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The conservation genetics of three cave-dwelling bat species in southeastern Europe and Anatolia

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Abstract: Genetic data from populations are currently being used in order to assess the conservation status of various species. In this study, the conservation implications of the genetic structure of 3 cave-dwelling bat species in southeastern Europe and Anatolia are discussed. These species are the greater horseshoe bat (*Rhinolophus ferrumequinum*), the bent-winged bat (*Miniopterus schreibersii*), and the long-fingered bat (*Myotis capaccinii*). The conservation status of the species is evaluated using 3 conservation unit approaches, specifically evolutionarily significant unit and management unit definitions and population aggregation analysis. These approaches are implemented simultaneously for the first time for any species in Turkey, through an evaluation of mitochondrial and nuclear DNA data previously generated for these species. Based on these data, both regional and cave-specific conservation recommendations are made. The results suggest that for *M. capaccinii*, the area to be protected in order to maximize the conservation of genetic diversity is around the border of Turkey with Bulgaria and Greece. For the other 2 species, these areas are within Anatolia.

Key words: Mitochondrial DNA, conservation unit, evolutionarily significant unit, management unit, population aggregation analysis

Güneydoğu Avrupa ve Anadolu'da yaşayan üç mağara yarasası türünün koruma genetiği

Özet: Günümüzde farklı türlerin koruma statülerin değerlendirilmesinde genetik veriler kullanılmaktadır. Bu çalışmada güneydoğu Avrupa ve Anadolu'da bulunan 3 yarasa türünde görülen genetik yapının, bu türlerin korunmasıyla ilgili önemi değerlendirildi. Bu türler büyük nalburunlu yarasa (*Rhinolophus ferrumequinum*), uzun kanatlı yarasa (*Miniopterus schreibersii*), ve uzun parmaklı yarasadır (*Myotis capaccinii*). Üç adet farklı koruma birimi yaklaşımı (evrimsel olarak önemli birim, koruma yönetimi birimi, ve popülasyon kümeleme analizi) kullanılarak bu türlerin koruma statüsü incelendi. Bu analizler Türkiye'de yaşayan türler için ilk defa uygulanmakta ve daha önceden bu türler için çıkartılmış olan mitokondrial ve çekirdek DNA verileri kullanılarak yapıldı. Bu sonuçlara göre hem bölgesel, hem de mağara bazlı olarak koruma stratejileri önerildi. Sonuçlar *M. capaccinii*'nin genetik çeşitliliğinin korunmasının en etkili olarak yapılması için Türkiye'nin Bulgaristan ve Yunanistan sınırına yoğunlaşılması gerektiğini göstermektedir. Diğer 2 tür için bu alan İç Anadolu Bölgesi'nin içerisinde yer almaktadır.

Anahtar sözcükler: Mitokondrial DNA, koruma birimi, evrimsel olarak önemli birim, koruma yönetimi birimi, popülasyon kümeleme analizi

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Introduction

Various unit criteria exist for defining and prioritizing populations for the purposes of their conservation using genetics methods. For animal species, these categorizations use mitochondrial DNA (mtDNA) data, nuclear DNA (nDNA) data, or both (Moritz, 1994a). The evolutionarily significant unit (ESU) (Moritz, 1994a) and the management unit (MU) (Crandall et al., 2000) concepts, as well as discrete character-based approaches such as population aggregation analysis (PAA) (Davis and Nixon, 1992), have generally been popular for delineating the boundaries of these units.

The ESU approach of Moritz (1994a) defines evolutionarily significant units in order to guide in maintaining the evolutionary potential of the units and is useful for long-term management issues. The basic criterion for defining an ESU is that it “should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at the nuclear loci” (p. 373). However, this ESU approach has been criticized on several grounds. Crandall et al. (2000) claimed that it does not incorporate ecological data and is restrictive in requiring reciprocal monophyly. Goldstein et al. (2000) criticized the ESU approach on the basis that tree-based approaches (as opposed to character-based ones) rely on distance measures that might erroneously retrieve 2 entities as highly divergent, although they are not characterized by any diagnostic feature. This stems from the consideration that any number of terminal nodes can be hierarchically organized into a tree.

