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GENETIC DIVERGENCE PATTERNS OF TWO SIBLING SPECIES (*MYOTIS MYOTIS,* MYOTIS *BLYTHII)* AND MYOTIS CAPPACINII (MAMMALIA: CHIROPTERA) IN CENTRAL ANATOLIA REGION

ABSTRACT

Myotis myotis (Borkhausen, 1797) and *Myotis blythii* (Tomes, 1857) are genetically close species of bats with very similar morphology and karyology. In this study, specific descriptive markers of 3 species (*Myotis myotis, Myotis blythii,* and *M. cappacinii*) belonging to the genus *Myotis* were determined by using ISSR technique for the first time. In total, 91 bands including 86 polymorphic bands were obtained from the 7 best-optimized primers out of the analyzed 16 primers. UPMGA cluster analysis showed 4 separate clusters. According to these results, the *M. myotis* and *M. blythii* were divided into two close groups, whereas a single individual belonging to *M. myotis* colony formed the third group. *M. cappacinii* was in a more distinct and remote group with respect to sibling species. Genetic diversity for all populations was calculated as 94.5%. ISSR-PCR method was shown to be a reliable and useful technique for detecting genotypic similarities/differences of *Myotis* species.

Keywords: Genetic Differentiation, ISSR, Chiroptera, Sibling, Myotis

1. INTRODUCTION

In order to understand the evolution of species diversity, extinction and speciation events, information about the phylogenetic history of species is needed. In recent years, studies on phylogeny and taxonomy have shown that PCR-based markers are useful in explaining polymorphism and evolutionary relationships between species [1]. Among these markers, ISSR (Sequence Repeat-Polymerase Chain Reaction) is a reliable and simpler method used as an alternative to other techniques (RAPD, AFLP, SSR) for genetic analysis [2]. ISSR analysis is useful for identifying the genetic differentiation of populations, including individuals who cannot be separated morphologically [3]. Bugarski-Stanojevic, et al., [4] described the relationship between problematic species and subspecies by identifying genetic distances with 6 optimized ISSR primers in closely related *Apodemus* populations. Especially in southern parts of Europe, sibling species *Apodemus flavicollis* (Melchior, 1934) and *A. sylvaticus* (Linnaeus, 1758), which cannot be separated morphologically, have been successfully identified by ISSR-PCR analysis [5]. In Turkey, the different chromosomal races/populations of another taxonomically problematic (because of chromosomal polymorphism) mammal species *Nannospalax xanthodon* were genetically differentiated using ISSR technique [6].

The use of DNA sequence data in taxonomy has led to the frequent detection of cryptic species in a variety of taxa, which has

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dramatically increased species diversity [7 and 8]. Among the mammals, one of the most diverse genera included in the Chiroptera army is *Myotis*, which contains 134 species [9]. Distributed particularly in the Palearctic region, *Myotis myotis* and *Myotis blythii* species of this genus are still controversial cryptic taxa, in point of their taxonomic status and distribution limits [10, 11, and 12]. Morphologically similar species are difficult to identify. The cryptic species are morphologically similar or identical. However, they exhibit substantial genetic differentiation and are generally considered sibling species [8 and 13]. In order to reveal certain differentiation between these species, distinctive genetic characters are needed. Mitochondrial DNA (mtDNA), as well as nuclear DNA (nDNA) based investigations, point out that genetic differentiation between *M. myotis* and *M. blythii* samples is very low, species share the same mtDNA haplotypes, and even hybrid individuals are present in some contact areas [14, 15, 16, 17, and 18]. *M. myotis* of Western Mediterranean origin is adapted to the mixed forest areas of the Mediterranean Region, whereas *M. blythii* of Asian origin is adapted to semi-arid and open areas [19 and 20]. On the other hand, both species exhibit sympatric speciation in Southern and Central Europe and form mixed colonies [21]. In Turkey, *Myotis myotis* is mostly distributed in the Mediterranean and the Black Sea Regions, while *Myotis blythii* is predominantly observed in the Eastern and Southeastern Anatolia Regions. In the case of Central Anatolia, both species are present as sympatric species [10 and 22]. Despite their ecological differences, sympatric life is another challenge that makes it difficult to separate species. The aim of this study was to determine and compare the genetic differences between the sympatric sibling species *M. myotis* and *M. blythii* (Central Anatolia) and *M. capaccinii* distributed in the Mediterranean region. Molecular polymorphism in bats, especially among sibling species, was first studied by selecting appropriate ISSR primers. Thus, the effectiveness of ISSR markers in the analysis of genetic variability of bat species was demonstrated.

