# Phylogenetic relationships of three bat species from Turkey

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**Summary:** The applicability of DNA sequencing of the Cytochrome b (encoded by mitochondrial DNA) gene was tested for species delineation and species identification in three bat species (*Miniopterus schreibersii, Myotis blythii* and *Myotis myotis*) sampled from Turkey as a geographic region. Morphologically identified species have also identified genetically. This study showed that DNA markers are valuable molecular methods for biodiversity monitoring programs in Turkey. Sequencing-based comparisons could provide more flexibility in large-scale studies for Turkish bat species.

**Key words:** Bats in Turkey, Cytochrome b, molecular differentiation, phylogenetic analyses, species identification.

### Türkiye'de bulunan üç yarasa türü arasındaki filogenetik ilişkiler

**Özet:** Türkiye'nin farklı iki ilinden örneklenen üç yarasa türünün (*Miniopterus schreibersii, Myotis blythii* and *Myotis myotis*) tarif ve tanımlanmasında mitokondrial Cytochrome b geninin kullanılabilirliği test edildi. Morfolojik olarak tanımlanmış olan türler genetik olarak da tanımlandı. Bu çalışma, Türkiye'de biyolojik çeşitliliğin izlenmesi programlarında DNA işaretleyicilerin, değerlendirilebilir moleküler metotlar olduğunu gösterdi. Dizin analizi tabanlı karşılaştırmaların, Türk yarasa türleri ile yapılacak geniş ölçekli çalışmalarda daha fazla esneklik sağlayabileceği sonucuna varıldı.

**Anahtar kelimeler:** Türkiye'de yarasalar, Cytochrome b, moleküler ayrımlaştırma, filogenetik analiz, tür tanımlama.

## Introduction

Up to now, 38 bat species have been recorded by various authors from Turkey (8, 26, 10). Of the 38 Turkish bats, one feeds on fruit while the others feed insects (10).

These species show variation with respect to their ecological, biological, karyological and molecular properties (1-3, 6, 7, 9, 13, 20). Morphological keys are available to assist the species identification allowing for the majority of species to be identified from external and cranial characters (1, 2, 15). However, molecular studies are also reliable and rapid tools for identifying even morphologically very similar species (23). Recently, molecular studies have enabled the determination of some specimens that were previously unconfirmed, as a new species (11, 14, 20-25).

Mitochondrial DNA (mtDNA) has long been treated as an ideal marker because of its convenience for reconstruction of gene genealogy and population history inference (16).

In this study, we tested the applicability of DNA sequencing of the Cytochrome b (encoded by mitochondrial DNA) gene for species delineation and species identification in three bat species sampled from Turkey. Cytochrome b gene is commonly used for species identification studies and phylogenetic analysis as a DNA marker (18, 19). This study is only a preliminary study for our future expectations and the sequence divergence estimation for Turkish bat species. We have just chosen three different species and analyzed cytochrome b gene of this species to show this study is feasible or not.

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## Materials and Methods

**Sample collection and preparation:** Different places visited to collect samples around Turkey (Fig. 1). A 3 mm biopsy from the wing membrane, blood and swab samples were taken from bats. A total of 55 specimens were collected from 9 different bat species but only three of them were used for in this study (Table 1). The species were *Miniopterus schreibersii, Myotis blythii* and *Myotis myotis*. *M. schreibersii* (Y7) sampled from Trabzon and *M.blythii* (Y17) and *M.myotis* (Y31) sampled from Balıkesir. Fieldworks were undertaken to avoid disturbing the colonies. Biopsy of specimens was applied as described by Worthington and Barratt (28). The 3 mm holes in wings are known to knit in four or five weeks.


**Figure 1.** The number of samples according to provinces.

**Table 1.** Samples collecting localities, their sizes and coordinates.

| Province | The Number of Samples | Decimal Coordinates | |
|---|---|---|---|
| | | LAT. | LONG. |
| Trabzon | 4 | 40,980000 | 39,770000 |
| Gümüşhane | 7 | 40,450000 | 39,430000 |
| Balıkesir | 17 | 39,650000 | 27,870000 |
| Ankara | 8 | 39,950000 | 32,850000 |
| Kırıkkale | 11 | 39,870000 | 33,620000 |
| Hatay | 1 | 36,220000 | 36,150000 |
| Adana | 7 | 37,000000 | 35,330000 |

**Morphological criteria for species identification:** Identification of bat species is achieved by using external, cranial measurements and baculum structure (1, 2, 4, 6, 15). Morphologically, three species were identified as *M.myotis, M.blythii* and *M.schreibersii*. Y31, Y17 and Y7 code numbers were given to each species, respectively.

