

## Taxonomic Rearrangement of the *Erebia tyndarus* Species Group (Lepidoptera, Nymphalidae, Satyrinae) Based on an Analysis of COI Barcodes, Morphology, and Geographic Distribution

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Despite numerous attempts to reveal the phylogenetic position and taxonomic status of formally described entities, a large number of unresolved taxonomic problems still persist in the *E. tyndarus* group, mostly due to incomplete species and population sampling, especially in the eastern part of the group's distribution. Here, we provide a COI barcode study based on essentially improved sampling of the taxa and populations from the Caucasus, including for the first time, data on one of the key taxa in this complex, *Erebia iranica*, described from the Demavend volcano in Iran. We also analyze the structure of valve in male genitalia and the geographic distribution of the taxa. Our analysis does not confirm the close relatedness and conspecificity of the taxa known in current literature as "*E. iranica iranica*" (North Iran) and "*E. iranica sheljuzhkoii*" (Great Caucasus). Instead, the obtained data indicates the need for the taxonomic reorganization of the *E. iranica* complex and its division into two species: monotypic *E. sheljuzhkoii* (Great Caucasus) and polytypic *E. iranica* with subspecies *E. iranica iranica* (North Iran), *E. iranica dromulus* (Turkey, Ararat Mt.), *E. iranica transcaucasica* (Lesser Caucasus), and *E. iranica graucasica* (Great Caucasus). In addition, our data do not support the recently proposed splitting of *E. callias* and *E. cassioides* into multiple allopatric species.

Key words: Lepidoptera, Nymphalidae, Satyrinae, *Erebia*, phylogeny, DNA barcode, COI, morphology.

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The group of ringlet butterflies similar to *Erebia tyndarus* (Esper, 1781) can be subdivided into four complexes of closely related cryptic species. (1) The Euro-Asia Minor complex (= the *E. ottomana* complex) includes the single species *E. ottomana* Herrich-Schäffer, 1847 distributed in the mountains of south Europa and in Turkey (HESSELBARTH *et al.*

1995). (2) The complex *E. tyndarus* sensu stricto is distributed in West Europe, and according to the last list of European butterflies (WIEMERS *et al.* 2018) includes 8 species: *E. hispania* Butler, 1868, *E. rondoui* Oberthür, 1908, *E. tyndarus* (Esper, 1781), *E. cassioides* (Hohenwarth, 1792), *E. nivalis* Lorković & de Lesse, 1954, *E. neleus* (Freyer, 1832),

*E. calcarius* Lorković, 1953 and *E. arvernensis* Oberthür, 1908. (3) The taxa of the *E. iranica* Grun-Grshimailo, 1895 species complex are distributed in the Russian, Georgian, Armenian, and Azerbaijani parts of the Caucasus, and in Turkey and North Iran (HESSELBARTH *et al.* 1995). (4) The taxa of the *Erebia callias* Edwards, 1871 species complex are distributed in Siberia and North America (DE LESSE 1960).

Species delimitation and identification in the *Erebia tyndarus* group is difficult. The morphology of male genitalia is not uniform within this group; however, the individual variations are very strong and are often comparable with the interspecific differences (e.g. see: DE LESSE 1960). The differences in wing pattern are very subtle or nearly lacking between many species (WARREN 1936, DE LESSE 1960).

In spite of morphological similarity, the taxonomic and identification problems within this group can be solved if chromosomal (LORKOVIĆ 1949; DE LESSE 1960; ROBINSON 1971; LUKHTANOV 1987) or molecular markers (MARTIN *et al.* 2002; ALBRE *et al.* 2008; LUKHTANOV *et al.* 2009; PEÑA *et al.* 2015; GRATTON *et al.* 2016; SCHMITT *et al.* 2016; NAKATANI *et al.* 2018) are applied.

An unusual diversity of karyotypes is the most remarkable characteristic of this group. Haploid chromosome numbers (n) range from n=8 in *E. calcarius* (LORKOVIĆ 1949; ROBINSON 1971) to n=51-52 in *E. iranica* (DE LESSE 1960; LORKOVIĆ 1972; LUKHTANOV 1987). These differences provide reliable characters for species delimitation, description, and identification (DE LESSE 1960). However, the parapatric *E. tyndarus* and *E. cassioides* (n=10), and the sympatric taxa of the *E. iranica* species complex (n=51-52) share the same chromosome numbers (ROBINSON 1971) and can be identified on the basis of molecular markers (ALBRE *et al.* 2008; LUKHTANOV *et al.* 2009; GRATTON *et al.* 2016) and subtle differences in morphology (WARREN 1936). The species status of *E. tyndarus* and *E. cassioides* (n=10) was also supported by hybridization experiments (LORKOVIĆ 1958).