PAA was proposed by Davis and Nixon (1992) and offers an alternative to the tree-based approach. PAA, as a character-based approach, uses diagnostic characters (from a conservation genetics perspective, base pair differences) between individuals to group them together into units or subpopulations without

the construction of a tree (DeSalle and Amato, 2004). This approach is considered to be a precursor to the phylogenetic species concept (Goldstein et al., 2000) and has been criticized both for protecting too much (Moritz, 1994a) and for potentially not protecting genetically distinct but not diagnosable populations (Waples, 1995).

The MU definition, as proposed by Crandall et al. (2000), stresses that ecological information should be used in addition to genetic information to define units. In this method, 4 different categories are considered, involving combinations of genetic and ecological exchangeability within recent and historical time frames, and the data at hand are evaluated to see which of these criteria are met. In each of the 4 categories, a null hypothesis (H_0) is framed as the lack of exchangeability. Subsequently, based on the combined presence of positive (exchange) and negative (no exchange) answers in each category (where a positive answer corresponds to a rejection of the H_0), conservation recommendations are made.

In the present study, these 3 criteria (ESU, MU, and the character-based PAA approach) were used to evaluate the conservation status of 3 cave-dwelling bat species in southeastern Europe and Anatolia. The species in question are the greater horseshoe bat (*Rhinolophus ferrumequinum*), the bent-winged bat (*Miniopterus schreibersii*), and the long-fingered bat (*Myotis capaccinii*). These species are included on the 2008 IUCN Red List of Threatened Species (<http://www.iucnredlist.org/>) under various threat levels. A general pattern of decrease in population trends is also visible (Table 1). The species are also threatened due to the potential effects of tourism. For instance, a cave hosting some of these species in Maronia, Greece, is currently under consideration for being opened to tourism (Papadatou, personal communication). These species were also chosen

Table 1. The conservation status of the 3 species of interest on the 2008 IUCN Red List of Threatened Species.

Species	Status	Population trend
<i>Miniopterus schreibersii</i>	Near Threatened	Decreasing
<i>Rhinolophus ferrumequinum</i>	Least Concern	Decreasing
<i>Myotis capaccinii</i>	Vulnerable A4bce	Decreasing

because they are 3 of the cave-dwelling species that have a widespread distribution in Turkey (Benda and Horacek, 1998; Bilgin et al., 2008b). In addition, although all bat species are officially under protection in Turkey, there exists no explicit action plan for their conservation. This study is also the first to use the above-mentioned conservation unit approaches for any species in Turkey.

The phylogeography and population genetics of these species have been investigated in various studies (Bilgin et al., 2006, 2008a, 2008b, 2009; Furman et al., 2009, 2010), using mtDNA (D-loop and *cyt-b*) and nuclear microsatellites. However, none of those studies focused on the conservation status and conservation genetics of these species. With this in mind, especially since the territory of Turkey hosts 3 biodiversity hotspots (Mittermeier et al., 2005), clear conservation recommendations based on sound science are necessary to inform present and future conservation management of these species.

Materials and methods

In order to undertake these analyses, data from previous studies were used. For *Miniopterus schreibersii*, data were based on reports by Bilgin et al. (2006), Bilgin et al. (2008a), Furman et al. (2009), and Furman et al. (2010). For *Myotis capaccinii*

and *Rhinolophus ferrumequinum*, data from Bilgin et al. (2008b) and Bilgin et al. (2009) were used, respectively. The tables for PAA analyses were produced by using MacClade v. 4.0 (Maddison and Maddison, 2000). The distribution maps for each species were produced with the software iMAP v. 3 (available from <http://www.bioevolution.com/imap/>).