**Nucleic Acid Preparation:** Total DNA was isolated from samples using the DNeasy Tissue Kit (Qiagen, Germany), following the 'Purification of Total DNA from Animal Tissue' protocol. Briefly, samples were lysed overnight using proteinase K after which the lysate was loaded onto a DNA Mini spin column. The DNA was then selectively bound to the column membrane by centrifugation and stored in elution buffer of kit (-20°C).

**Sequence amplification:** Previously published primers were used (18). BarbF1 (5'-CCT CAA ATA TTT CAT CAT GAT G-3') and BarbR2 (5'-GTC CTC CAA TTC ATG TTA GG-3') primer pairs were used to amplify cytochrome b.

Super Script III One-Step RT-PCR with Platinum Taq was used for PCR amplification (Invitrogen, USA). PCR mastermixes were prepared according to manufacturer instructions as follows: 17,8 µl of HPLC H20, 25 µl of 2XBuffer, 1 µl of forward primer (Barb F1) (0.2 µM), 1 µl of reverse primer (Barb R2) (0.2 µM) and 0.20 µl of Platinum

Taq DNA Polymerase (0.5 U/ml). Template DNA (5 µl at 100 ng/µl) was added to the aliquot (45 µl) of mastermix. Thermocycling conditions included an initial denaturation at 95°C (15 min), followed by 37 cycles at 94°C (50 s), 45.6-56.5°C (50 s) and 72°C (60 s) with a final 10 min extension at 72 °C using a PTC 100 machine (MJ Research, USA).

Amplified products were separated in a 1% agarose gel and visualised under ultraviolet illumination following ethidium bromide staining. All successfully amplified products were purified using the QIAquick® Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). Sequencing reactions on purified PCR products included primer pairs used for initial amplification (at 0.5 initial concentration), and the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) was used according to the manufacturer's instruction. Sequenced products were cleaned with DyeEx 2.0 Nucleospin nucleotide removal kit (Qiagen GmbH, Hilden, Germany). Automated fluorescence sequencing was performed with an ABI PRISM® 310 Genetic Analyser (Applied Biosystems, Foster City, USA).

**Sequence Analysis:** The sequences were edited and aligned using CLC Main Workbench software (22). Multiple sequence alignments were generated using CLC Main Workbench program (22). Cytochrome b gene haplotype consensus sequences were generated for each species (811 bases) covering the 142-952 base nucleotide region of the cytochrome b gene, with an out group (GenBank Accession number is EU751000) also aligned. Additional bat species sequences (GU817369, GU817368, EU153108, AF376830, AF376841, AF376840) were obtained from the National Centre for Biotechnology Information (NCBI) and used for comparison. Phylogenetical tree was built by Neighbor-Joining method (Fig. 2). Reliability of clades was checked with bootstrap analysis with 1000 replications (18).