2. RESEARH SIGNIFICANCE

In this study, two sibling vespertilionid bat species distributed in Turkey, were separated by using ISSR primers and new DNA markers for the first time.

3. MATERIALS USED IN THE EXPERIMENTS

3.1. Specimen Examined

M. myotis (5♂), *M. blythii* (7♂), and *Myotis capacinnii* (1♂) examined in this study were taken from Konya Sızma village, Kırıkkale Keskin and Hatay Hassa regions, respectively (Figure 1). The samples were taken from the roosts with special bat nets and a gloved hand. The samples were brought to the laboratory and the standard four external dimensions and weights were recorded according to Bogdanowicz, [16]. The head skeletons were boiled in a 15% ammonia solution at 70-80°C, then dried and numbered according to Southern, [23] and Mahoney, [24]. The abrasions in the canine and incisor teeth were examined under a binocular microscope for the determination of age groups. Standard external dimensions of the samples were taken with a millimeter ruler. Internal character measurements were conducted with 0.1 mm precision caliper. The identification of species was accomplished according to Arlettaz, et al., [25] and Dietz and Kiefer, [26].

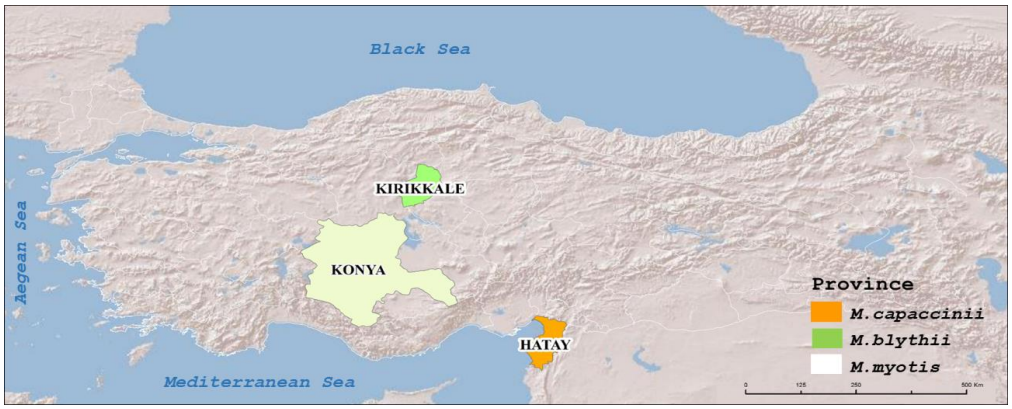

Figure 1. Locations of the examined *Myotis* species in Turkey

3.2. DNA Extraction and ISSR-PCR Amplification

DNA was isolated from liver tissues from a total of 13 samples using DNA extraction kit (Thermo Scientific GeneJET Genomic DNA Purification Kit). DNA purity was determined by NanoDrop and the degree of DNA degradation was measured using 0.1% agarose gels with 0.5 µg/ml ethidium bromide. ISSR-PCR amplification was performed from the obtained genomic DNA. The 16 primers (UBC807, 809, 811, 813, 818, 827, 828, 830, 841, 842, 864, 866, 868, 873, 876, 880) were randomly chosen from UBC (British Columbia University) primer set for ISSR-PCR analysis. The 7 primers producing reproducible loci and prominent bands with good resolution quality were selected from these 16 primers. The total number of bands, the total number of polymorphic bands and the rate of polymorphism were given for each primer (Table 1). PCR amplifications were conducted in a thermal cycler (Bio-Rad T100™) with a total volume of 25 uL using 0.3 ul Taq polymerase (5u/ul, Thermo fisher Taq DNA polymerase), 2.5 ul 10x PCR buffer, 1.5uL $MgCl_2$ (25 mM), 0.4 uL dNTP (10 mM), 1 u1 DNA (30 ng), 1 ul primer, and 16.8 ul ddH_2O.The PCR program started with an initial phase of 5 min at 94°C, followed by 40 cycles of 45 s at 94°C, 45 s at 40°C, 2 min at 72°C and 10 min final elongation at 72°C. ISSR amplification products were eluted at 90 V with 1X TBE buffer on 1% agarose gel electrophoresis stained with ethidium bromide. Molecular sizes of amplified products were estimated using a 100-10000 bp DNA ladder marker (Gene Ruler DNA Ladder Mix, Thermo Scientific, USA).

3.3. Data Analysis

DNA band profiles were scored based on the DNA ladder from gel photographs. Scoring was done by writing (1) in the presence of the band and (0) in the absence of the band. The genetic distance matrix generated by the presence and absence of bands was calculated according to Nei, [27]. The dendrogram was generated by UPGMA analysis based on the Jaccard coefficient. The genetic diversity for all populations was calculated using POPGENE v.1.32 (Population Genetic Analysis) [28].

4. FINDINGS AND DISCUSSIONS

4.1. ISSR AND PHYLOGENETIC ANALYSIS

In this study, 7 ISSR primers giving the best amplification out of total 16 primers were selected for the analysis of *Myotis* species. These 7 primers amplified 86 polymorphic loci, of estimated sizes from 150 to 2200 bp, varying from 19 polymorphic loci for primer $(GGAGA)_3$ to 6 locus for the primer $(AG)_8G$. The polymorphism rates of the primers ranged from 85% to 100%., while the total polymorphism rate was

recorded as 95% (Table 1). The average number of polymorphic bands per primer was calculated as 12.28. According to these data, $(AG)_8T$, $(TG)_8A$, $(GACA)_4$, $(GGAGA)_3$ primers produce the highest and most distinctive bands for *Myotis* species with 100% polymorphism rate (Figure 2).

Table 1. The list of ISSR primers used to detect polymorphism in *Myotis* species

Primer Sequence	Number of Bands	Number of Polymorphic Bands	Polymorphism %
UBC 807 $(AG)_8T$	13	11	100%
UBC 809 $(AG)_8G$	6	6	85%
UBC 813 $(CT)_8T$	7	7	93%
UBC 828 $(TG)_8A$	15	14	100%
UBC 873 $(GACA)_4$	17	17	100%
UBC 876 $(GATA)_2(GACA)_2$	12	12	90%
UBC 880 $(GGAGA)_3$	21	19	100%
ISSR Total	91	86	95%

