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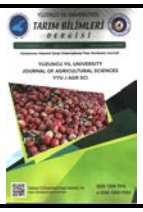

Araştırma Makalesi (Research Article)

Molecular Evaluations and Genetic Divergence of *Erynnis tages* and *Erynnis marloyi* (Lepidoptera, Hesperiidae) Based on mtCOI Gene with Turkey Populations

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Abstract: *Erynnis tages* and *Erynnis marloyi* were known as European species until recent years. Due to their narrow distribution areas, the morphological similarities of the two species were very high, and their status was controversial. However, as the records of these species came from the new regions, their distribution areas turned out to be wide, contrary to what is known. With the mtCOI gene barcode, there was a chance to identify genetic variations hidden between inter-species and intra-species. The present study was the first time the barcode characterization of populations in Turkey and other registered population of barcodes with the genetic variation were assessed. Phylogenetic trees based on mt COI gene sequences were created using Neighbor-joining, Bayesian inference, and maximum-likelihood algorithms. Genetic divergence was confirmed by Automatic Barcode Gap Analysis using the Kimura 2 parameter. It is genetically confirmed that *E.tages* and *E.marloyi* are two distinct species independent from each other. *E.tages* population of Turkey was found genetically similar to that of the population belonging to southern Italy. Southern Russia was also genetically similar. However, *E. marloyi* Turkey's population was genetically similar to the population of Iran.

Türkiye Populasyonlarıyla mtCOI Genine Dayalı *Erynnis tages* ve *Erynnis marloyi*'nin (Lepidoptera, Hesperiidae) Moleküler Değerlendirmeleri ve Genetik Divergensi

Makale Bilgileri

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Anahtar kelimeler
Barkod,
Erynnis,
Filogeni,
Genetik varyasyon,
Türkiye.

Öz: *Erynnis tages* ve *Erynnis marloyi* son yıllara kadar Avrupa türleri olarak bildirilmiştir. Dar yayılış alanları nedeniyle bu iki türün morfolojik benzerlikleri çok fazla olmasından dolayı tür statüleri tartışma konusu olmuştur. Ancak yeni bölgelerden bu türlere ait kayıtlar geldikçe yayılış alanlarının bilinenin aksine geniş olduğu ortaya çıkmıştır. mtCOI gen barkodu ile türler arası ve tür içi gizlediği genetik varyasyonları da belirleme şansı yakalanmıştır. Sunulan çalışmada ilk kez Türkiye populasyonlarının barkod karakterizasyonu yapılmış ve diğer kayıtlı populasyonların barkodları ile genetik varyasyonları değerlendirilmiştir. mt COI gen dizilerine dayanan filogenetik ağaçlar, Komşu birleştirme, Bayesian çıkarım ve Maksimum Olabilirlik algoritmaları kullanılarak oluşturulmuştur. Genetik varyasyon Kimura-2-parametresi kullanılarak Otomatik Barkod Boşluğu Bulma analizleriyle onaylanmıştır. *E.tages* ve *E.marloyi*'nin birbirinden bağımsız iki farklı tür olduğu genetik olarak doğrulanmıştır. *E.tages*'in Türkiye populasyonu ile Güney İtalya ve Güney Rusya populasyonları ve *E. marloyi*'nin Türkiye populasyonu ile de İran populasyonu genetik olarak çok benzediği görülmüştür.

1. Introduction

The genus *Erynnis* (Schrank, 1801), also known as the dusky-wing butterflies of the Hesperiidae family, includes more than 27 species. According to current data records, there is the highest number of species in Nearctic (Zakharov et al., 2009). However, new records and species descriptions are also available in Neotropics and Palearctic in recent years. Among the members of this genus, the two are morphologically similar species that have long been known as European butterflies. These contain minor differences in darker color tones Dingy Skipper for *Erynnis tages* (Linnaeus, 1758) and Inky Skipper for *Erynnis marloyi* (Boisduval, 1834) with states of out-morphology (Mazzei et al., 1999). Species boundaries and variations between populations of the two morphologically very similar species could not be evaluated in detail due to habitat restrictions. Recently, the new data regarding the distribution area of these two species (with new records coming from different geographies), necessitates the assessments at the species and population levels. New records have been reported belonging to Turkey, Lebanon, southern Iran, Armenia, and Pakistan (Koçak and Kemal, 2011).

