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Species Delimitation of Some *Melanargia* Species (Lepidoptera, Nymphalidae, Satyrinae) in The Southeastern Anatolia Region Based On The mtCOI Gene

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Abstract

Among the Palearctic species, butterflies of the genus *Melanargia* are known for their black and white wing patterns. The morphological character polarization of this genus is full of varying combinations of subgenus, species complex, and subspecies status. Its taxonomy is open to debate, especially in species and subspecies categories, with definitions mostly based on wing color. In recent years, cryptic species, phenotypically masked species, and species with intense intraspecific variation have been identified through the determination of lineages under the leadership of molecular systematics. The mtCOI gene, which is especially described as a species signature, is an important DNA barcode used for Lepidoptera.

In the presented study, the mtCOI gene sequence of the populations of *Melanargia larissa, M.grumi, M.hylata, M.syriaca*, and *M.russiae* species in the South-eastern Anatolia region was determined for the first time. To determine the boundaries of these species, gene characterization and genetic distances were carried out according to the Kimura-2 Parameter, and putative species analyses were carried out by the ABGD method. Trees were constructed with Maximum likelihood and Bayesian inference algorithms to determine the phylogenetic relationships between species of the genus. In light of these analyses, it has been shown that the genetic distance of morphological species *M. larissa, M. grumi, M.hylata*, and *M. syriaca* is not at the species level and that *M.larissa* maintains its species status according to the principle of priority. In addition, the *M.russiae* population presented in this study forms a monophyletic clade with other populations of the same species in the phylogenetic tree, proving that this taxon is a distinct species

1. Introduction

The *Melanargia* genus was established by Meigen in 1828 and belongs to the family Satyridae. It is distributed from Europe to the easternmost part of Russia and is represented by 24 valid species [1]. Members of the genus have been classified under three subgenera for a long time due to their distribution in these geographical regions: *Melanargia*, *Parce* and *Turcargia*. *Melanargia* subgenus is known for its single species, *M. galathea*. This species is the type species of *Melanargia* and is found in Europe, southwestern Russia and in our country only in the Northern Anatolian forest belt. The type species of the second subgenus, *Parce*, is *Melanargia russiae*. This species has an intermittent distribution in the alpine zone in Europe, Anatolia and Central Asia and is found locally in the high mountains in eastern Turkey. Although the type species of the *Turcargia* subgenus is *Melanargia larissa*, this subgenus also includes *M.grumi*, *M.hylata*,

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M.syriaca, and *M.titea*, and these species are recorded in some countries in the southwestern Palearctic [2]. The species of genus *Melanargia*, commonly known as 'marbled whites' due to their checker black-white wing patterns, are characterized by a wide vein at the base of the forelimbs and are easily distinguished from other genera in the Satyrini subtribe [3]. The taxonomic structure of the genus, and evaluation of taxa as subgenera or species groups is still debated. In particular, the existing morphological variations of these butterflies are quite confusing in determining their taxonomic status. The density of black patterns, especially on the upper wings of individuals examined between different species of the genus or within the same species, makes it difficult to determine species delimitation. Individuals within populations of nearly every species contain variations with very light or very dark patterns. Although new species were added to this genus until recently, the character polarization of the species has not been fully established. As the importance of molecular taxonomy in systematic research is realized, the phylogenetic relationships between members of this genus have begun to be understood. Nazari et al. [4] investigated the taxonomic and phylogenetic relationships using the genital morphologies and mtCOI, ribosomal 18S rRNA and nuclear wg genes of all known Palaearctic butterfly species of the genus Melanargia. Thus, the taxonomic structure of the genus and the evaluation of taxa as subgenera or species groups have been opened to discussion at the molecular level. With the results of this study, they proposed three subgenera of the genus as *Melanargia*, *Halimede* and *Argeformia* with revized species/subspecies. A new *Melanargia* species (*M.sadjadii*) was recognized in northern Iran, near the Caspian Sea, by Carbonel and Naderi [5], which is very similar to *Mtitea wiskotti* with its external morphology and sparse black spots on its wings, and to Mevartianae with its genital structure. Later, Nazari et al. [4], they gave new status as a subspecies of *M.evartianae* for *M.sadjadii* with molecular analysis.

