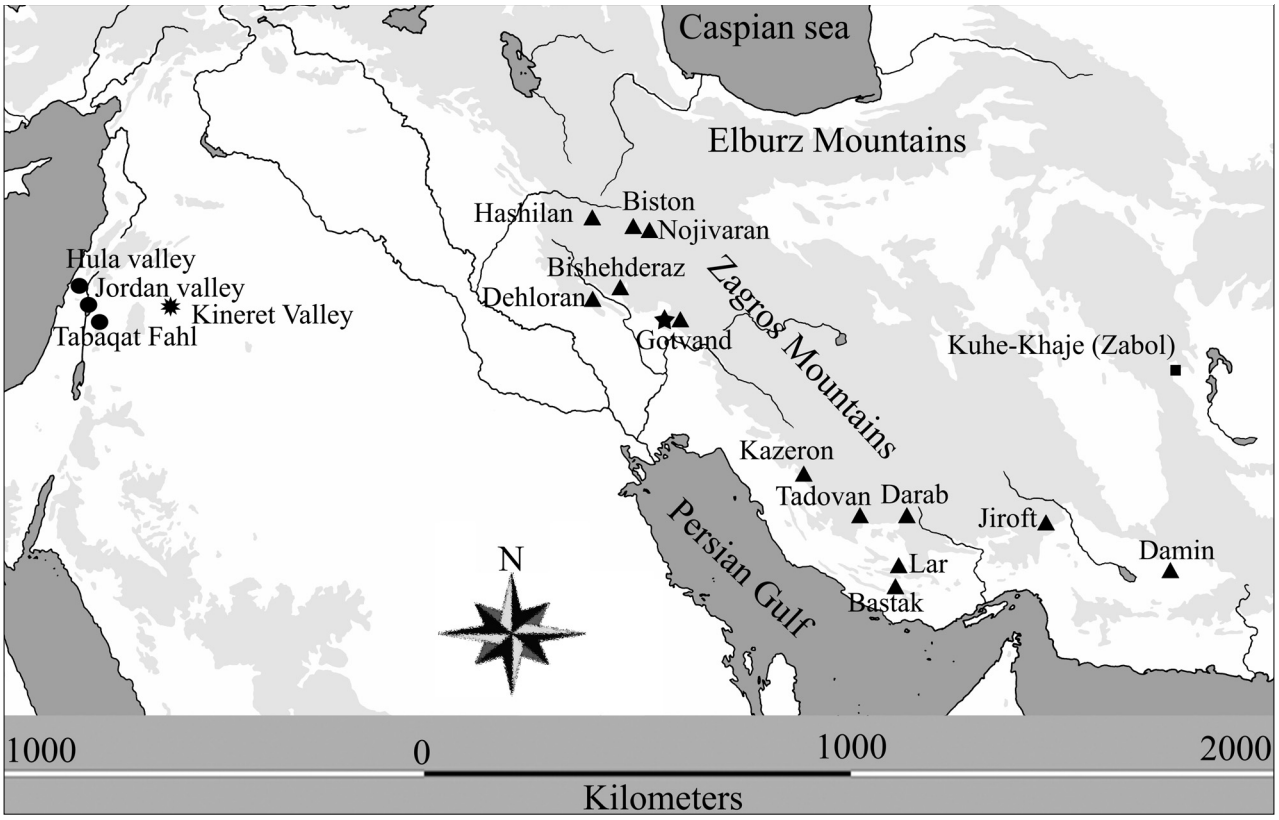

FIG. 1. Map of *R. microphyllum* sampling localities in Iran and Levant with outgroups. ▲ — *R. microphyllum* (Iran), ● — *R. microphyllum* (Levant), ■ — *R. muscatellum*, ★ — *R. hardwickei*, ✷ — *R. cystops*

DNA Amplification and Sequencing

Genomic DNA was isolated from the specimens using Tissue Kits (GenNetBio™), following the manufacturer's instructions (Seoul, South Korea). Amplification and sequencing of the mitochondrial control region was performed using the primers D1-Pro5′-CCACCATCAGCACCCAAAGC-3′ and Dloop-R1 5′-TACCARAGCCATGACACCACAGTT-3′ (Levin *et al.*, 2008). In the Dloop-R1 primer we used A nucleotide instead of R. Polymerase chain reactions (PCRs) in a final volume of 25 µl contained optimized amounts of PCR water, 2.5 µl of 10X PCR buffer (100 mM Tris-HCl (pH 8.8), 500 mM KCl), 0.2 µl of each primer (10µM), 0.5 µl of dNTP (10 mM), 0.75 µl $MgCl_2$ (50 mM), 50–100 ng of genomic DNA template, and 1 unit of *Taq* DNA polymerase. Cycling parameters were as follows: 94°C for 5 min, 35 cycles of 94°C for 30s, 56°C for 40s, 72°C for 45s, 72°C for 10 min. Sequencing reactions were done in Macrogen Korea Laboratories. DNA sequencing was performed on an ABI 3700 DNA sequencer (Applied Biosystems). The obtained sequences were edited and aligned using ClustalW (Thompson *et al*., 1994), as implemented in the Bioedit program sequence alignment editor (Hall, 1999) using the default settings. The sequences were deposited in GenBank with the accession numbers JF769752 to JF769776.