With the advanced development of molecular biology techniques in the last decade, it has become applicable in systematic and taxonomic studies for tests of taxonomic characterization with molecular characters. A genetic identity (DNA barcodes) for the organism is created using short DNA regions. It is the Mitochondrial Cytochrome Oxidase I (mtCOI) gene region that shows both intra-species variations and species-level variations most reliably for butterflies. The non-overlapping nature of mutations in the populations and species-level of this gene region have made it the most suitable molecular character in determining inter-species and intra-species categories. Particularly morphologically similar species, cryptic species, and diagnostic (phenotypic plasticity) features are suitable for determining hidden taxa and also phylogeny estimations (Li et al., 2011; Mitchell and Gopurenko, 2016; Wang et al., 2018).

Nowadays, the butterfly taxonomy has been shaped according to the barcode information of different populations from different geographies with an understanding of the importance of barcoding. In this study for the first time Turkey's populations of *Erynnis tages* and *Erynnis marloyi* were barcoded and determined genetic diversity and species boundaries were determined with the other registered populations.

2. Materials and Methods

The butterfly specimens of both species were collected in 2016 from Bahçesaray-Mukus valley (Van, Turkey) by Muhabbet Kemal and Ahmet Ömer Koçak and stored in the Entomological Research Center Ankara (Cesa) Collection.

The legs of the *Erynnis* specimens were washed with ethanol. The RED Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, Missouri, USA) was used to purify Total Genomic DNA (tgDNA) (Kemal et al., 2018). PCR reaction was performed using LepF1 and LepR1 primers to amplify a 658 bp fragment of mt-COI gene from tgDNA. The PCR products were sent to Macrogen (Macrogen, Amsterdam, Netherlands) for bidirectional sequencing.

The other population's barcodes were retriewed from GenBank (Anonymous, 2020b) and Boldsystem (Anonymous, 2020a) database for determination of variations between *E.tages* and *E.marloyi* species. *E.brizo* species was used as outgroup and a total of 85 barcodes were used for phylogenetic analysis.

Genetic distances between populations and species were calculated using the Kimura 2-parameter distance model (Kimura, 1980). The aligned DNA barcodes by MEGA 7.0 software were separated into hypothetical species using Automatic Barcode Gap Discovery (ABGD) method with a prior P that ranges from 0.005 to 0.1 and the K2P model with the default settings (Anonymous, 2020c). The neighbor-joining (NJ) tree was constructed using the Kimura 2-Parameter distance model in MEGA 7.0 software. Maximum-likelihood (ML) bootstrapping analyses were achieved with 1000 replicates using RAxML Blackbox on XSEDE v.8.2.4 (Stamatakis et al., 2008) on the CIPRES Science Gateway. A Bayesian inference (BI) analysis was performed in MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003) with the Markov chain Monte Carlo algorithm. The program JModeltest v.2.1.7 (Posada, 2008) selected the TIM2+I evolutionary model as the best model according to the Akaike

information criterion for Bayesian inference. The program was run for 5 000 000 generations, with a sample frequency of 100 and a burn-in of 12 500.

3. Results

The aligned 85 COI fragments have the full barcode length. *E. marloyi* and *E. tages* barcodes have 31 variable sites (4.71%), of which 20 (3.03%) have parsimony informative. The most variation was determined at the first codon position. AT-deviation was high in the first position with an average AT-base pairs of 93.9% (Table 1).

Table 1. Barcode characterizations of the mtCOI gene in *E. tages* and *E. marloyi* populations

Nucleotide Position	Variable Site (%)	Informative Site (%)	T (%)	C (%)	A (%)	G (%)	AT (%)	GC (%)
1st	83.7	69.5	47.0	5.2	46.9	0.5	93.9	5.7
2nd	9.6	27.6	29.0	14.2	30.6	26.0	59.6	40.2
3rd	6.4	5.1	43.0	25.1	15.5	16.0	58.5	41.1
All	4.7	95.2	40.0	14.8	31.0	14.1	71.0	28.9

According to Kimura -2-parameter, the genetic distance between *E. tages* and *E. marloyi* was 2.98%. When the genetic distances of the two species at the intraspecies levels are evaluated, the genetic difference of Turkey's population compared with other populations of *E. tages* ranged from 0.46% at -1.23%. In addition genetic distance between populations of *E. marloy*i obtained from Turkey and Iran was 0.30%. Inter-species and intra-species variations were also determined as histogram and graphics with ABGD using the K2P model (Figure 1). According to ABGD analysis results, *E. tages* includes 5 intraspecific sections and *E. marloyi* 4 intraspecific sections (Figure 1). The cladistic analaysis in the presented phylogenetic tree were compatible with ABGD results (Figure 2).