Results

Reviewing the distribution of the mtDNA diversity in these 3 species shows the presence of 2 well-supported mitochondrial clades within each (Figures 1a-1c). Again, in each species, the geographic distribution of the individuals belonging to these clades follows an east/west orientation and defines potential hybrid zones for the different species (Figure 2). In *Miniopterus schreibersii* and *Rhinolophus ferrumequinum*, these zones are observed to pass approximately through central Anatolia (Figures 2a-b). This pattern suggests differentiation during the Pleistocene or Pliocene in the Balkans for the western clade, and possibly the Caucasus or the south of the Caspian Sea for the eastern clade, with postglacial secondary contact in the middle, within Anatolia. In *Myotis capaccinii*, the contact zone occurs farther to the west, passing through Turkey's border with Bulgaria and Greece in southeastern Europe (Figure 2c). For *M. capaccinii*, its

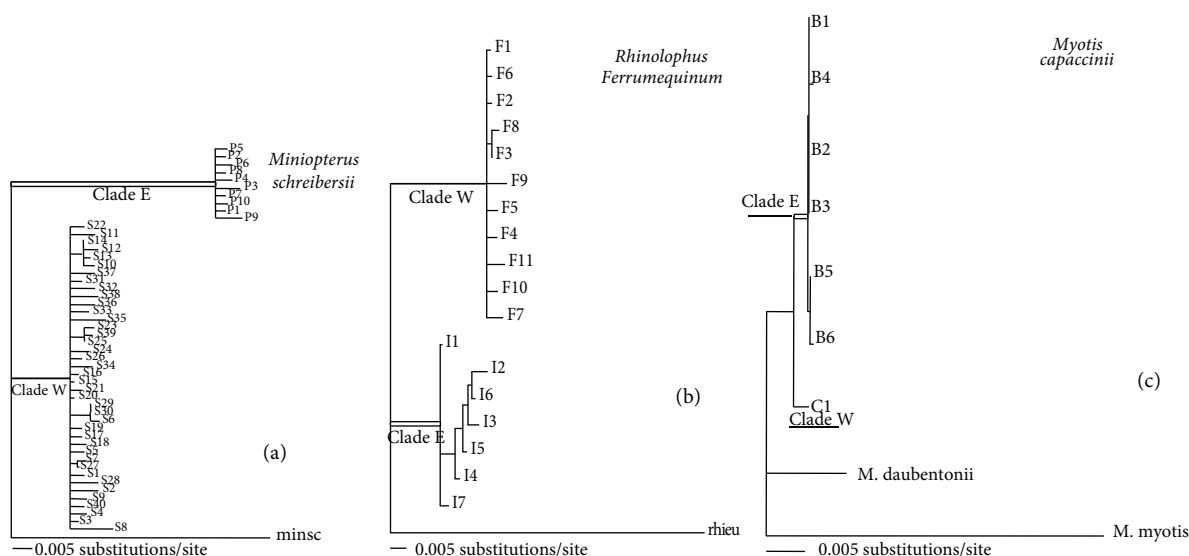


Figure 1. Neighbor-joining trees showing the presence of 2 main clades in a) *M. schreibersii* (Bilgin et al., 2006, 2008a), b) *R. ferrumequinum* (Bilgin et al., 2009), and c) *M. capaccinii* (Bilgin et al., 2008b). The bootstrap values for clades E and W are 81 or higher.