Although this group has attracted the attention of numerous researchers (WARREN 1936; LORKOVIĆ 1949; 1958, 1972; DE LESSE 1960; LUKHTANOV 1987; MARTIN *et al.* 2002; CHURKIN 2003; ALBRE *et al.* 2008; BOGDANOV 2008; DESCIMON & MALLETT 2009; YAKOVLEV 2012; RUBIN & YAKOVLEV 2013; PEÑA *et al.* 2015; GRATTON *et al.* 2016; SCHMITT *et al.* 2016; LUCEK 2018; NAKATANI *et al.* 2018), a large number of unresolved taxonomic problems still persist in this group, especially among the taxa distributed in Turkey, Iran, Caucasus, and Siberia.

Here we provide an analysis of this group based on new *COI* barcodes, morphology, and geographic distribution.

## Material and Methods

38 specimens (*E. iranica iranica*, *E. iranica transcaucasica*, and *E. sheljuzhkoi*) were processed at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) using standard high-throughput protocol described in DEWAARD *et al.* (2008) and resulted in a 658 bp fragment of *COI*. The BOLD accession numbers of these specimens and their geographic data are presented in Fig. 1. The sequences, pictures, and collection data of these specimens are deposited and can be freely downloaded at the BOLD Public Data Portal (<http://www.boldsystems.org/index.php/databases>). *Erebia iranica transcaucasica* was found to be represented by two haplotypes in Turkey (Ispir): *h1* (27 specimens, ##LOWAB234-09, LOWAB243-09 to LOWAB254-09, LOWAB256-09 to LOWAB260-09, LOWAB262-09 to LOWAB271-09 and LOWAB293-09) and *h2* (2 specimens, ## LOWAB246-09 and LOWAB269-09). The set of voucher specimens of the butterflies is kept at the Zoological Institute of the Russian Academy of Science (St. Petersburg) and at the McGuire Center for Lepidoptera and Biodiversity (University of Florida).

Further 78 specimens available from the GenBank database (VILA & BJORKLUND 2004; LUKHTANOV *et al.* 2009; DINČA *et al.* 2011, 2015; HAUSMANN *et al.* 2011; PEÑA *et al.* 2015; MUTANEN *et al.* 2016; LITMAN *et al.* 2018; NAKATANI *et al.* 2018) and three specimens available from the Public Data Portal of BOLD ([http://www.boldsystems.org/index.php/Public\\_SearchTerms](http://www.boldsystems.org/index.php/Public_SearchTerms)) (HUEMER & WIESMAIR 2017) were also included in our analysis.

We excluded the GenBank samples LC340508 (Russia, North Caucasus, Dombai) and LC340477 (Armenia, Aragats) from the analysis because of their extremely strong deviation in the nucleotide composition not supported by other sequences from the same localities.

The sequences FJ663620 (*Hyponephele dysdora*) and FJ66326 (*Boeberia parmenio*) (LUKHTANOV *et al.* 2009) were used to root the phylogeny.

Sequences were aligned using BioEdit software (HALL 1999) and edited manually. Phylogenetic hypotheses were inferred using Bayesian inference as described previously (SAHOO *et al.* 2016; LUKHTANOV 2017; LUKHTANOV & DANTCHENKO 2017). The Bayesian analysis was performed using the program MrBayes 3.2 (RONQUIST *et al.* 2012) with default settings as suggested by Mesquite (MADDISON & MADDISON 2015): burnin=0.25, nst=6 (GTR + I + G). Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed. The consensus of the obtained