Population Genetic Analysis

Genetic diversity was calculated for all colonies based on haplotype diversity (h) and nucleotide diversity (π). Values for the numbers of polymorphic sites, parsimony informative polymorphic sites, number of transitions and transversions, and the mean numbers of pairwise differences among sequences were also estimated. These diversity indices were computed with the software DnaSP, version 4.0 (Rozas *et al.*, 2003).

Molecular variance was assessed using separate analyses of molecular variance (AMOVA) with 10,000 permutations (Excoffier *et al.*, 1992; Excoffier, 2007) at several possible population groupings of *R. microphyllum* (among four regions, among two regions (Iran and Levant) and among colonies in Iran) in Arlequin version 3.1 (Excoffier *et al.*, 2005). They were assessed according to the degree of differentiation among regions (θ_{CT}), among populations within regions (θ_{SC}) and within populations (θ_{ST}).

We tested the model of isolation by distance among all populations and the Iranian population by plotting pairwise genetic differentiation index (θ_{ST}) against geographic distance (Rousset, 1997). To assess the statistical significance of the correlation between genetic and geographic distances across the entire range and Iranian range of the species, a Mantel test (10,000 permutations) was performed with Arlequin version 3.1 (Excoffier *et al*., 2005). The geographic distance between each pair of sampling localities was derived from coordinates and was calculated by the Geographic Distance Matrix Generator program, version 1.2.3 (Ersts Internet).

To assess intraspecific divergence within *R. microphyllum* in different regions and also interspecific divergence between the three other species of *Rhinopoma*, genetic distances were calculated using MEGA ver. 4 (Tamura *et al.*, 2007). Using the

net evolutionary divergence method, the diversity between *R. microphyllum* in different regions was subtracted from overall divergence between them. Net average distances and standard error estimates were computed using Kimura two parameter (Kimura, 1980) with 1,000 bootstrap replicates in MEGA ver. 4 (Tamura *et al*., 2007). Uncorrected pairwise K2P distances for 29 haplotypes were also calculated using the maximum composition likelihood method.

Phylogenetic Analyses

Individual haplotypes were used to construct a Neighbor-joining (NJ) and Maximum parsimony tree (MP) in PAUP*, version 4.0b10 (Swofford, 2002), Maximum likelihood (ML) with PHYML, version 3.0 (Guindon and Gascuel, 2003) and a Bayesian inference (BI) tree in MRBAYES, version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). A distance-based analysis was implemented by estimating the Kimura two parameter model to estimate distances and a neighbor-joining (NJ) tree was constructed to examine the relationship between all identified haplotypes. Statistical support for branching patterns was estimated by 1,000 bootstrap replications. A maximum parsimony (MP) analysis was implemented; weighting all nucleotide change positions equally. Heuristic searches were run using random addition of taxa and tree bisection and reconnection algorithm (TBR). Bootstrap support was estimated using 1,000 replicates with heuristic mode parsimony. The maximum likelihood (ML) analysis used the GTR model of nucleotide substitution. The GTR model (base frequencies: A = 0.3662; C = 0.2216; G = 0.0971; and T = 0. 3150; rate parameter estimates ([A-C] = 4.2647; [A-G] = 12.8076; [A-T] = 3.0709; [C-G] = 1.2067; [C-T] = 20.9915; [G-T] = 1.0000), Rates = equal was selected as the most appropriate substitution model using the Akaike information criteria (lnL = 1143.8785; AIC = 2303.7571) implemented in MODELTEST, version 3.7 (Posada and Crandall, 1998) and jModelTest, version 0.1.1 (Posada, 2008). In ML analysis a starting tree was obtained by BIONJ and nodal support was estimated from 1,000 bootstrap replicates. Bayesian Inference was performed with two simultaneous runs and four search chains within each run (three heated chains and one cold chain) for 2,000,000 generations, sampling trees every 100 generations. The first 5,000 sampled trees were discarded as burn-in, and subsequent tree likelihoods were checked for convergence in Tracer 1.5.0 (Rambaut and Drummond, 2009). After discarding, a consensus tree with posterior probabilities was generated and visualized using the Treeview software (Page 1996). In this method the same substitution model was used as the ML analyses. In all analyses, the tree was rooted with *R. hardwickei*, *R. muscatellum* and *R. cystops*. To complement the tree-based approaches we also implemented 95% statistical parsimony haplotype network (Templeton *et al.*, 1992) using the program TCS version 1.21 (Clement *et al.*, 2000).

RESULTS

Genetic Variation and Population Analysis

We identified 29 different haplotypes (Table 2) from a total of 83 *R. microphyllum* individuals based on 567 base pairs of the mitochondrial control region. Seven haplotypes were identified from GenBank and only one of them was shared with our sequences. A total of 43 polymorphic sites were recorded including 37 transitions, six transversions, and one indel. Also, 525 characters were constant, 21 variable characters were parsimony informative, and 21 variable characters were parsimony uninformative. Mean nucleotide composition in the greater mouse-tailed bat control region was A: 34.76%, C: 22.30%, T: 30.44% and G: 12.50%. No haplotype found in Iran populations was shared with populations in Levant, India or Morocco. Of the 22 different haplotypes in the Iranian population, 68.18% haplotypes from 13 localities were not shared, and 31.82% were shared among localities. The most common haplotypes were 9, 10 and 11; found in 47 individuals. Haplotypes 12, 16 and 20 were discovered in six localities. Haplotype 19 was found in three individuals from two localities and other haplotypes comprised only one individual (Table 2). The dominant Iranian haplotype (H9) of *R. microphyllum* differed from the Indian haplotype by 15 nucleotide substitutions and from Levant (H1) and Moroccan haplotypes by 14 nucleotide substitutions (Table 2). The Iranian haplotypes were further distinguished by an insertion of a single nucleotide that was absent in Levant, Moroccan and Indian haplotypes; this is the single indel of the alignment. Haplotype diversity values (h) in the Iranian population ranged from 0.0 in Bastak, where only one individual and one haplotype was recorded, to 1.0 in Tadovan and Jiroft populations. Nucleotide diversity values (π) varied from 0.002 to 0.0045, showing a close relationship among the haplotypes. The average number of nucleotide differences in the Iranian population was 2.067 and the average number of nucleotide substitutions per site (Jukes and Cantor) was 0.004. In Levant with 10 samples, h and π values were 0.667 and 0.0014, respectively (Table 2).