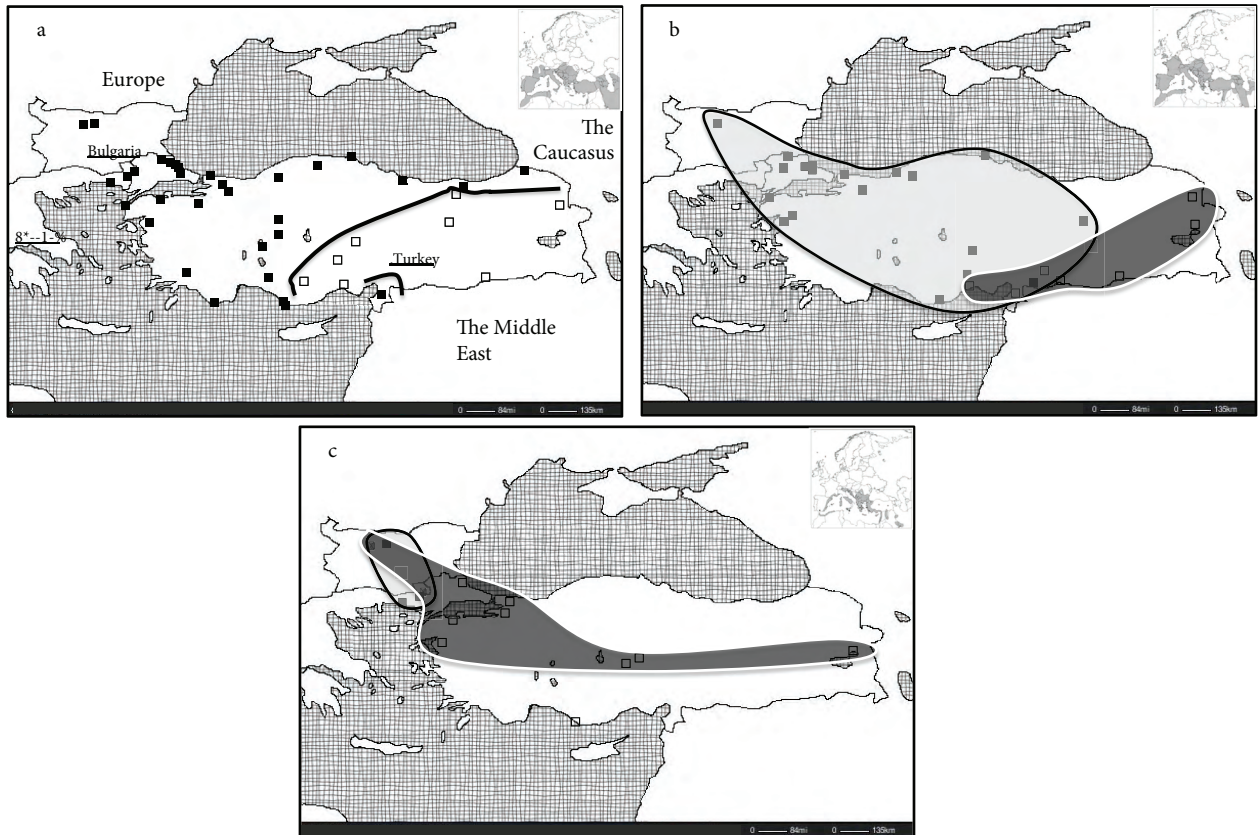


Figure 2. The geographic distribution of individuals belonging to eastern (in white squares) and western (in black circles) clades for a) *M. schreibersii*, b) *R. ferrumequinum*, and c) *M. capaccinii*. The stars in the map of *M. capaccinii* represent the 2 caves where bats with the eastern and western haplotypes are found in sympatry. In addition, the contact zone between the eastern and western clades has been indicated with a line. Minimum spanning polygons have been drawn for the eastern and western clades for the other 3 species, and their overlap indicates the parapatric contact zones for each species. The inlaid figures in the top-right corners show the distribution of each species in the Mediterranean Basin, the Caucasus, and part of the Middle East (EUROBATS, 2009).

wide distribution in Anatolia suggests that the ice age refugium might have existed in Anatolia, whereas the western clade might have expanded to the Balkans from central Europe. The genetic monomorphism of the individuals in the western clade (all had the same haplotype) suggests that they could be representative of a founder population, which might have originated in Europe.