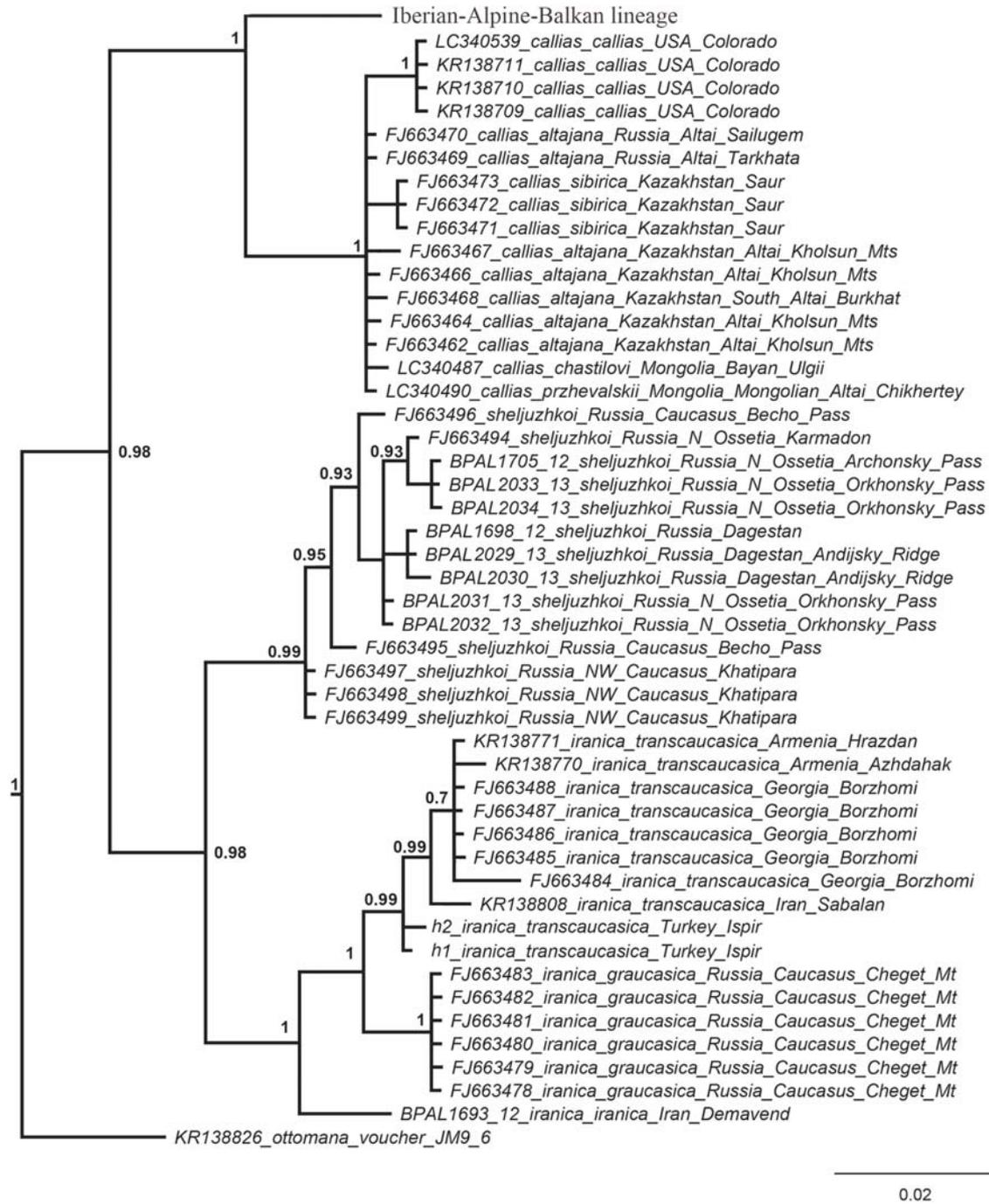


Fig. 1. Bayesian tree of the *E. tyndarus* group based on *COI* barcodes. The sequences FJ663620 (*Hyponephele dysdora*) and FJ66326 (*Boeberia parmenio*) (LUKHTANOV *et al.* 2009) were used to root the phylogeny (not shown). Numbers at nodes indicate Bayesian posterior probability.

trees (Figs 1 and 2) was visualized using FigTree 1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

DNA barcode gaps (=minimum *COI* uncorrected p-distances) between the taxa (Table 1) were calculated manually. The data on geographic distribution and cohabitation (=sympatry) were ex-

tracted from available literature (WARREN 1936; DE LESSE 1960; LUKHTANOV 1987; MARTIN *et al.* 2002; ALBRE *et al.* 2008; BOGDANOV 2008; YAKOVLEV 2012; RUBIN & YAKOVLEV 2013; GRATTON *et al.* 2016; SCHMITT *et al.* 2016).