The AMOVA results suggested that a significant genetic difference was attributable to two levels of all three hierarchical levels tested (among groups, among populations within groups, and within populations). To assess whether the high geographic distance impeded gene flow, we classified all populations into four regions to analyze genetic structure by AMOVA. Results showed that no significant genetic difference was detected among populations within region (0.22% genetic variation, $\theta_{SC} = 0.01765$, $P = 0.46$), and most genetic variation (87.77%) was explained by differences among regions. When we used Iranian colonies to assess the position of subspecies, there was no significant

TABLE 2. Variable nucleotide positions and molecular diversity within the partial sequences (567 bp) of the control region for 29 haplotypes of 83 *R. microphyllum* in different regions. New haplotypes (H8 to H29) are reported here. The alignment of haplotypes H1 to H7 is given in Levin *et al.* (2008)

Haplotype	Polymorphic sites 1111111222222222222222333333333445555 3466736788880001445555566789023355555240256 9346175601250891690123712783270324789896466	Iran cave populations Dehloran	Bishehderz	Darab	Nojivaran	Biston	Hashilan	Damin	Jiroft	Gotvand	Lar	Tadovan	Kazeron	Bastak	Iran	Levant	Morocco	India	Total
1	GGGCCTCCCCTATAGTCTCAAAG-TCGTACCAGCATTAGTGAA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6	–	–	6
2	............C..........-..................	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	1
3	................C.....-..................	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	1
4	.....................-.................C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	1
5	.....................-..T..............	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	1
6	........T....A........-.............GA....	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	1
7	..ATTC.TTTC...A.......A-CT.....G.T.CC.A....	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1
8	..A.T..T.T..C.AC...TGGAACT...TT............	1	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	1
9	..A.T..T.T....AC...TGGAACT...TT............	2	2	2	3	1	2	1	–	3	–	1	2	–	20	–	–	–	20
10	..A.T..T.T....AC...TGGAACT...T..A......C...	2	–	1	–	1	1	1	1	1	2	1	2	–	13	–	–	–	13
11	..A.T..T.T....AC...TGGAACT...TT.......C....	1	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	1
12	..A.T..T...G..AC...TGGAACT...TT............	1	–	–	–	–	1	–	–	–	–	–	–	–	2	–	–	–	2
13	..A.T..T.T....AC...TGGAACT.C.TT............	1	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	1
14	..A.T..T.T....AC...TGGAACT....T............	–	–	–	–	–	–	–	–	1	–	–	–	–	1	–	–	–	1
15	..A.T..T.T....AC...TGGAACT...T.............	–	1	1	–	2	1	3	1	1	2	1	1	–	14	–	–	–	14
16	..A.T..T.T..C.AC...TGGAACT...T.............	–	–	–	–	–	–	–	–	1	–	1	–	–	2	–	–	–	2
17	A.A.T..T.T....AC...TGGAACT...T.............	–	1	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	1
18	..A.T.TT.T....AC...TGGAACT...T..A......C...	–	–	–	–	–	–	–	–	–	1	–	–	–	1	–	–	–	1
19	..A.T..T.T....AC...TGGAACT..............T..	–	–	1	–	–	1	–		–	–	1–	–	–	3	–	–	–	3
20	.AA.T..T.T....AC...TGGAACT...T.............	–	–	–	–	_	–	–	1	–	1	–	–	–	2	–	–	–	2
21	..A.T..T.T...GAC...TGGAACT...TT............	–	–	–	–	–	–	–	–	–	–	1	–	–	1	–	–	–	1
22	..A.T..T.T....AC...TGGAACT...T..A......C.C.	–	–	–	–	–	–	–	–	–	–	–	1	–	1	–	–	–	1
23	..A.T..T......AC...TGGAACT...T.............	–	–	–	–	–	–	–	–	–	–	–	–	1	1	–	–	–	1
24	..A.T..T......ACT..TGGAACT...TT............	–	–	–	1	–	–	–	–	–	–	–	–	–	1	–	–	–	1
25	..A.T..T.T....AC...TGGAACT...T..A..........	–	–	–	1	–	–	–	–	–	–	–	–	–	1	–	–	–	1
26	..A.T..T.T....AC..TTGGAACT...TT............	–	–	–	1	–	–	–	–	–	–	–	–	–	1	–	–	–	1
27	..A.T..T.T....AC...TGGAACT..G...........T..	–	–	–	–	1	–	–	–	–	–	–	–	–	1	–	–	–	1
28	..A.T..T.T....AC...TGGAACT...TT...G........	–	–	–	–	–	1	–	–	–	–	–	–	–	1	–	–	–	1
29	..A.T..T.T.G..AC...TGGAACT...T.............	–	–	–	–	–	–	11	–	–	–	–	–	1	–	–	–	–	1
Sample size		8	4	5	6	5	7	6	4	7	7	5	6	1	71	10	1	1	83
Haplotype diversity (h)		0.929	0.833	0.900	0.800	0.900	0.952	0.800	1.000	0.857	0.905	1.000	0.867	–	0.860	0.667	–	–	0.894
Nucleotide diversity (π)		0.0045	0.002	0.004	0.0029	0.004	0.0045	0.0024	0.0035	0.003	0.004	0.0039	0.0036	–	0.0036	0.0014	–	–	0.0092
Number of polymorphic sites		8	2	5	5	6	8	4	4	5	6	5	4	–	19	4	–	–	42
Number of transitions		7	2	4	5	5	7	4	4	5	5	5	3	–	16	2	–	–	36
Number of transversions		1	0	1	–	1	1	–	–	–	1	–	1	–	3	2	–	–	6
Number of parsimony informative sites		3	1	1	–	–	1	–	–	1	2	1	3	–	9	–	–	–	21
Average number of nucleotide difference		2.536	1.167	2.200	1.667	2.400	2.571	1.333	2.000	1.714	2.286	2.200	2.067	–	2.058	0.800	–	–	5.204