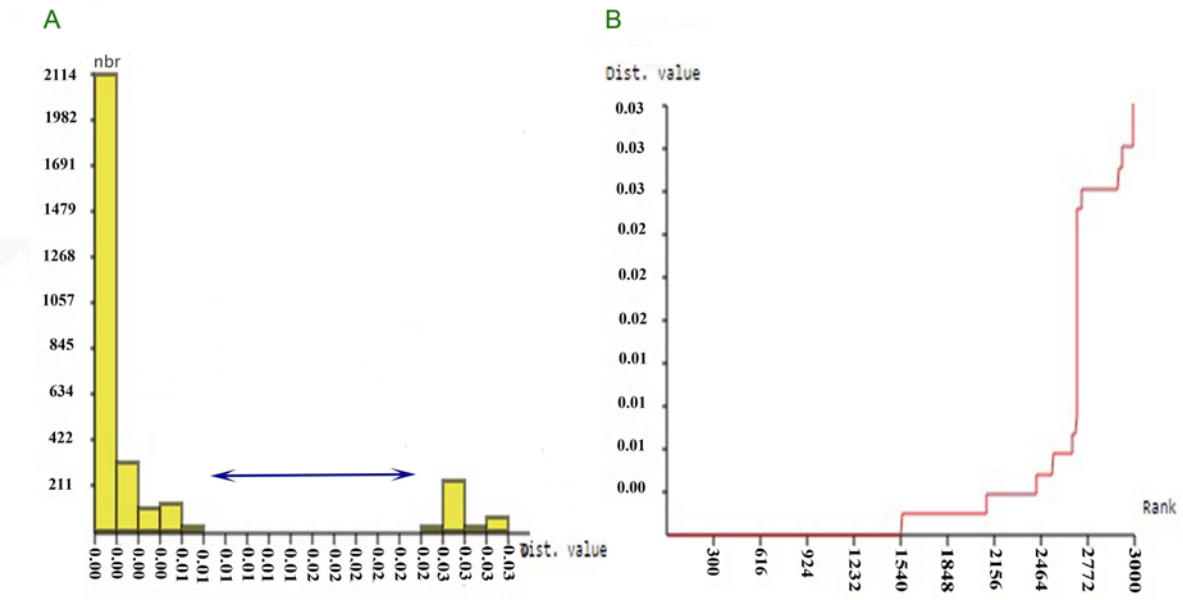

Figure 1. Histogram of pairwise K2P distances of 79 (except outgroup) aligned sequences (A) and ranked distances of two species (B).

The support values are shown on the NJ tree since the phylogenetic tree constructed with the NJ /BI/ML algorithms showed similar topology (Figure 2). Both species were phylogenetically separated based on strong values (NJ/BI/ML; 100/1.00/91). It has been shown that, Turkey's

population with Russian-EZHBA473-07/74-07 (the genetic distances 0.61%) and Italian BIBSA1360/MN141295 populations (the genetic distance value 0.46%) of *E. tages* were clustered in the same sub-clade and, Spanish-EZSPC1139-10/HM901339 (the genetic distance value 0.92%), British-OXB248-15/MN139663 (the genetic distance value 0.92%) and Iriesh-WMB4030-14/MN145401 (the genetic distance value 1.23%) populations have a sister position within the sub-clade.