Table 1

The type of distribution (S is sympatry; A is allopatry) and barcoding gap between the taxa of the *E. iranica* and *E. callias* species complexes. *ira* is *E. iranica iranica*, *gra* is *E. iranica graucasica*, *tra* is *E. iranica transcaucasica*, *she* is *E. sheljuzhkoi*, *cal* is *E. callias callias*, *alt* is *E. callias altajana*, *cha* is *E. callias chastilovi*, *prz* is *E. callias przhivalskii*, *sib* is *E. callias sibirica*

Taxon	<i>ira</i>	<i>gra</i>	<i>tra</i>	<i>she</i>	<i>cal</i>	<i>alt</i>	<i>cha</i>	<i>prz</i>	<i>sib</i>
<i>ira</i>	–	A 2.2%	A 2.0%	A 2.8%	A 4.9%	A 4.8%	A 4.8%	A 5.0%	A 5.9%
<i>gra</i>		–	A 1.6%	S 3.2%	A 5.1%	A 5.0%	A 4.9%	A 4.9	A 5.5 %
<i>tra</i>			–	A 3.1%	A 5.0%	A 4.9%	A 4.9%	A 5.0%	A 5.5%
<i>she</i>				–	A 5.0%	A 5.0%	A 4.9%	A 4.9%	A 5.6%
<i>cal</i>					–	A 0.5%	A 0.7%	A 0.5%	A 0.8%
<i>alt</i>						–	A 0.2%	A 0%	A 0.3%
<i>cha</i>							–	A 0.2%	A 0.5%
<i>prz</i>								–	A 0.3%
<i>sib</i>									–