TABLE 3. Hierarchical analysis of molecular variance (AMOVA) among mtDNA control region sequences of *R. microphyllum* in different geographical groupings. Percentage of variation is provided for three hierarchical levels. θ_{CT} indicates the degree of differentiation among groups; θ_{SC} indicates the degree of differentiation among populations within groups; and θ_{ST} indicates the degree of differentiation within populations

Structure	Source of variation	Variation (%)	Fixation indices	*P*-value
Four regions (Iran, Levant, Morocco and India)	Among regions	87.77	$\theta_{CT} = 0.8777$	< 0.01
	Among populations/Within regions	0.22	$\theta_{SC} = 0.0177$	0.46
	Within populations	12.01	$\theta_{ST} = 0.8804$	< 0.001
Two regions (Iran vs. Levant)	Among regions	87.91	$\theta_{CT} = 0.8792$	< 0.01
	Among populations/Within regions	0.12	$\theta_{SC} = 0.0103$	0.42
	Within populations	11.97	$\theta_{ST} = 0.8803$	< 0.001
Iranian colonies	Among colonies	0.13	$\theta_{ST} = 0.0014$	0.46
	Within colonies	99.87	–	–

differentiation among colonies (0.13%); most genetic variation (99.87) existed within colonies (Table 3).

The mean of pairwise distance between all haplotypes of control region sequences was 1.41%. Also, the average genetic distances between *R. microphyllum* from Iran compared to Levant, Morocco and India were 2.14%, 2.19% and 2.38%, respectively (Table 4). Pairwise uncorrected K2P genetic distances between all haplotypes in different regions ranged from 0.18–3.29%. Similar values for the Iranian haplotypes were 0.18–1.07% . However, the average genetic distances within *R. microphyllum* from Iran and Levant were 0.55% and 0.28%, respectively. The average genetic distances between *R. microphyllum* and the three outgroup species are also shown in Table 4. Pairwise genetic distance plotted against geographic distance (Fig. 2A) indicated significant isolation by distance across the species' range (Mantel test: $R^2 = 0.48$, $P < 0.001$), however, no significant correlation was detected between the populations across the entire range in Iran (Mantel test: $R^2 = 0.04$, $P > 0.05$) (Fig. 2B). Results from the present study indicate that strong gene flow exists in various populations of this species within its Iranian range.

Phylogenetic Analyses

All phylogenetic analyses (Maximum likelihood, Bayesian inference, Maximum parsimony and Neighbor joining) of *R. microphyllum* haplotypes from western and eastern populations yielded similar topologies, consisting of three major lineages. The topology resulting from Bayesian inference analysis is presented in Fig. 3. These analyses produced highly concordant trees, each revealing that *R. microphyllum* form a monophyletic lineage with respect to the congeneric species *R. hardwickei*, *R. muscatellum* and *R. cystops*. In the Bayesian tree, all haplotypes grouped into three major groups (Levant/Morocco, India and Iran) that broadly corresponded to the distinct geographic regions and statistical parsimony haplotype network. The clades are all well-defined geographically, with neither shared haplotypes among regions nor evidence of contact zones (Figs. 3 and 4). For the NJ, ML, MP and BI trees, all Iranian samples were grouped in a single monophyletic clade with strong bootstrap support > 90% (Fig. 3). The statistical parsimony haplotype network (Fig. 4), based on 95% statistical parsimony, showed three disconnected haplotype subnetworks, which were in agreement with the

TABLE 4. The average K2P genetic distances with standard errors between populations of *R. microphyllum* and three species of this genus