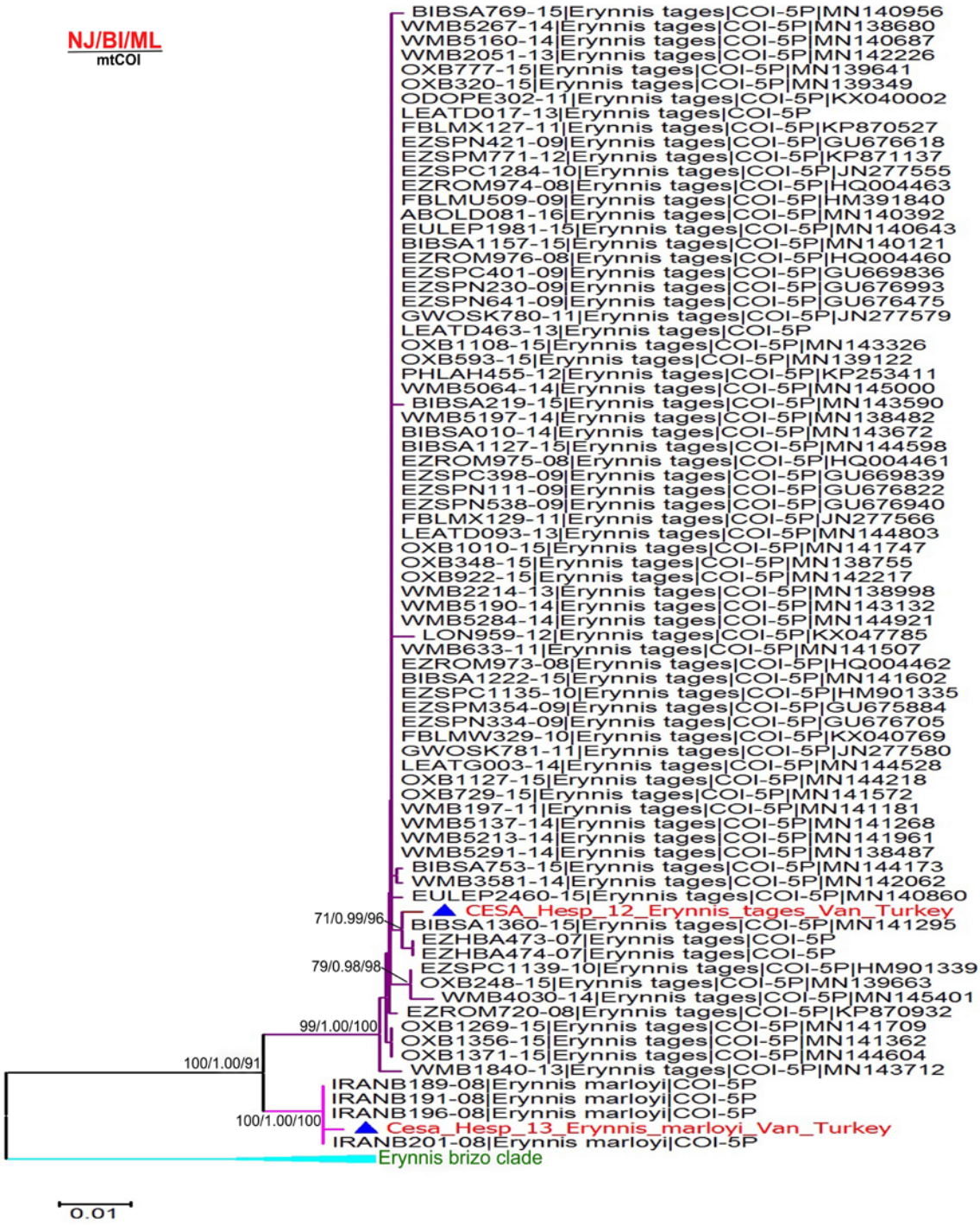

Figure 2. The phylogenetic tree based on mtCOI gene sequences of *E. tages* and *E. marloyi* populations. Numbers at the nodes indicate the NJ bootstrap values, the BI posterior probability and the ML bootstrap values. Bar, 1 substitutions per 100 nucleotide positions.

4. Discussion and Conclusion

Species that share close or common habitats may show similar morphological characteristics, depending on phenotypic plasticity, regional adaptation, or coevolution mechanisms (Forsman, 2015). Therefore, the phenotypes of these species in the ecosystem can mask true diversity. The best way to solve such a species problem is to identify the population units available. If the distribution areas of the species are known, adaptation to different environments and genetic variations can be revealed. Thus, it provides an understanding of the morphological, phylogenetic, and even ecological separation between closely related species obtained from different geographies. In recent years, with the development of molecular biology, the concept of species identification based on ecological species and genes has been revising taxonomy established by morphological characters. In particular, the mtCOI gene offers very successful distinctions at the species and intraspecies levels in Lepidoptera (Lassance et al., 2019). In this study, to determine species delimitation of *E.tages*, for the first time genetic distances of Europe, Russia, and Turkey populations were compared by adding new data. According to the results of the presented phylogenetic analysis, the populations of southern Italy and southern Russia were closest to the population of Turkey and the farthest was the Irish population. When the genetic distance variability between *E. tages* populations is supported by morphological characters, subspecies can be suggested for some populations.

The population records of *E. marloyi* from Southeast Europe and Turkey, Lebanon, Iran to South Pakistan are available. However, only the barcodes of Iranian populations have been made and recorded until this study. In the present study, was compared only the genetic barcodes of Iran and Turkey populations because there was not any barcode record in Genbank or Boldsystem for the populations reported from the mentioned geographies.The genetic distance between *E. marloyi* populations obtained from very close geographies (Kermanshahan / Iran and Van / Turkey) was quite low. Molecular data from a large number of different geographies is needed to determine a reliable variation.

In this study, Turkey's populations of *E. tages* and *E. marloyi* were firstly barcoded and made genetic distance analysis. Two species were evaluated used the tree-based method for phylogenetically delimiting species. According to the molecular analysis results, *E. tages* and *E. marloyi* are morphologically similar but genetically distant (at the species level) that is, whose are two distinct species. As the information about the spreading areas of these species increases, it will be easier to understand phylogenetic relationships and biodiversity.

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