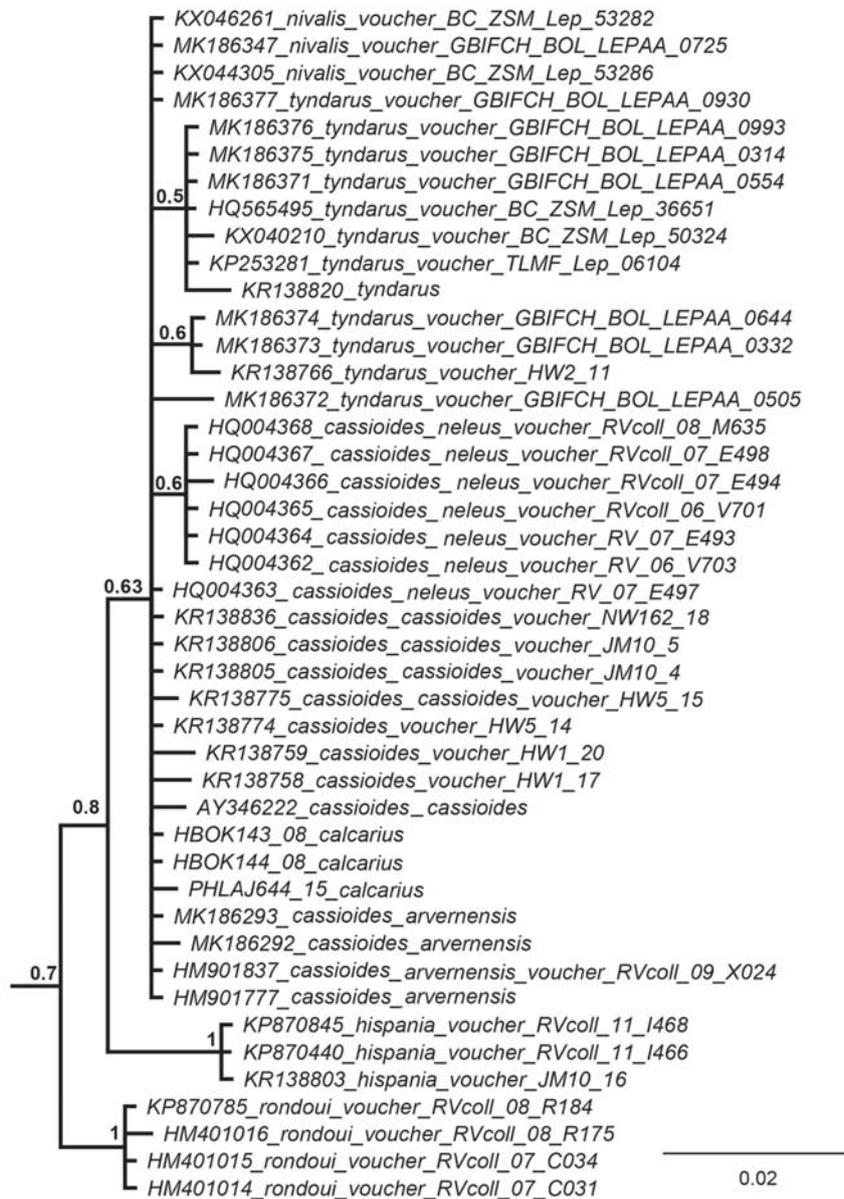


Fig. 2. Bayesian tree of the *E. tyndarus* group based on COI barcodes (fragment showing the taxa of the Iberian-Alpine-Balkan lineage). Numbers at nodes indicate Bayesian posterior probability.

## Results and Discussion

Despite numerous attempts to reveal the phylogenetic position and taxonomic status of formally described entities, a large number of unresolved taxonomic problems still persist in the *E. tyndarus* group, mostly because of incomplete species and population sampling, especially from the eastern part of the group's distribution (MARTIN *et al.* 2002; PEÑA *et al.* 2015; GRATTON *et al.* 2016; SCHMITT *et al.* 2016). Here we provide a *COI*-barcode analysis based on essentially improved sampling of the taxa and populations from the Caucasus, including, for the first time, data on one of the key taxa in this complex, *Erebia iranica*, described from the Demavend volcano in Iran.

The analysis revealed *E. ottomana* as the most basal lineage (clade) within the group (Fig. 1). Then, the Caucasian (II) and the European-Siberian-American (III) clades were revealed to be highly supported monophyletic groups (Fig. 1).

The Caucasian clade has been shown to include two lineages: the lineage of *E. sheljuzhkoii* distributed in the Great Caucasus only, and the lineage of *E. iranica* sensu lato distributed in the Great and Lesser Caucasus as well as in Turkey and North Iran. Thus, these two lineages are sympatric in the Great Caucasus and separated by a significant barcode gap (Table 1) which correlates with a gap in morphology (Fig. 3) and, therefore, represent two different biological species.

Our analysis does not confirm the close relatedness and conspecificity of the taxa known in current literature as “*E. iranica iranica*” (North Iran) and “*E. iranica sheljuzhkoii*” (ALBRE *et al.* 2008, LUKHTANOV *et al.* 2009). Instead, it demonstrates that these taxa are quite distant with respect to their DNA barcodes. Their conspecificity has been claimed on the basis of identity in chromosome number ( $n=51-52$ ) (LORKOVIĆ 1972; LUKHTANOV 1987). However, molecular studies demonstrate that this character ( $n=51-52$ ) has a plesiomorphic nature, and, thus, is not evidence for creating any taxonomic or phylogenetic conclusions.

Differentiation in the valve shape between allopatric forms of the *E. iranica* complex (Figs 3 and 4) is correlated with a relatively low (1.6-2.2%) barcoding gap between them (Table 1). In accordance with the criteria formulated in our work (allopatry + barcoding gap less 3% + no evidence for reproductive isolation, LUKHTANOV *et al.* 2016) they should be classified as a subspecies, not as a different species.

In general, the analyzed data indicates the need for taxonomic reorganization of the *E. iranica* complex and its division into two species: monotypic *E. sheljuzhkoii* (distributed in the Great Caucasus) and polytypic *E. iranica* with subspecies *E. iranica iranica* (North Iran, Demavend Mt), *E. iranica dromulus* (Turkey, Ararat Mt), *E. iranica transcaucasica* (Lesser Caucasus), and *E. iranica graucasica* (Great Caucasus).