Species	*R. cystops*	*R. hardwickei*	*R. muscatellum*	*R. microphyllum*		
				Levant	Morocco	India
R. cystops						
R. hardwickei	16.68 ± 1.83					
R. muscatellum	24.51 ± 2.41	24.91 ± 2.37				
R. microphyllum (Levant)	21.01 ± 2.13	22.57 ± 2.15	17.46 ± 1.93			
R. microphyllum (Morocco)	21.34 ± 2.14	22.54 ± 2.12	17.68 ± 1.87	0.72 ± 0.37		
R. microphyllum (India)	21.58 ± 2.14	22.17 ± 2.17	18.38 ± 1.96	2.76 ± 0.68	2.38 ± 0.63	
R. microphyllum (Iran)	21.82 ± 2.19	22.77 ± 2.18	17.72 ± 1.96	2.14 ± 0.57	2.19 ± I0.60	2.38 ± 0.34

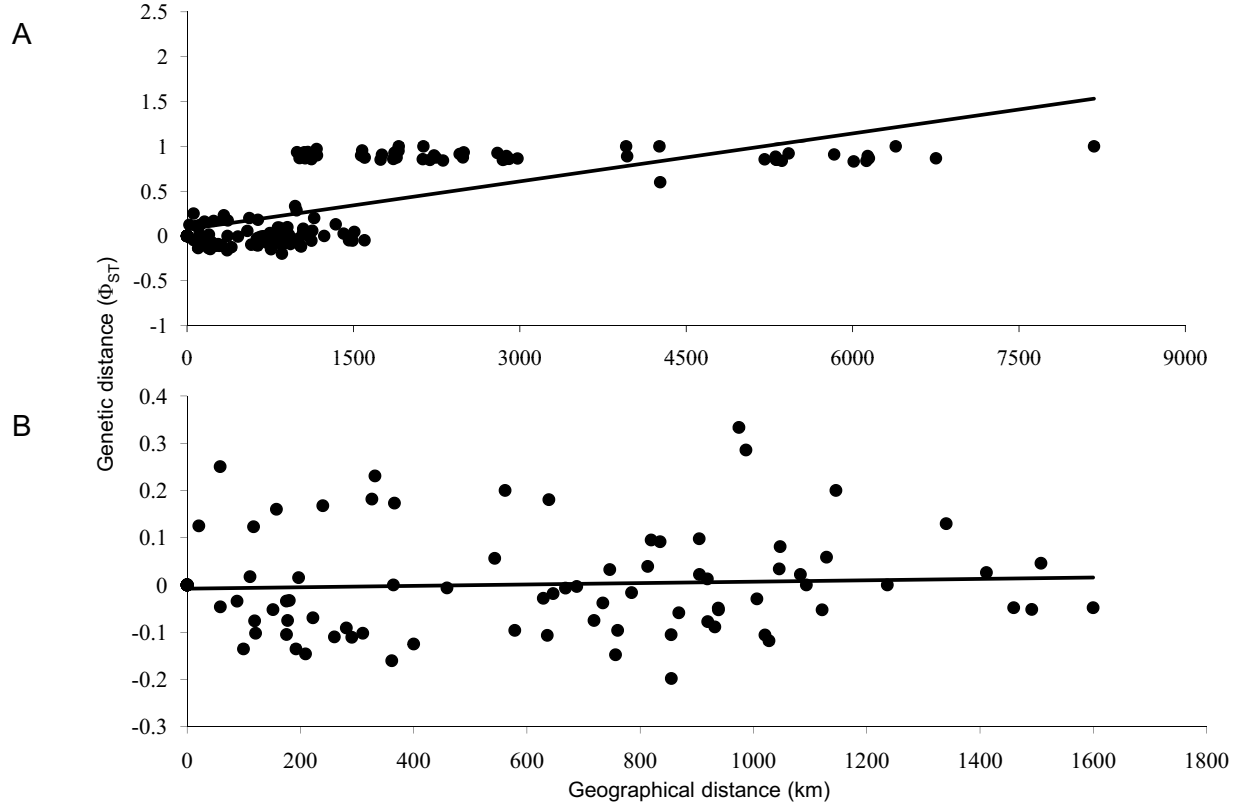

FIG. 2. Pairwise population differentiation plotted against geographical distance (km) using θ_{ST}. A — among all populations, B — among all Iranian populations

topology described in the Bayesian tree. Haplotypes from different locations within Iran linked to each other and did not form two distinct subnetworks consistent with traditional subspecies designations. In the Iranian samples, 47 out of 71 individuals exhibited one of the three main haplotypes (9, 10 and 15). Haplotypes in Moroccan individuals linked to Levant by three missing haplotypes. The Indian haplotype was not linked to other haplotypes.

DISCUSSION

In this study, using mtDNA sequences, we investigated the taxonomic validity of subspecies designations of *R. m. microphyllum* and *R. m. harrisoni* in Iran. Our results revealed high genetic variability in all samples of *R. microphyllum* over its range (π = 0.0092). However, a lower degree of genetic diversity has been identified in populations of *R. microphyllum* in Iran (π = 0.0036), as inferred from mtDNA in this study. This reduced genetic diversity means that genetic drift may be driving diversity within these populations and the biodiversity and evolutionary potential of *R. microphyllum* has been diminished. The genetic variability obtained in the present study (π = 0.0092) is lower than similar values obtained from other studies of bat control region sequences such as those from *Mystacina tuberculata* (Lloyd, 2003), *Tadarida brasiliensis mexicana* (Russell *et al.*, 2005), *Rhinolophus monoceros* (Chen *et al.*, 2006), *Corynorhinus townsendii pallescens* (Piaggio *et al.*, 2009), *Musonycteris harrisoni* (Ortega *et al.*, 2009), and *Rhinolophus armiger* (Xu *et al.*, 2010) with π values of 0.0360, 0.045, 0.0129, 0.0104, 0.036, and 0.019, respectively. Values lower than *R. microphyllum* include 0.0056 for *Leptonycteris curasoae curasoae* (Wilkinson and Fleming, 1996), 0.0030 for *Nyctalus azoreum*, 0.0020 for *Nyctalus leisleri* (Salgueiro *et al.*, 2004), 0.0062 for *Myotis myotis* (Ruedi and Castella, 2003), 0.0055 and 0.0028 for *C. t. townsendii* and *C. t. virginianus* (Piaggio *et al.*, 2009) and 0.005 for *Miniopterus schreibersii* (Furman *et al.*, 2010).

