DNA barcoding reveals twelve lineages with properties of phylogenetic and biological species within *Melitaea didyma* sensu lato (Lepidoptera, Nymphalidae)

Elena A. Pazhenkova¹,², Evgeny V. Zakharov³, Vladimir A. Lukhtanov¹,²,⁴

¹ Department of Karyosystematics, Zoological Institute of the Russian Academy of Sciences, Universitetskaya emb. 1, 199034 St. Petersburg, Russia ² Department of Entomology, Faculty of Biology, St. Petersburg State University, Universitetskaya emb. 7/9, 199034 St. Petersburg, Russia ³ Biodiversity Institute of Ontario, University of Guelph, Guelph, ON, Canada N1G 2W1 ⁴ McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History, McGuire Hall, 3215 Hull Road, PO Box 112710, University of Florida, Gainesville FL 32611-2710, USA

Corresponding authors: Elena A. Pazhenkova (pazhenkova.e@gmail.com); Vladimir A. Lukhtanov (lukhtanov@mail.ru)

Academic editor: S. Grozeva | Received 16 September 2015 | Accepted 27 October 2015 | Published 19 November 2015

Citation: Pazhenkova EA, Zakharov EV, Lukhtanov VA (2015) DNA barcoding reveals twelve lineages with properties of phylogenetic and biological species within *Melitaea didyma* sensu lato (Lepidoptera, Nymphalidae). In: Lukhtanov VA, Kuznetsova VG, Grozeva S, Golub NV (Eds) Genetic and cytogenetic structure of biological diversity in insects. ZooKeys 538: 35–46. doi: 10.3897/zookeys.538.6605

Abstract

The complex of butterfly taxa close to *Melitaea didyma* includes the traditionally recognized species *M. didyma*, *M. didymoides* and *M. sutschana*, the taxa that were recognized as species only relatively recently (*M. latonigena, M. interrupta, M. chitralensis* and *M. mixta*) as well as numerous described subspecies and forms with unclear taxonomic status. Here analysis of mitochondrial DNA barcodes is used to demonstrate that this complex is monophyletic group consisting of at least 12 major haplogroups strongly differentiated with respect to the gene COI. Six of these haplogroups are shown to correspond to six of the above-mentioned species (*M. didymoides*, *M. sutschana*, *M. latonigena, M. interrupta, M. chitralensis* and *M. mixta*). It is hypothesized that each of the remaining six haplogroups also represents a distinct species (*M. mauretanica, M. occidentalis, M. didyma, M. neera, M. liliputana* and *M. turkestanica*), since merging these haplogroups would result in a polyphyletic assemblage and the genetic distances between them are comparable with those found between the other six previously recognized species.

Keywords

Biodiversity, butterflies, COI, cryptic species, mitochondrial DNA, Nymphalidae, phylogeography, taxonomy

Copyright Elena A. Pazhenkova et al. This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Introduction

The complex of butterfly taxa close to *Melitaea didyma* (Esper, 1779) is widely distributed in the Palaearctic region. This complex includes the traditionally recognized species *M. didyma*, *M. didymoides* Eversmann, 1847 and *M. sutschana* Staudinger, 1892, the taxa that were recognized as species only recently (*M. latonigena* Eversmann, 1847, *M. interrupta* Colenati, 1846, *M. chitralensis* Moore, 1901 and *M. mixta* Evans, 1912) as well as numerous described subspecies and forms with unclear taxonomic status (Higgins 1941, 1955, Hesselbarth et al. 1995, Kolesnichenko 1999, Kolesnichenko et al. 2011). All these taxa are similar in male and female wing pattern and genitalia structure (Higgins 1941). In our opinion, this complex does not include the species *M. deserticola* Oberthür, 1909, *M. ala* Staudinger, 1881, *M. enarea* Frühstorfer, 1917 and *M. persea* Kollar, 1849 which are similar to *M. didyma* in wing color and pattern but were shown to be distinctly different with respect to genitalia structure (Higgins 1941). The first significant review of this complex was published by Higgins (1941, 1955) in frame of a complete revision of the genus *Melitaea*. Recently the genus *Melitaea* was revised by Oorschot and Coutsis (2014). The taxa within the *M. didyma* complex have a strong morphological variation between individuals of different generations and indistinct clinal variability in wing size and color from north to south (Lvovsky and Morgun 2007). Available cytogenetic (Lukhtanov and Kuznetsova 1989), morphological (Lvovsky and Morgun 2007, Kolesnichenko et al. 2011, Oorschot and Coutsis 2014) and molecular (Wahlberg and Zimmermann 2000, Lukhtanov et al. 2009, Dincă et al. 2015) data show that the *M. didyma* species complex requires a more detailed taxonomic revision.