The Mitochondrial Cytochrome Oxidase I (COI) gene is an important barcoding sequence widely used for species identification, delimitation, and also used nucleotide characterization of the gene to reveal variation in species or subspecies categories [6], [7], [8]. The aim of this study was to barcode some *Melanargia* species in Southeastern Turkey for the first time and to contribute to the molecular taxonomy of the *Melanargia* genus through species delimitation analyses. In this region, the *Melanargia* genus is represented by the species larissa, syriaca, grumi, hylata and russiae [9], [10], [11], [12], [13]. The Van population of *M.russiae* was compared with other populations reported from different geographies. In addition, the barcodes of the species within the "larissa" complex, whose morphological distinction is still controversial, were evaluated for the first time with new recording data in Turkey, and their phylogenetic species levels were discussed with molecular analyses.

2. Material and Method

Melanargia specimens used for molecular analysis were collected, morphologically identified, and then preserved by Muhabbet Kemal and Ahmet Ömer Koçak from the South-eastern Anatolia Region between 2015 and 2018.

Table 1. Label information of materials whose species delimitation are evaluated.

No	Taxon name	Collected location and date with collectors	Location code*	CESA ID**
1	*Melanargia grumi*	TR- Adıyaman Pr Kahta, Aydınpınar 6.5.2018, M.Kemal & A.Koçak leg. (Cesa)		LepDNA Saty01–Cesa,
2	*Melanargia grumi* ssp.*grumi*	TR – Mardin Pr., Nusaybin Kalecik 560m, 10.5. 2015, I. Akdeniz leg. (Cesa)	47Gd	LepDNA Saty 08-Cesa
3	*Melanargia grumi* ssp.	TR – Bitlis Mutki, Kavakbaşı 1670m, 9.6.2018, M.Kemal & A.Koçak leg. (Cesa)	13F22	LepDNA Saty 09-Cesa
4	*Melanargia hylata karabagi*	TR- Hakkari Pr. Ağaçdibi, 15.6.2018, M.Kemal &A.Koçak leg. (Cesa)	30B	LepDNA Saty02–Cesa

5	*Melanargia hylata*	TR- Van Pr., Bahçesaray Paşaköy 1600m, 6.7.2016, M. Kemal & A.Koçak leg. (Cesa)	65Ai	LepDNA Saty 04-Cesa
6	*Melanargia larissa masageta*	TR- Malatya Pr., Beydağı TP 1275m, 20.6.2015, M. Kemal & A.Koçak leg. (Cesa)	44L	LepDNA Saty 03-Cesa
7	*Melanargia syriaca* ssp. *kocaki*	TR- Bitlis Pr., Güroymak 1600m, 4.7.2015, M.Kemal & A.Koçak leg. (Cesa)	13D	LepDNA Saty 06-Cesa
8	*Melanargia syriaca* ssp. *kocaki*	TR-Muş Pr., Buğlan Pass 1640m, 21.7.2019, M.Kemal & A.Koçak leg. (Cesa)	49Ea	LepDNA Saty 07-Cesa
9	*Melanargia russiae* ssp.	Van Pr., Gürpınar, Başet Mt., Dijik 2710m, 10.7.2019, M.Kemal & A.Koçak leg. (Cesa)	65Gv	LepDNA Saty 10-Cesa

* The location codes of the materials used in the study are taken from the study of Koçak and Kemal (2019) [14].
** Informations of the sequenced barcodes were keeped in the Cesa barcode store. (https://entcesa.tripod.com/Cesacollection.pdf)

Legs from each individual belonging to nine populations of these butterflies were cleaned with ethanol. Total genomic DNA (tgDNA) was isolated from the muscle tissue of the hindlimb femur of each sample using the RED Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, Missouri, USA) [15]. The mitochondrial COI gene 658 bp sequence was amplified by PCR reaction using LepF1 and LepR1 primers for each individual. Used cycling parameters were as follows: Initial denaturation at 94 °C for 2 min, followed by 5 cycles of 40 s denaturation at 95°C, 40 s annealing at 45°C, 1 min extension at 72°C, and additional 36 cycles of 40 s denaturation at 94°C, 40 s annealing at 51°C, 1 min extension at 72°C, with a final extension of 72 °C for 10 min. Purification and bidirectional sequencing of PCR products were performed by the Macrogen company (Macrogen, Amsterdam, Netherlands).