In these 3 species, after polarizing the data set based on the individuals with the eastern and western mtDNA haplotypes, microsatellite nuclear DNA data displayed different patterns of genetic differentiation. In *M. capaccinii*, there was no evidence for differentiation; the microsatellite data suggested complete panmixia (Bilgin et al., 2008b). In *M. schreibersii*, there was significant genetic

differentiation, in both mtDNA (Bilgin et al., 2008a; Furman et al., 2010) and nuclear DNA (Furman et al., 2010). Finally, in the geographic region of interest, using D-loop sequences, Bilgin et al. (2009) showed the presence of 2 mtDNA clades in *R. ferrumequinum* within central Anatolia. Although they worked with different samples, the microsatellite study of Rossiter et al. (2007) and Bilgin et al. (2009) suggests that the 2 mtDNA clades in this species could also be exhibiting high levels of nuclear differentiation. It should also be noted that a study by Flanders et al. (2009) on the global phylogeography of *R. ferrumequinum*, although lacking samples from Anatolia, showed similarities between the mtDNA of European and Syrian populations, with the latter potentially representative of eastern Anatolian populations.

This lack of differentiation could be due to the use of the ND2 region by Flanders et al. (2009); the ND2 region evolves more slowly than the D-loop region examined by Bilgin et al. (2009). D-loop analysis of the Syrian samples and ND2 analysis of the Anatolian populations of this species will be necessary to draw firmer conclusions.

Classification of conservation units

The information outlined above was used for making the ESU, MU, and PAA-based conservation unit classifications for each species. These classifications under each category are described below.

Evolutionary significant unit (ESU)

In each species, 2 mtDNA clades, retrieved by the tree approaches, were seen to be reciprocally monophyletic. This meets the first criteria for ESU definition. However, by definition, an ESU also requires significant differentiation at the nuclear loci. In *Myotis capaccinii*, the 2 mtDNA clades of this species may not be classified as separate ESUs, as the

mtDNA differentiation was not coupled with nuclear differentiation. In *Miniopterus schreibersii*, although at lower levels, there was evidence for significant nuclear differentiation. *Rhinolophus ferrumequinum* also showed nuclear differentiation at high levels. Hence, based on the ESU criteria of Moritz, the 2 mtDNA clades within both *M. schreibersii* and *R. ferrumequinum* should be classified as ESUs.

Management unit (MU)

The results of the MU approach are given in Tables 2, 3, and 4 for *M. schreibersii*, *R. ferrumequinum*, and *M. capaccinii*, respectively. In all of the species, the mtDNA differentiation is a reason to reject the H_0 of historical genetic interchangeability. On the other hand, with their higher rate of mutation, microsatellite data can be used to evaluate the presence or absence of genetic interchangeability for recent time scales. Using this perspective, there was no evidence for recent exchangeability for *M. capaccinii*, and the H_0 therefore cannot be rejected. For *R. ferrumequinum* and *M. schreibersii*,

Table 2. MU exchangeability table for *M. schreibersii*; (+) represents rejection and (-) represents failure of rejection of the corresponding null hypothesis.

Genetic exchangeability	Ecological exchangeability	
+	-	Recent time scales
+	-	Historical time scales

Table 3. MU exchangeability table for *R. ferrumequinum*; (+) represents rejection and (-) represents failure of rejection of the corresponding null hypothesis.

Genetic exchangeability	Ecological exchangeability	
+	-	Recent time scales
+	-	Historical time scales

Table 4. MU exchangeability table for *M. capaccinii*; (+) represents rejection and (-) represents failure of rejection of the corresponding null hypothesis.

Genetic exchangeability	Ecological exchangeability	
-	-	Recent time scales
+	-	Historical time scales

the presence of significant differentiation makes it possible to reject the H_0 for recent time scales. In all 3 species, as there is geographic range overlap, including complete sympatry for *M. capaccinii*, there is no evidence for rejecting the H_0 of historical or recent ecological exchangeability. The conclusions based on the case representations of Crandall et al. (2000) for the different species are as follows:

- 1) *M. schreibersii*: Allow gene flow consistent with current population structure; treat as a single population.
- 2) *R. ferrumequinum*: Allow gene flow consistent with current population structure; treat as a single population.
- 3) *M. capaccinii*: Treat as a single population; if inexchangeability is a result of anthropogenic effects, restore to historical condition; if inexchangeability is natural, allow gene flow.