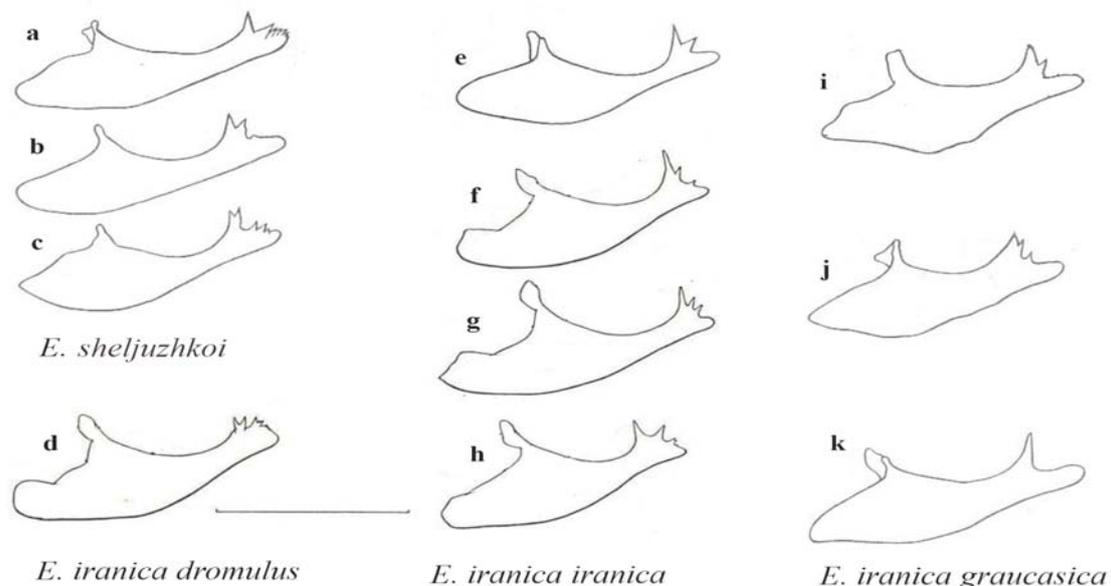


Fig. 3. Valve shape in the male genitalia of *E. sheljuzhkoii*, *E. iranica dromulus*, *E. iranica iranica*, and *E. iranica graucasica* (lateral view). Scale bar corresponds to 1 mm. a-c – Russia, Great Caucasus, Teberda, Khatipara; d – Turkey, Ararat, lectotype of *E. iranica dromulus* (coll. Staudinger, Humboldt Universität, Berlin); e – Persia, Demavend, 26 June 1894, syntype of *E. iranica* (coll. Zoological Institute RAS, St. Petersburg); f-g – Iran, Demavend; h – Iran, Demavend, syntype of *E. iranica* (Natural History Museum, London); i-k – Russia, Great Caucasus, Bukabashi Mt. a, b, c, e, i, j, k are based on figures from LUKHTANOV (1987); d, f, g, h are based on figures from DE LESSE (1960).

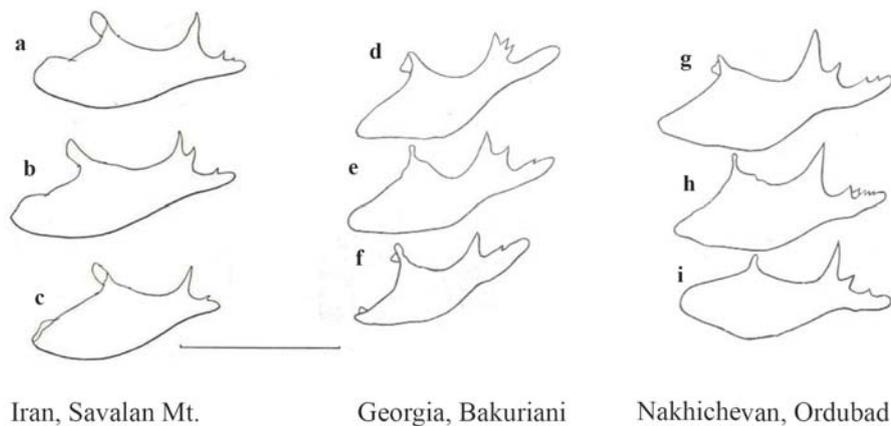


Fig. 4. Valve shape in the male genitalia of *E. iranica graucasica* (lateral view). Scale bar corresponds to 1 mm. a, b, c are based on figures from DE LESSE (1960); d, e, f, g, h, i are based on figures from LUKHTANOV (1987). a – holotype of *Erebia iranica savalanica* de Lesse, 1956 (Muséum national d'histoire naturelle, Paris).