Forward and reverse DNA fragments of each bidirectionally sequenced sample in Ab1 format were created contigs after nucleotides controlled, and also converted to .fasta format in the CodonCode Aligner v.8.0.2. A data set was created with used the CLUSTALW alignment algorithm in MEGA 7.0 software by all possible pairwise aligning 9 *Melanargia* barcodes obtained from Southeastern Turkey with other 93 *Melanargia* barcodes registered in online portals. [16], [17]. In the same program, nucleotide compositions were computed for gen characterization of available five species and genetic distances between *Melanargia* populations and species were measured using the Kimura-2 parameter [18]. In the ALTER online portal, the alignment sequences were converted from fasta format to philip and nexus format and then used for Maximum-likelihood (ML) and Bayesian inference (BI) algorithms in the respectively. The ML tree was constructed in CIPRES Science Gateway XSEDE v.8.2.4 using RAxML Blackbox efficiency with 1000 replicates [19]. TPM1uf substitution model was selected as the best evolutionary model according to Akaike criteria in the program JModeltest v.2.1.7 [20], but TPM1uf substitution model were replaced by the GTR model for the closest over-parameterized model [21]. Therefore, BI analysis nucleotide change commands were arranged according to GTR +I +G model with the Markov chain Monte Carlo algorithm in MrBayes 3.2.6 software [22]. Markov chain Monte Carlo (MCMC) simulations were run for 3 000 000 generations, sampling every 100 generations. In the context of Bayesian statistics, summarized tree was obtained through the posterior probability distribution after the first 7500 trees were burned. The topology of both algorithm trees was visualized using FigTree v.1.4.2. DNA barcodes were identified as putative species by the Automated Barcode Gap Discovery (ABGD) method based on a preliminary P value ranging from 0.005 to 0.1 [23].

3. Results and Discussion

Gene characters of current populations of *Melanargia* spp. were determined according to the first, second and third nucleotide positions of the mtCOI gene sequences of *Melanargia* species (Table2). The AT deviation was calculated at the first nucleotide position in the variable region of the mtCOI gene nucleotide characters of *M.hylata* and *M.russiae* populations. The second nucleotide positions were conserved in the variable region of

M.larissa, and the third nucleotide positions in the variable region of *M.russiae* and *M.grumi*. In addition, both the second and third nucleotide positions of *M.hylata* and *M.syriaca* variable regions were conserved. The most numbers of singletons in the mtCOI gene characterization variable region were determined in *Mhylata* populations and the lowest in *M.grumii* populations.

Table 2. Barcode characterizations of the COI gene from different populations of *Melanargia* spp.

Species	Nucleotide Position	Variable Site (%)	Informative Site (%)	T (%)	C (%)	A (%)	G (%)	AT (%)	GC (%)
	1st	92.3	66.5	49.0	7.7	42.3	0.9	91.3	8.6
M. grumii	2nd	7.6	25.5	25.0	16.9	32.0	26.0	57.0	42.9
	3rd	0.0	6.8	43.0	24.7	16.0	16.0	59.0	40.7
	All	1.9	98.1	39.2	16.4	30.1	14.3	69.3	30.7
	1st	100.0	66.2	49.0	7.7	42.7	0.5	91.7	8.2
M. hylata	2nd	0.0	25.5	25.0	16.9	31.5	26.5	56.5	43.4
	3rd	0.0	6.8	43.0	24.7	16.0	16.0	59.0	40.7
	All	1.6	98.3	39.1	16.5	30.0	14.4	69.1	30.9
	1st	94.7	66.4	48.0	9.1	42.3	0.9	90.3	10.0
M. larissa	2nd	0.0	25.8	25.0	16.9	31.5	26.5	56.5	43.4
	3rd	5.2	6.3	43.0	24.7	15.5	16.4	58.5	41.1
	All	2.8	97.1	39.1	16.5	29.9	14.5	69.0	31.0
	1st	100.0	66.5	50.0	7.3	42.7	0.5	92.7	7.8
M. syriaca	2nd	0.0	25.8	25.0	16.9	31.5	26.5	56.5	43.4
	3rd	0.0	6.5	43.0	24.7	16.0	16.0	59.0	40.7
	All	2.1	97.8	39.4	16.3	30.1	14.3	69.5	30.6
	1st	88.8	66.1	49.0	8.2	40.9	2.3	89.9	10.5
M. russiae	2nd	11.1	25.3	26.0	16.4	31.1	26.9	57.1	43.3
	3rd	0.0	5.9	43.0	24.7	16.0	16.0	59.0	40.7
	All	2.7	97.2	39.3	16.3	29.3	15.1	68.6	31.4

When the genetic distance relations between the *Melanargia* populations obtained from Southeastern Turkey, and constituting the materials of this study are examined, the four morphological species do not diverge at the species level (Table3). It has been determined that the 1.40-1.88% genetic distance between the *Melanargia syriaca kocaki* populations and other larissa-hylata-syriaca populations is sufficient at the molecular level for the subspecies level of the *syriaca kocaki* population. It is clear that the *M.russiae* population is a strong species with a genetic difference range of 6.20-6.74% from other populations.