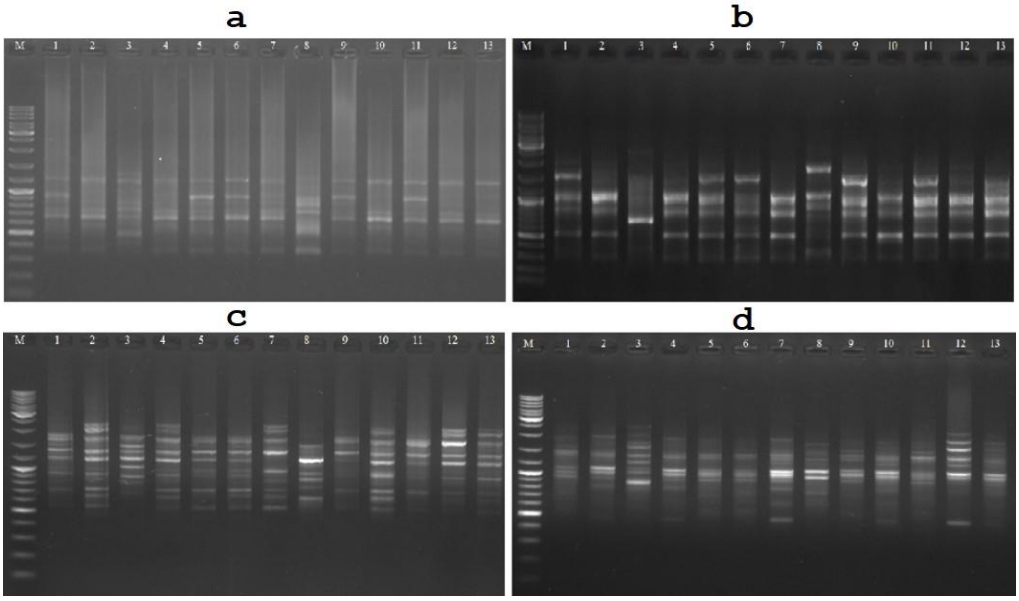

Figure 2. Gel electrophoresis profiles of PCR products by using ISSR primers for *Myotis* specimens (a:UBC 828, b:UBC 807, c:UBC 873, d:UBC 880)

The genetic distance matrix was calculated using the polymorphism ratios determined by the markers given in Table 1. In the UPGMA analysis based on the Jaccard coefficient, two main clusters/groups were identified (Figure 3). These groups consisted of the samples of *Myotis myotis* (3, 8, 10, 11, 12, 13) from Konya and *Myotis blythii* (1, 9, 4, 5, 7) from Kırıkkale. These two sibling groups are clearly separated from each other. Despite being a member of *Myotis* colony, specimen 6 was represented as a separate clad in the dendrogram. When the band patterns of this sample are examined, the presence of similar bands with both species (*Myotis blythii* and *Myotis myotis*) was revealed, which increases the probability of being a hybrid individual. As expected, *M. capaccinii* sample was represented as a separate group (2) away from the other samples. When the calculated average similarity was evaluated, the highest similarity (89%) was found between the *Myotis myotis* samples (numbers: 10, 11; 11, 12) taken from Konya. However, the highest average distance (78)

was found between the *Myotis myotis* (11) ve *Myotis cappacinii* (2) samples (Table 2).

Table 2. Nei's original measures average of genetic identity (above diagonal) and genetic distance (below diagonal)

S	1	2	3	4	5	6	7	8	9	10	11	12
1	****	0.5217	0.7283	0.7826	0.7609	0.6304	0.7174	0.7065	0.7717	0.6957	0.6957	0.6957
2	0.6506	****	0.5543	0.5652	0.5000	0.5435	0.5870	0.4891	0.5109	0.5217	0.4565	0.5217
3	0.3171	0.5900	****	0.7065	0.7065	0.7500	0.6196	0.8043	0.6739	0.7500	0.7717	0.7500
4	0.2451	0.5705	0.3474	****	0.8478	0.6739	0.7609	0.6848	0.7717	0.6304	0.6522	0.6739
5	0.2733	0.6931	0.3474	0.1651	****	0.6957	0.8043	0.7065	0.7500	0.6522	0.6739	0.6522
6	0.4613	0.6098	0.2877	0.3947	0.3629	****	0.6522	0.6848	0.6196	0.6739	0.6739	0.6522
7	0.3321	0.5328	0.4787	0.2733	0.2177	0.4274	****	0.6630	0.7717	0.6739	0.6957	0.7391
8	0.3474	0.7151	0.2177	0.3787	0.3474	0.3787	0.4109	****	0.7174	0.8370	0.8152	0.8152
9	0.2591	0.6716	0.3947	0.2591	0.2877	0.4787	0.2591	0.3321	****	0.7283	0.7283	0.7717
10	0.3629	0.6506	0.2877	0.4613	0.4274	0.3947	0.3947	0.1780	0.3171	****	0.8913	0.8261
11	0.3629	0.7841	0.2591	0.4274	0.3947	0.3947	0.3629	0.2043	0.3171	0.1151	****	0.8913
12	0.3629	0.6506	0.2877	0.3947	0.4274	0.4274	0.3023	0.2043	0.2591	0.1911	0.1151	****
13	0.3023	0.5705	0.2877	0.3321	0.3629	0.3947	0.3321	0.2591	0.3171	0.2451	0.1911	0.1398