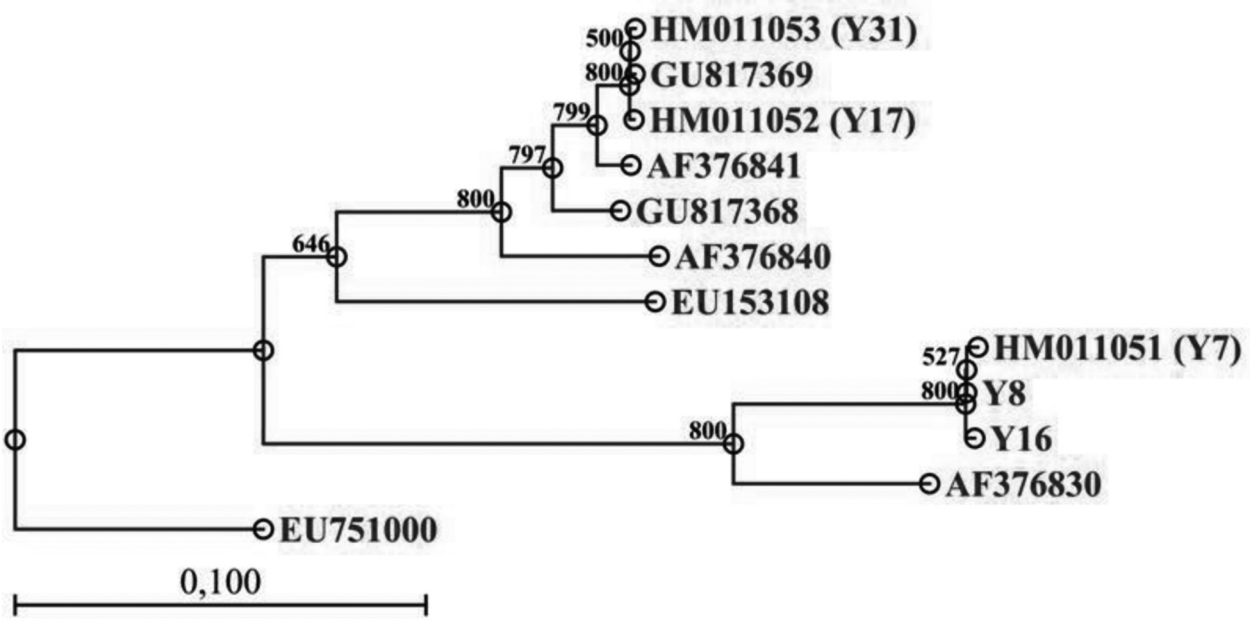


**Figure 2.** Phylogenetic relationships of three different bat species. Y8 and Y16 are used for additional data. Bat species sequences (GU817369, GU817368, EU153108, AF376830, AF376841, AF376840) were obtained from NCBI and used for comparison.

## Findings

Cytochrome b sequences were obtained from three species, the sequence length 811 bases. These sequences have been deposited to GenBank with the accession numbers HM011051, HM011052 and HM011053. A sample of *Eptesicus serotinus* (EU751000) from Azerbaijan (27) was included in the analysis as an outgroup. The species consensus sequences (811bases) were built using nucleotide region of the cytochrome b gene. This consensus demonstrated that they aligned across 811 bases,

with no sites of insertion or deletion. The neighbor joining tree was generated and given in figure 2. Our analyses, based on the mitochondrial genes cytochrome b (a common species-level marker), suggest that this kind of markers are capable of discriminating bat species with high accuracy. Morphologically identified species have also identified genetically in this study.

## Discussion and Conclusion

Up to now, 38 bat species were recorded from Turkey (10). Predominantly, researchers from Turkey prefer morphological studies about bats and they published their findings. Morphological characterization of bat species should be applied as an essential protocol (5). But, identification of bat species using molecular tools will also be possible in conjunction with bat characterization of morphological features. Especially, samples are damaged or identification from morphological criteria is not possible or if the specimen is degraded or visual inspection is not possible or the morphological expertise is not available or large-scale studies are needed.

The rapid and accurate identification of species is a critical component of large-scale biodiversity monitoring programs (17). Substantial sequence divergence suggests an unexpected high number of undiscovered species (24).

The phylogenetic studies were done for two bat species by different research groups in recent years in Turkey. Only, *M.schreibersii, M.capaccinii* and *Plecotus kolombatovici* have been studied (12, 13, 20). Therefore we need more molecular works and comparative studies on all bat species.

Three different species were chosen and analyzed cytochrome b gene of this species to show for future expectation. Study showed that DNA markers are valuable molecular methods for biodiversity monitoring programs in Turkey. Therefore, sequencing-based comparisons could provide more flexibility in large-scale studies for Turkish bat species. Also, results gave us an idea about sequence divergence estimation in bat population in Turkey, because there is no completed similar study.

The cytochrome b gene sequences for the three Turkish bat species in this study were compared with sequences available from GenBank. All of the sequences are highly similar to those of published specimens. The sequence similarity is ranging between 86% and 99%.

We present a framework for future characterization of the impact of sequence detection, morphological characterization and building a DNA library for Turkish bat species.

## Author's Contributions

HU carried out the fieldwork and molecular genetic studies, performed analyses and drafted the manuscript. IA coordinated the fieldwork and helped in drafting the manuscript. NA and NU helped drafting the manuscript. OA carried out the fieldwork, supervised the laboratory works and helped in drafting the manuscript. TM and CF helped in drafting manuscript. All authors read and approved the final manuscript.

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