Table 3. Genetic distances of the mtCOI sequence between *Melanargia* populations using the Kimura 2-parameter model.

The populations in the study	**K2P-Genetic distances**							
M. grumi-Adıyaman								
M. grumi-Mardin	**0.00**							
M. grumi-Bitlis	0.46	**0.46**						
M. larissa masageta-Malatya	0.61	0.61	**1.08**					
M. hylata karabagi-Hakkari	0.77	0.77	0.61	**0.77**				
M. hylata-Van	1.08	1.08	0.92	1.08	**0.31**			
M. syriaca kocaki-Muş	1.88	1.88	1.40	1.88	1.40	**1.71**		
M. syriaca kocaki-Bitlis	1.88	1.88	1.40	1.88	1.40	1.71	**0.00**	
M. russiae-Van	6.38	6.38	6.56	6.74	6.20	6.56	6.74	**6.74**

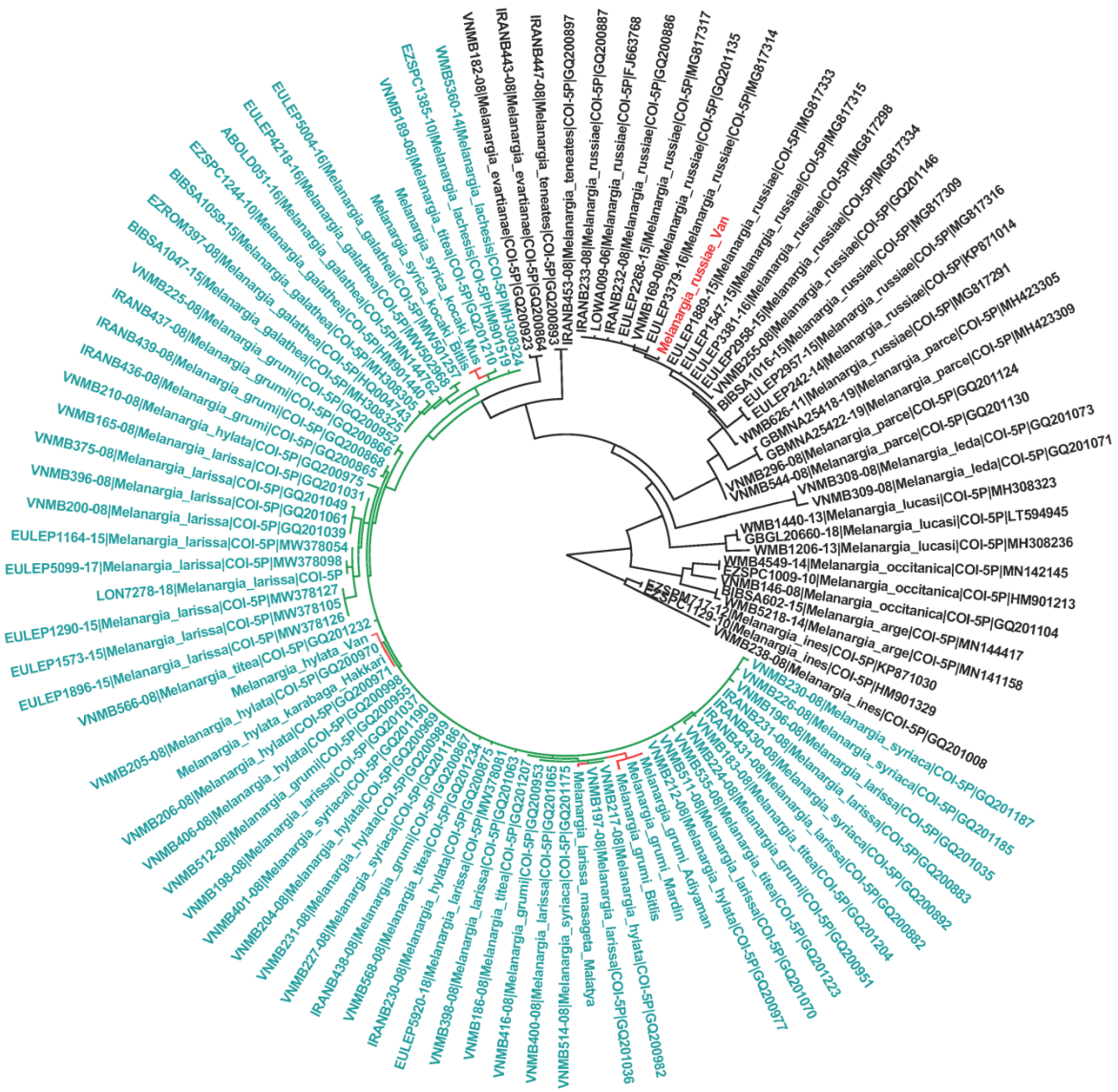

Figure 1. Circular phylogenetic tree created by ML algorithm when viewed from inside to outside, blue taxa (grumi+hylata+syriaca+titea=larissa) lineage representing a single species (red branches represent Turkish populations). In the M.russiae clade, the Turkish population written in red is shown.

Because maximum likelihood (ML) and Bayesian inference (BI) trees show similar tree topologies, the phylogenetic relationships of Melanargia species are shown in the circular ML tree without support values given in the text. (Figure1). Both phylogenetic analyses were identified well-supported major clades of *Melanargia*. The first major clade includes of *Melanargia ines* (100/1.00). The second major clade (100/1.00) consists of two distinct groups. The first group includes the *M.arge* and *M.occitanica* clade (100/1.00). The other group consisted of monophyletic and non-monophyletic taxa. Monophyletic taxa (*M.lucasi*, *M.leda*, *M.parce*, *M.russiae*, *M.tenetates*, *M.evartianae*, *M.galathea*, *M.lachesis*) formed branches with strong support values. Non-monophyletic taxa (*M.grumi* , *M.hylata*, *M.syriaca*, *M.titea*, *M.larissa*) are crowded ingroups indicated by the blue text.