Character-based (population aggregation analysis) approach

According to the discrete character-based PAA approach, the 2 clades in each species should be considered as separate units in terms of protection. There were 15, 17, and 24 fixed differences between the 2 intraspecific clades in *M. schreibersii*, *R. ferrumequinum*, and *M. capaccinii*, respectively. These differences are presented in Tables 5-7. Any one of these positions is diagnostic for classifying any given individual of these species into either the west or east clade for each species. For *M. schreibersii* and *R. ferrumequinum*, the 2 haplotypes that were possibly the most ancestral (due to their central position and high frequencies in haplotype networks; Bilgin et al., 2008a, 2009) were used for comparison. Under this approach, each mtDNA clade qualifies as a separate conservation unit.

Table 5. The variable sites between clade W (S15) and clade E (P6) haplotypes for *M. schreibersii*. S15 and P6 were the most likely ancestral haplotypes in each clade.

	147	153	187	200	202	203	206	207	208	209	210	211	214	215	218	220	223	265
S15	C	A	C	T	C	-	T	C	A	A	G	T	G	C	T	C	G	A
P6	T	G	T	C	T	A	C	T	G	G	A	C	A	T	C	T	A	G

	295	312	339	349	364	371	372	384
S15	T	A	G	A	A	C	G	G
P6	C	G	A	G	G	T	A	A

Table 6. The variable sites between clade W (F1) and clade E (I1) haplotypes for *R. ferrumequinum*. F1 and I1 were the most likely ancestral haplotypes in each clade.

	198	241	265	270	308	351	354	360	375	378	384	385	395	400	405	420	428	434	440	448	463	464
F1	C	A	C	C	G	G	T	A	C	C	C	T	T	T	T	G	C	T	C	A	A	A
I1	T	G	T	T	A	T	C	C	T	T	T	C	C	C	C	A	T	C	T	G	G	G

Table 7. The variable sites between clade W (B1) and clade E (C1-C7) haplotypes for *M. capaccinii*.

	41	45	50	93	110	116	139	144	150	162	174	176	177	197	200	203	236	248	272	296	353	374	375	410	452	485	497
B1	C	C	T	G	C	T	G	T	G	T	A	C	A	C	C	A	T	T	G	T	T	A	C	T	A	C	C
C1	T	T	.	A	T	C	A	C	A	.	G	T	G	T	T	.	C	.	A	C	C	G	T	C	G	T	T
C2	T	T	.	A	T	C	A	C	A	.	G	T	G	T	T	G	C	.	A	C	C	G	T	C	G	T	T
C4	T	T	C	A	T	C	A	C	A	.	G	T	G	T	T	.	C	.	A	C	C	G	T	C	G	T	T
C3	T	T	.	A	T	C	A	C	A	.	G	T	G	T	T	G	C	.	A	C	C	G	T	C	G	T	T
C5	T	T	.	A	T	C	A	C	A	C	G	T	G	T	T	.	C	C	A	C	C	G	T	C	G	T	T
C6	T	T	.	A	T	C	A	C	A	C	G	T	.	T	T	.	C	C	A	C	C	G	T	C	G	T	T