The mtDNA sequence data presented here confirm the monophyly of *R. microphyllum* as a sister taxon of *R. muscatellum* (Fig.3). Phylogenetic analysis of *R. microphyllum* haplotypes from western and eastern populations (Fig. 3) revealed two major clades (A and B) and four subclades (C, D, E and F). Clade A included Iranian and Indian bats and

Clade B included Levant and Moroccan bats. Phylogenetic reconstruction suggests that subclades E and F (BI: 0.71, BPML = 92; Fig. 3) are more closely related compared to subclades C and D (BI: 0.58, BPML = 80; Fig. 3). The number of substitutions between the Levant (H1) and Moroccan haplotypes (4 substitutions, K2P-distances = 0.71%) is much lower compared to that of Iranian (H9) and Indian haplotypes (15 substitutions and one indel, K2P-distances = 2.53%). Subclade D is an Oriental cluster that corresponds to *R. m. kinneari* subspecies identified by Van Cakenberghe and De Vree (1994). E and F subclades seem to be a Palaearctic cluster comprising Moroccan and Levant populations

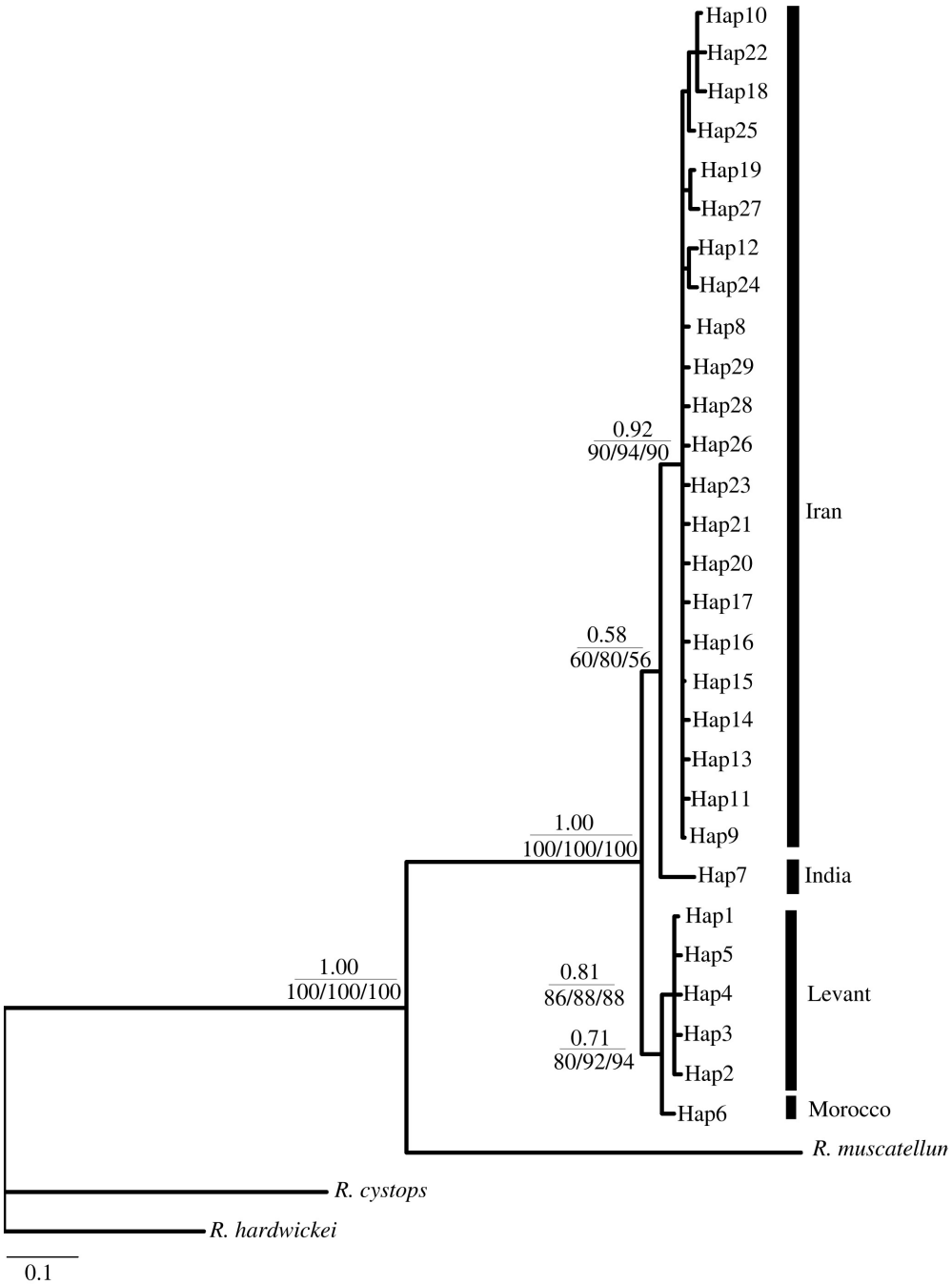

FIG. 3. Bayesian tree of haplotypes with three species based on 567 bp of the mitochondrial control region sequence. Maximum parsimony, Maximum likelihood and Neighbour joining topologies were identical. Number above line indicates the posterior probabilities of the nodes in the Bayesian inference analysis. Numbers below line are the bootstrap values of the nodes in the Maximum likelihood, Maximum parsimony and Neighbour joining analyses, respectively