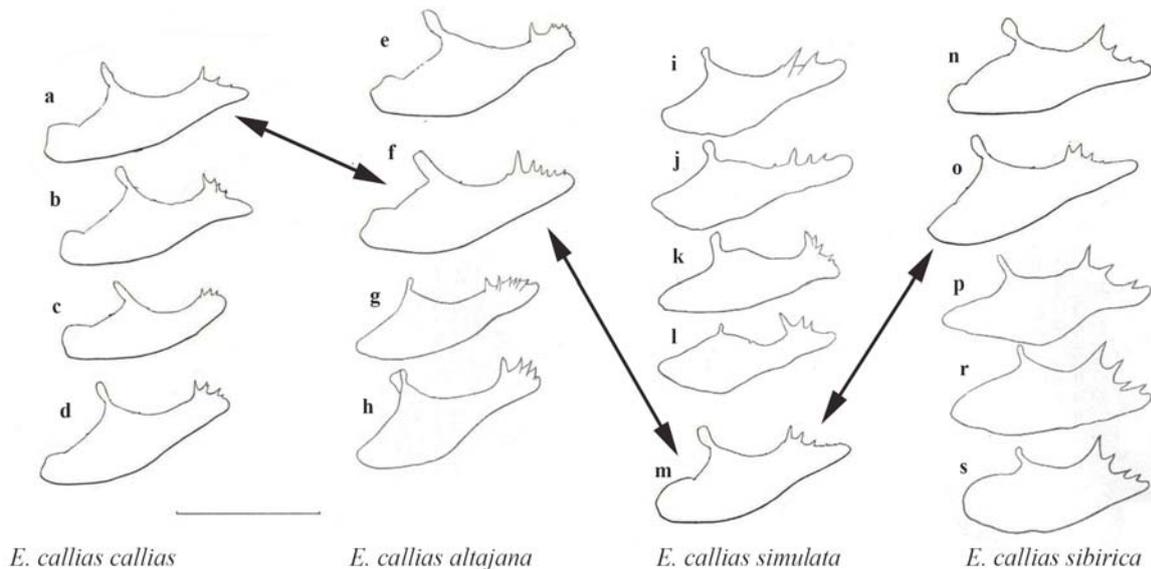


Fig. 5. Valve shape in the male genitalia of *E. callias* (lateral view). Although the structure of the valve is not identical in the four different subspecies (and is especially specialized in *E. callias sibirica*), there are clear transition forms between the subspecies (shown by arrows). Scale bar corresponds to 1 mm. a–d – USA, Colorado; e – Russia, Altai, Bashkaus, lectotype of *E. callias altajana* (Natural History Museum, London); f – Russia, Altai, Tchuja; g, h – Russia/Kazakhstan border, Altai, Kholsun Mts; i–k – Russia, Siberia, East Sajon; l – Mongolia, Urga; m – Russia, “Irkutsk”; n – Kazakhstan, Tarbagatai, lectotype of *E. callias sibirica* (coll. Staudinger, Humboldt Universität, Berlin); o – Kazakhstan, Tarbagatai; p–s – Kazakhstan, Saur. a, b, c, d, e, f, m, n, o are based on figures from DE LESSE (1960); g, h, i, j, k, l, p, r, s are based on figures from LUKHTANOV (1987).

The European-Siberian-American clade has been shown to include two assemblages: the lineage of *E. callias* distributed in Siberia and North America (Colorado) (Fig. 1) and the lineage represented by the taxa distributed in the mountain systems of Southern Europe (the Iberian-Alpine-Balkan lineage) (Figs 1 and 2).

The Siberian-American lineage includes the single species *E. callias*. It has been split recently into

several allopatric species such as *E. callias sensu stricto*, *E. sibirica* Staudinger, 1881, *E. chastilovi* Churkin, 2003, and *E. przhivalskii* Yakovlev, 2012 on the basis of differences in male genitalia (RUBIN & YAKOVLEV 2013). Indeed, the structure of male genitalia is not identical in different populations within this lineage (Fig. 5). The population from the Saur-Tarbagatai mountain system (*E. callias sibirica*) is especially different, in that it

has a wider shape of valve in male genitalia. Similarly, the wide valve is found in some populations in Mongolia (*E. callias przhivalskii*). The almost complete absence of differentiation in DNA barcodes (Table 1), along with the presence of transitional forms in male genitalia (Fig. 5), seems to support the subspecies rather than species status of the studied taxa. However, the number of genital preparations and DNA barcodes studied is insufficient to draw definitive taxonomic conclusions in this case. Further research based on more intensive sampling of specimens and multiple molecular markers may shed light on the status and phylogenetic relationships of the Siberian and Mongolian taxa.