Nei's original genetic distances were calculated independently for the specimens separated on the basis of sibling species *M. blythii*

and *M. myotis*. In these species, the average similarity was calculated as 0.67% and the average distance was calculated as 0.36% using 7 ISSR markers.

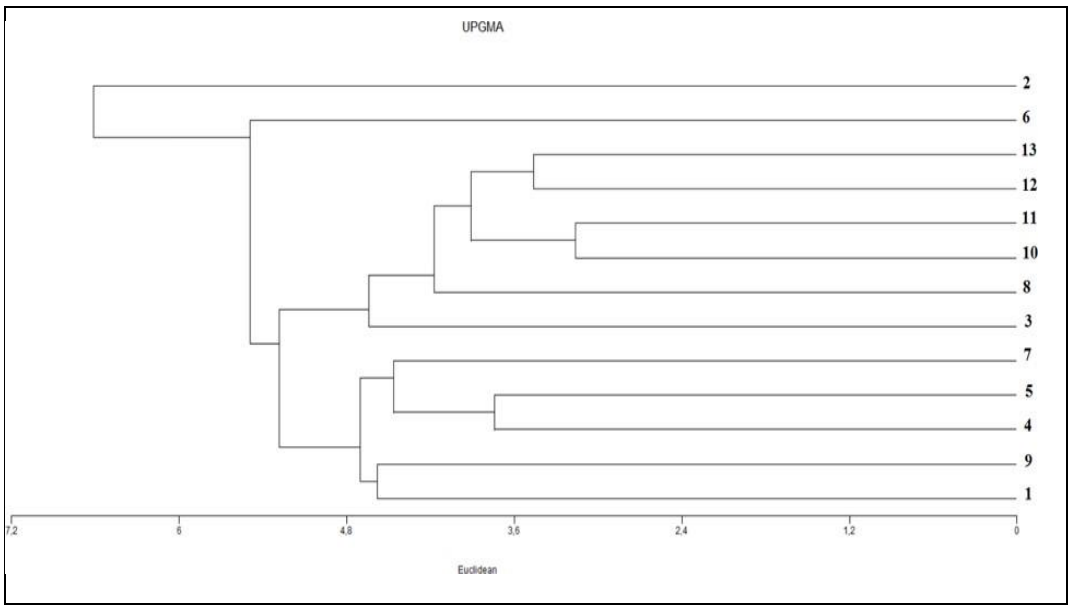

Figure 3. Dendrogram of genetic similarity among *Myotis* specimens (2: *Myotis capacinnii; 6:* belong to *Myotis myotis* colony; 3, 8, 10, 11, 12, 13 *Myotis myotis*;1, 9, 4, 5, 7: *Myotis blythii*)