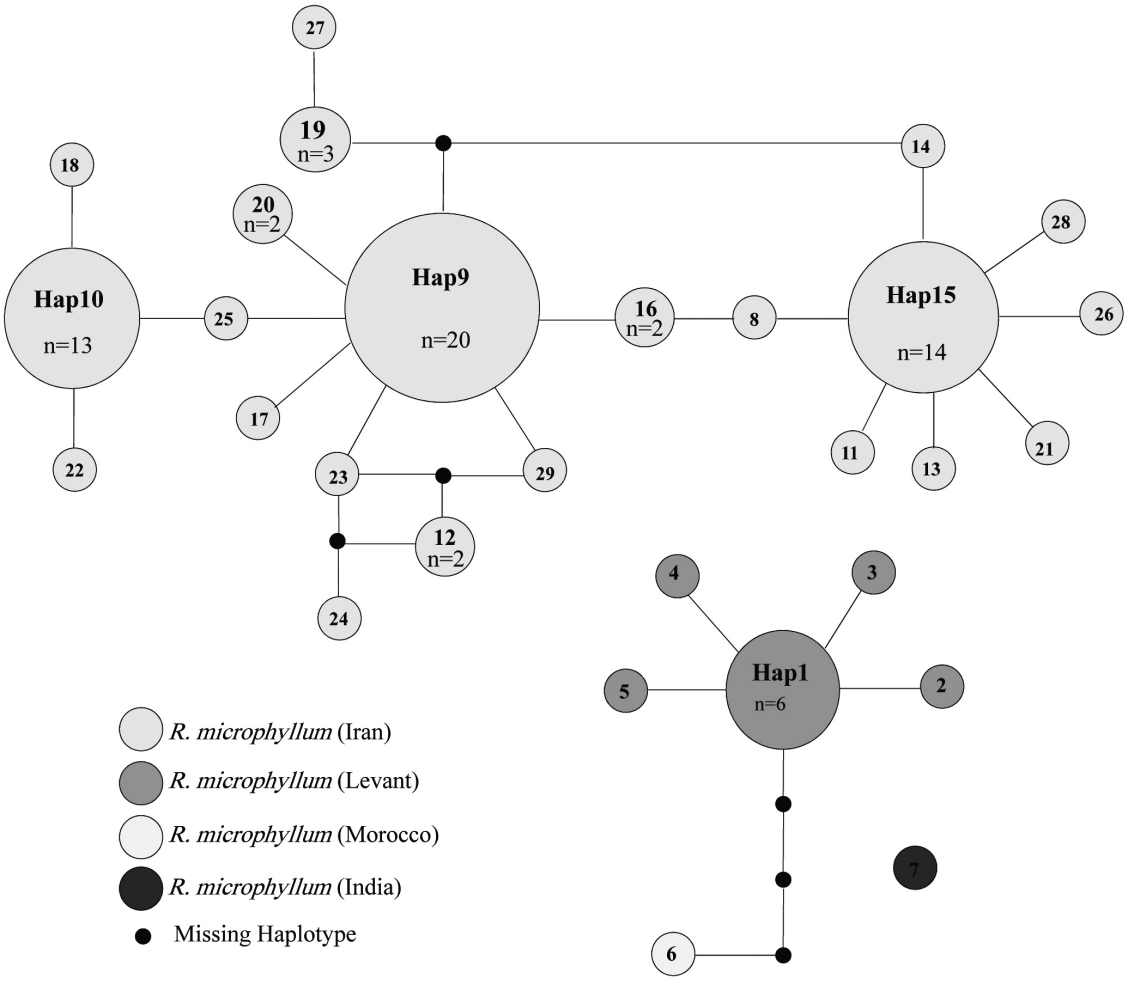

FIG. 4. Statistical parsimony network of 29 *R. microphyllum* control region haplotypes, obtained from 83 individuals. Numbers within circles are haplotype numbers

corresponding to *R. m. microphyllum* subspecies identified by Van Cakenberghe and De Vree (1994). Also, Subclade C is a Palaearctic cluster comprising the Iranian bats and corresponds to two traditional subspecies identified by Schlitter and DeBlase (1974) but this subclade is a monophyletic group that had bootstrap support ≥ 90 in four analyses and therefore cannot be considered as two distinct lineages. Our sampling across the ranges of the Iranian population is sufficient to allow comparison between the mtDNA control region phylogeny and the Schlitter and DeBlase (1974) subspecies designations. Therefore the well-supported monophyletic subclade C (Fig. 3) within Iranian samples discredits the two subspecies designated by Schlitter and DeBlase (1974) and confirms the one subspecies designated by Van Cakenberghe and De Vree (1994).