Discussion

Considering the conservation of the bats in southeastern Europe and Anatolia as a whole, the data at hand suggest different conservation strategies based on species and conservation unit definitions. For *Rhinolophus ferrumequinum* and *Miniopterus schreibersii*, the ESU and PAA criteria suggest the eastern and western mtDNA clades to be treated as different conservation units. The MU criterion, however, because of the lack of any evidence for rejecting ecological exchangeability, does not support a conservation unit definition. I disagree with the MU approach in these cases. In *M. schreibersii*, for instance, significant forearm length differences have been recorded between the 2 clades (Furman et al., 2009). The average forearm length of the western clade is 45.4 ± 1.0 mm, and the eastern clade is 46.6 ± 0.8 mm; the difference between them is significant at the 0.001 level (t-test). Hence, the 2 clades should at least qualify as separate subspecies, and the MU designation of Crandall et al. (2000), which suggests treating them as a single entity, conflicts with the morphological data at hand. Similarly, had any morphological differentiation been found between the 2 mtDNA clades of *R. ferrumequinum*, it could be used to propose 2 separate biological species, with the support of the microsatellite and mtDNA differentiation and the parapatry of the 2 mtDNA clades (Bilgin et al., 2009). Therefore, in my opinion, treating 2 genetic entities as a single population is too relaxed for a conservation perspective. Conversely, for *Myotis capaccinii*, the eastern and western clades do not qualify as ESUs or MUs due to the lack of nuclear genetic differentiation. This is in opposition to the results of the PAA approach, which diagnoses each clade as a separate unit.

The necessity for MU and ESU concepts to require significant differences in nuclear gene flow (or in contemporary time scales) is overly stringent in terms of unit definitions. This mirrors the debate over species concepts as discussed by Moritz (2002); in this case, the difference is analogous to the interpretation of these data in terms of phylogenetic versus biological species concepts, where the 2 mitochondrial clades in each species, although qualifying as phylogenetic species, are not necessarily biological species due to the absence of evidence for reproductive isolation.

Moritz (1994b) suggested that, although requiring significant nuclear differentiation might be overly restrictive for the definition of ESUs, it is necessary to avoid misclassifying populations, which are differentiated in nuclear genomes but not in organellar genomes. On the other hand, however, this approach precludes unit definitions for populations that are differentiated in organellar genomes but not in nuclear genomes. Hence, I agree with the interpretation of Eggert et al. (2004), who noted that the definition of an ESU is still an evolving concept itself, and there might be cases in which it is appropriate not to require nuclear differentiation in ESU definitions in order to make it more comprehensive.

The use of character-based approaches (Vogler and Desalle, 1994; Goldstein et al., 2000) for ESU definition is able to capture the differences in mtDNA for diagnosing units and is the most appropriate for these 3 species. By the Moritz (1994b) definition of MU (although not by the definition of Crandall et al. (2000)), these clades also qualify as MUs, since differentiation in either nuclear or mitochondrial DNA is sufficient for Moritz's MU recognition.

As the present study attempts to demonstrate, the 2 clades in each species qualify as ESUs according to certain criteria and not by others. The same is true for MUs. For these 3 bat species, adopting the PAA approach permits the most comprehensive and geographically meaningful unit classification and conservation strategy. Hence, for the species in question, the best strategy would be to start protecting the caves that host, or areas that could host, individuals belonging to the different eastern and western clades. This would maximize the genetic diversity preserved with a relatively small amount of effort. In prioritizing the conservation of the cave populations in central (e.g., Zindan cave) and northeastern (e.g., Çatak and Cehennemdere caves) Anatolia, a maximum of genetic diversity could be conserved in a relatively small area for *M. schreibersii*. For *R. ferrumequinum*, this area would be along the Mediterranean coast of Turkey and southeastern Anatolia (e.g., Karanlık cave). For *M. capaccinii*, individual caves where bats from 2 different clades are found together (such as Parnitzite in Bulgaria and Koufovouno in Greece), should be prioritized and targeted for protection. In this regard,

caves that are under consideration for being opened to tourism in the Balkans should be checked for the presence of *M. capaccinii*. If present, the populations should be genetically assessed to evaluate the sympatry of the western and eastern clades of this species. Accordingly, any intention of encouraging tourism to the caves, especially those that sympatrically host individuals belonging to both the eastern and western clades, should be reconsidered.

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