Recently, detection of species-diagnostic markers has become very important in the identification and maintenance of genotypes, especially in animals [29]. In this research, the ISSR primers were utilized to identify the genetic variations between the two sibling bat species in Turkey. According to the results, the best optimized 7 ISSR primers are seen as a good differential marker in the genetic diagnosis of *Myotis* species (Table 1, Figure 3). Considering the genetic distances between species; The genetic distance ratio between *M. myotis* and *M. blythii* (36%) is successful in defining the phylogenetically separate species, while the genetic proximity ratio (67%) is successful in defining the close genetic relationship between sibling species. Thus, according to data obtained from mitochondrial genes (*Cytb* and *nd1*) of 10 sibling species of European *Myotis* samples, at least divergent taxa were *M. myotis* and *M. blythii*, which showed about 2.5% sequence distinction [14]. In addition, 33 *Myotis* taxa, including American species, are strictly divided into 5 main clades by using molecular data. While *M. myotis* and *M. blythii* were located in the same clade, the phylogenetic positions of some individual taxa such as *M. capaccinii* were uncertain. Considering the results obtained in this study, *M. myotis* and *M. blythii* represent genetically closely related groups, while a single individual belonging to *M. cappacinii* represents a separate group away from other samples.

Berthier, et al., [15] in their research on mitochondrial DNA (mtDNA), stated the absence of hybrid gametes in line with the existence of several common alleles in European *M. myotis* and *M. blythii* specimens exhibiting sympatric spread. When the Anatolian and European samples were compared, intraspecific level of genetic diversity was noted in the bat fauna of Anatolia [18]. Being composed of 41 species, the bat fauna of Turkey is genetically rich [30]. In this study, the samples were also found to exhibit high genetic diversity (94.57%) (Table 3).

Table 3. Genetic diversity according to *% P*, the percentage of polymorphic loci; *h*, Nei's gene diversity; *I*, Shannon's diversity; *ne*, the effective number of alleles; *na*, average allele number per locus

Specimens	n_a	n_e	*I*	*h*	*%P*
All populations (M. myotis, M. blythii, M. cappacinii)	1.945	1.487	0.293	0.451	%94.57

Hybridization events between *M. myotis* and *M. blythii* have led to controversy about their taxonomic status. Although sympatric species are likely to be seen in hybrid individuals, this phenomenon is rare for bats. Hoffmann, et al., [31] revealed hybridization events between the chromosomal races of Peters' tent-making bat (*Uroderma bilobatum*) species in contact zones by using mitochondrial *Cytb* gene analysis. Previously, 160 multilocal genotypes of two species from the mixed nursed colony were examined in sympatric areas of Italy and Switzerland. It has been shown that approximately 25% of *M. blythii* possess genes of *M. myotis* origin and hybrids can be observed frequently [15]. In another study, the hybridization rate and the composition of a maternity colony of *M. myotis* and *M. blythii* in the French Alps were evaluated [32]. Researchers identified 140 *M. myotis*, 12 *M. blythii* and 13 hybrids among 250 samples by multilocal gene analysis. In a study in which the morphometric methods were also utilized in addition to molecular approaches, *M. myotis* and *M. oxygnathus* species were clearly separated from each other [17]. However, intermediate values were identified in the dimensions of the skull and mandibular neck for 6 samples living as sympatric species. Accordingly, these specimens were characterized as possible hybrid individuals by the authors of the research.

5. CONCLUSIONS AND RECOMMENDATIONS

In this study, the samples separated in terms of morphological and geographic features were also separated in terms of their genetic characteristics based on the dendrogram produced by ISSR analysis (Figure 3). However, the dendrogram's profile of undiagnosed specimen (Sample 6) from *M. myotis* colony (in total 6 samples) may imply the presence of hybridization among sympatric colonies living in Central Anatolia. Nonetheless, in order to claim on the consistency of ongoing nuclear gene integration pattern between *M. myotis* and *M. blythii* in Anatolian samples and to explain the hybridization levels of these species, larger sampling sites and the greater number of molecular markers with careful evaluation are needed. To conclude, ISSR primers were evaluated as genetic markers that can be used in the diagnosis of cryptic taxa since they provide similar data to previous studies in determining the genetic relationships between *Myotis* species.

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