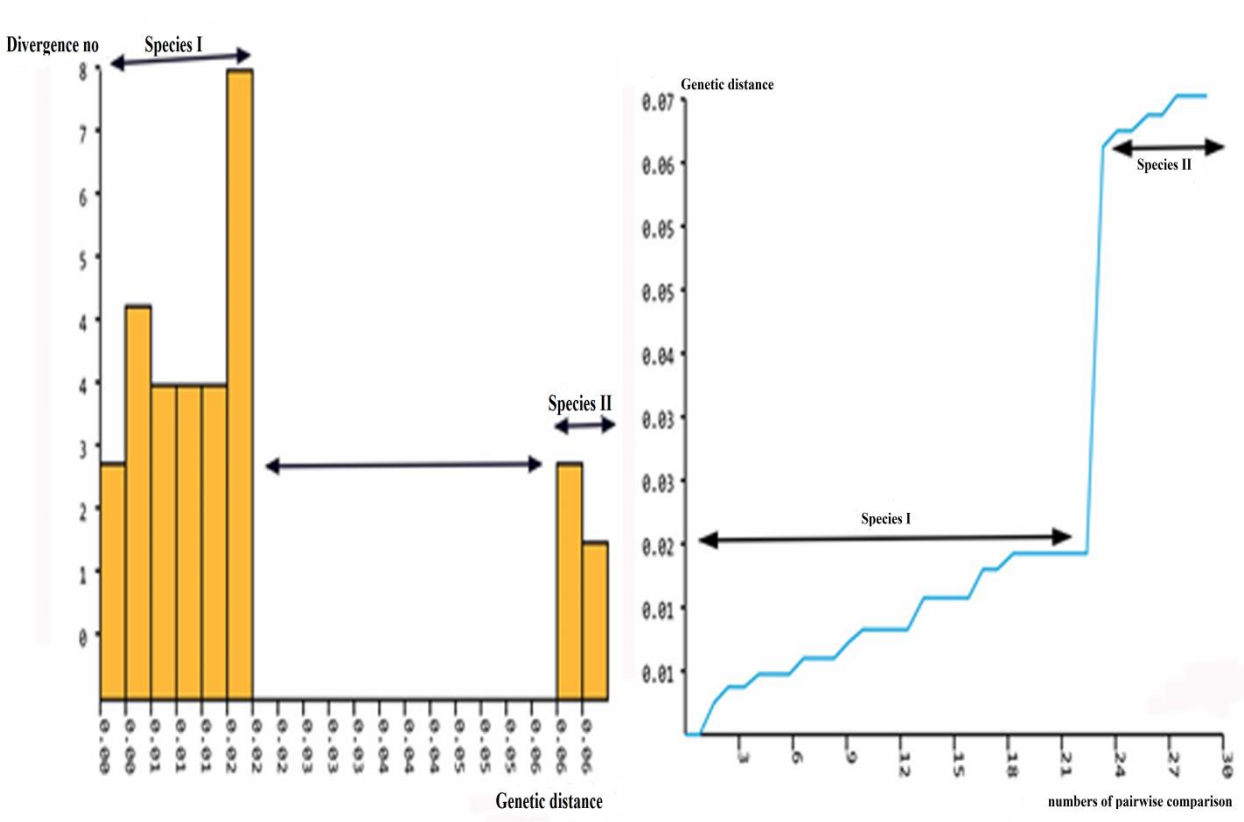

Figure 2. Histogram of pairwise K2P distances of nine Turkish populations aligned sequences (A) with ranked distances (B).

In the presented study, it was determined that the genetic distance range of individuals belonging to *Melanargia* populations consisted of two groups (Figure2). The first group is in the range of 0.00-1.88 and the second group is in the range of 6.38-6.74. In the ABGD test, in both pairwise and multiple comparisons of 9 populations, the nucleotide change for group I does not exceed 2% for 4 morphological species (*M.larissa*, *M.grumi*, *M.hylata* and *M.syriaca*). The *M.russiae* population in the second group was genetically separated from other populations in the first group as a distinct species with an average genetic distance of 6.5% according to its lower and upper limits.

4. Conclusion and Suggestions

In the study, as a result of genetic distance, species delimitation and phylogenetic analysis of the mtCOI gene sequence among 9 Turkish populations belonging to five morpho-species, these were determined that these populations belonged to only two species. The first species is *M.russiae*, which is morphologically defined as the same, and the second species, which is *M.larissa* according to the principle of priority, is a group of populations belonging to four morphological species (grumi+hylata+larissa+syriaca). For this reason, we support the evaluation of *M.syriaca kocaki* and *M.hylata karabagi* populations within/under the *M.larissa* species. In our study, populations within the larissa group showed very limited genetic differences. Although insufficient phylogenetic signal in this complex cannot be ruled out for mtCOI, Nazari et al. [4] reported that the signal in the 18S rRNA and nuclear wg genes was weak in the phylogeny resolution of this complex. Thus, the lack of molecular divergence for Lepidoptera is not limited to mitochondrial genes. In same study, they conducted with *Melanargia* samples from different geographies, except for the populations of the southeastern Anatolia region, they reported that *M.larissa* is a strong species, and its eleven subspecies, most of them with new statuses.