The statistical parsimony haplotype network (Fig. 4) confirms the classical division of *R. microphyllum* subspecies based on morphological characters (e.g., Van Cakenberghe and De Vree, 1994). The Levant and Moroccan populations belong to the subspecies *R. m. microphyllum*, while the Indian populations belong to the subspecies *R. m. kinneari.* Lack of support for traditional subspecies designations in Iran is emphasized by the network phylogeny of haplotypes that shows no correlation with geography, because all Iranian haplotypes linked to each other and constructed one subnetwork. The populations of *R. microphyllum* reveal moderate differentiation in the control region sequences accompanied by correlation between the genetic and geographic distances. Significant structure between samples from different regions was also detected using population genetic analyses. The AMOVA revealed significant variance among the four regions (Table 3: $\theta_{CT} = 0.8777$, $P < 0.01$) whilst variance within groups was not significant ($\theta_{SC} = 0.01765$, $P > 0.05$). The Iranian populations of *R. microphyllum* did not show visible differences

in their population genetic structure and there was no significant correlation between the genetic differentiation and geographic distance (Fig. 2B). The AMOVA results for the Iranian population indicate that variation among colonies was very low (0.13%) while within colony variation was very high (99.87%). Our results indicate the existence of gene flow between Iranian populations and do not confirm the traditional subspecies in Iran.

Based on partial cytochrome b sequence analysis, Hulva *et al.* (2007) and Levin *et al.* (2008) suggested that gene flow exists between the Indian and Levant population, but Levin *et al.* (2008) based on partial control region sequences, show contrasting results, with substantial differences between Indian and Levant populations, thus supporting the isolation of these populations. Our results based on control region show that the lack of genetic variation in the cytochrome *b* sequences (Hulva *et al.*, 2007 and Levin *et al.*, 2008) does not reflect the existence of contact between the Indian and Levant populations; but rather the insufficient variability of this marker to address the population structure of *R. microphyllum*.

Rhinopoma m. harrisoni was first described by Schlitter and DeBlase (1974) on the basis of several specimens collected from southeastern Kazeron in Fars Province in Iran. The authors also reported the presence of this subspecies in the western part of the country up to Meshraghe in Khuzestan province in the northern Mesopotamian plain, more mountainous areas in the Fars province and northern littorals of the Persian Gulf (see Fig. 1). Although the authors proposed that *R. m. harrisoni* is a smaller taxon occurring in the most southern parts of the distribution range, they also presented a distribution for *R. m. harrisoni* that overlapped with *R. m. microphyllum* and hence indicated the two subspecies were sympatric. Their conclusions were based on morphological analysis of a limited number of specimens. For example, in selected measurements of the two subspecies of *R. microphyllum* from southern Iran, there were only four male and seven female specimens. Similar values for *R. m. harrisoni* were limited to three females and 19 males. Their small sample sizes and the lack of statistical analysis could have contributed to the artificial recognition between different segments of the same population.

The conclusion of Schlitter and DeBlase (1974) on the taxonomic status of *R. microphyllum* populations in various parts of its distribution in southern Iran also lack any explanation for possible reproductive barriers bringing about these differences in the sympatric populations. Although there have been reports of large, stable, seasonal aggregations of male *R. microphyllum* in summer roosts in temperate areas of the Zagros range far away from their original arid range in the Persian Gulf littorals and northern Mesopotamian plain (Hemmati, 2001), these bats are mainly residents of the areas where the weather conditions are characterized by hot, prolonged summers similar to those of most northern African countries. These areas are typically lowlands at northern coasts of the Persian Gulf and the Oman Sea well away from the Iranian Plateau with no geographic barrier able to divide the bat populations into southern and northern segments. In fact, the narrow shores of the Persian Gulf could have acted as a corridor through which movement toward west and east did not encounter any geographic barrier. Assuming the extent of genetic variability as a measure of connectivity between various populations, results obtained in the current study imply that the degree of genetic variability is fairly constant over the narrow lands of the littorals of the Persian Gulf, indicating that various populations of this species have had adequate gene flow to prevent any divergence in its Iranian range.

The validity of *R. m. harrisoni* as a distinct taxon also has been questioned by other authors. Van Cakenberghe and De Vree (1994) not only casted doubt on the sufficiency of the sample size used by Schlitter and DeBlase (1974) but also put forward the possibility of a misidentification. They found that a limited number of specimens used by Schlitter and DeBlase (1974) could be re-examined and with the exception of one specimen, all came from one locality from which only one specimen was in a condition to provide external measurements (Van Cakenberghe and De Vree, 1994). Finally, these authors questioned the validity of the characteristics used by Schlitter and DeBlase (1974) for recognition of *R. m. harrisoni.* These characteristics included a smaller body size than the nominal form, absence of a prominent sagittal crest on the skull, and a triangular rostrum. They further suggested that the skull characters used to identify *R. m. harrisoni* could actually distinguish *R. hardwickei* rather than *R. microphyllum*.

Based on the present information we, therefore, suggest that there are not two subspecies of *R. microphyllum* living in sympatry in Iran. Therefore, *R. m. microphyllum* and *R. m. harrisoni* should be synonymized as the same subspecies with the name *R. m. harrisoni*, because molecular results indicate that Iranian samples differ from Levant and Moroccan samples (*R. m. microphyllum*).

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