The Iberian-Alpine-Balkan lineage consists of the populations that are weakly differentiated with respect to DNA barcodes (Fig. 2), except for the clearly distinct *E. hispania* and *E. rondoui*. However, chromosomal analysis, hybridization experiments, and thorough analysis of the distribution in zones of sympatry and parapatry (LORKOVIĆ 1958) demonstrated that the rest of the populations represented four different biological species, *E. tyndarus*, *E. cassioides*, *E. nivalis*, and *E. calcarius*. Based on differences in allozymes, SCHMITT *et al.* (2016), split *E. cassioides* into three allopatric species; *E. cassioides sensu stricto*, *E. neleus*, and *E. arvernensis*. Although there is a certain logic to this action (SCHMITT *et al.* 2016) based on the use of the phylogenetic species concept, it contradicts the logic of LORKOVIĆ (1958) and DE LESSE (1960), who divided the complex into species based on the data of reproductive isolation, i.e. based on the biological species concept.

Thus, acceptance of the changes proposed by SCHMITT *et al.* (2016) makes the system of the *Erebia tyndarus* group eclectic, partly based on the phylogenetic species concept and partly based on the biological species concept. In this situation, adhering to the biological species concept, we consider it more reasonable to use the traditional system (LORKOVIĆ 1958) and do not support the splitting of *E. cassioides* into three species.

### Taxonomic conclusion

We propose the following taxonomic arrangement of the *E. tyndarus* group (haploid chromosome numbers are in parentheses). Recently, a population of the *E. tyndarus* group was discovered in the Polar Urals (Russia) and was described as a new species, *E. churkini* Bogdanov, 2008. However, it has not been studied so far in respect to molecular markers and, therefore, has not been included in the species list below.

#### *Erebia ottomana* clade

1. *Erebia ottomana* Herrich-Schäffer, 1847 (n=40)

#### Caucasian clade

##### *Erebia sheljuzhkoii* lineage

2. *Erebia sheljuzhkoii* Warren, 1935 (n=ca51-52)

##### *Erebia iranica* lineage

3. *Erebia iranica* Grum-Grshimailo, 1895
  - 3a. *Erebia iranica iranica* Grum-Grshimailo, 1895 (n=51)
  - 3b. *Erebia iranica dromulus* Staudinger, 1901 (n unknown)
  - 3c. *Erebia iranica transcaucasica* (= *savalanica* de Lesse, 1956) (n=51)
  - 3d. *Erebia iranica graucasica* Jachontov, 1909 (n=ca51-52)

#### Euro-Siberian-American clade

##### Siberian-American lineage

4. *Erebia callias* Edwards, 1871
  - 4a. *Erebia callias callias* Edwards, 1871 (n=15)
  - 4b. *Erebia callias tsherskiensis* Dubatolov, 1992 (n unknown)
  - 4c. *Erebia callias altajana* Staudinger, 1901 (n=16)
  - 4d. *Erebia callias simulata* Warren, 1933 (n unknown)
  - 4e. *Erebia callias chastilovi* Churkin, 2003 (n unknown)
  - 4f. *Erebia callias przhivalskii* Yakovlev, 2012 (n unknown)
  - 4g. *Erebia callias sibirica* Staudinger, 1881 (n unknown)

##### *Erebia rondoui* lineage

5. *Erebia rondoui* Oberthür, 1908 (n=24)

##### *Erebia hispania* lineage

6. *Erebia hispania* Butler, 1868 (n=25)

##### Pyrenean-Alps-Balkan lineage

7. *Erebia tyndarus* (Esper, 1781) (n=10)
8. *Erebia nivalis* Lorković & de Lesse (n=11)
9. *Erebia calcarius* Lorković, 1953 (n=8)
10. *Erebia cassioides* (Hohenwarth, 1792) (n=10)
  - 10a. *Erebia cassioides cassioides* (Hohenwarth, 1792) (n=10)
  - 10b. *Erebia cassioides neleus* (Freyer, 1832)
  - 10c. *Erebia cassioides arvernensis* Oberthür, 1908 (n=10)

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## Author Contributions

Research concept and design: V.L.; Collection and/or assembly of data: V.L., A.S., V.T., E.Z.; Data analysis and interpretation: V.L., E.Z.; Writing the article: V.L.

## Conflict of Interest

The authors declare no conflict of interest.

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