Molecular analysis of *Melanargia* populations according to the mtCOI gene sequence showed that *M.lucasi*, *M.leda*, *M.parce*, *M.russiae*, *M.tenetates*, *M.evartianae*, *M.galathea*, *M.lachesis*

and *M.larissa* are the strong species. In the presented phylogenetic tree, *M.parce* demonstrated two different branches. These two distinct lineages represent two morphologically masked but genetically distinct species. Although the morphological species *M.titea* shares common habitats with the larissa complex only with *M.syriaca* in Hatay [4], it was clustered in the phylogenetic tree with the Muş and Bitlis populations of *M.syriaca* presented in this study. As an appearance, *M.titea* is easily distinguished from the larissa complex.

In this study, the effectiveness of DNA barcoding was tested for the identification and discovery of species from the species-rich fauna of south-eastern Turkey. According to our results, the variations in wing color taken into account in *Melanargia* taxonomy do not lead to a meaningful conclusion with molecular analyses. In our phylogenetic tree, the stable placement of taxa that have completed their speciation and the cladistic chaos of taxa that have not yet reached species saturation (grumi+hylata+syriaca+titea in larissa) can be clearly seen. Moreover, the results of mtCOI gene characterization of *Melanargia* Turkish populations were also showed signals like admixture or subdivisions, inbreeding, or introgression. Although these taxa, which have a wide distribution area, may change in appearance due to ecological pressures in different geographies, their genetic distance from each other is not at the species level because gene flow continues between them.

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Conflict of Interest Statement

There is no conflict of interest between the authors.

Statement of Research and Publication Ethics

The study is complied with research and publication ethics

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