Here analysis of mitochondrial DNA barcodes is used to demonstrate that this complex is a natural (monophyletic) group consisting of at least 12 major haplogroups strongly differentiated with respect to the gene *COI*. Then the taxonomy of the *M. didyma* species complex is discussed.

Material and methods

Standard *COI* barcodes (658-bp 5’ segment of mitochondrial *cytochrome oxidase subunit I*) were studied. *COI* sequences were obtained from 85 specimens collected in Afghanistan, Armenia, Austria, Bulgaria, China, Israel, Kazakhstan, Kyrgyzstan, Mongolia, Morocco, Russia, Syria, Tajikistan, Turkey and Uzbekistan. Collection data of these samples are presented in the Suppl. material 1.

Legs from 24 specimens (KT792884–KT792908, see the Suppl. material 2) were processed at the Department of Karyosystematics of the Zoological Institute of the Russian Academy of Sciences. The set of voucher specimens of these butterflies is kept in the Zoological Institute of the Russian Academy of Science (St. Petersburg). DNA was extracted from a single leg removed from each voucher specimen. For DNA extraction we used the GeneJet Genomic DNA Purification Kit (Fermentas)
DNA barcoding reveals twelve lineages with properties of phylogenetic...

in accordance with the manufacturer’s instructions. Extracted DNA samples were stored at -20 °C.

For DNA amplification we used primers LepF 5’- ATTCAACCAATCATAAA-GATATTGG-3’ and LepR (5’-TAAACTTCTGGATGTCCAAAAATCA-3’ (de-Waard et al. 2008). The polymerase chain reaction (PCR) was carried out in 25-mL reactions using a DNA Engine thermal cycler (Eppendorf Mastercycler personal), and typically contained 0.5 mM of each primer, 0.8 mM dNTPs, Fermentas PCR buffer with additional MgCl2 to a final concentration of 2 mM and 1.25 units Fermentas Taq DNA polymerase. All reactions were initially denatured at 94 °C for 2 min, and then subjected to 30 cycles of 60 s at 94 °C denaturation, 60 s at 47 °C and 90 s at 72 °C extension. After amplification, double-stranded DNA was purified using GeneJet PCR Purification Kit (Fermentas). Sequencing of double-stranded product was carried out at the Research Resource Center for Molecular and Cell Technologies.

Legs from 61 specimens of *Melitaea* (HM404715–HM404718, KT874693–KT874751, see the Suppl. material 2) 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). The set of voucher specimens of these butterflies is kept at the McGuire Center for Lepidoptera and Biodiversity (University of Florida), at the Zoological Institute of the Russian Academy of Science (St. Petersburg) and in Museum for Insects, Pyatigorsk, Russia (Suppl. material 1).

The analysis involved 148 COI sequences (including outgroup). Among them there were 63 published sequences (Wahlberg and Zimmermann 2000, Vila and Bjorklund 2004, Leneveu et al. 2009, Lukhtanov et al. 2009, Dincă et al. 2011, 2015, Hausmann et al. 2011, Ashfaq et al. 2013) collected from GenBank (Suppl. material 2). Sequences were aligned using BioEdit software (Hall 1999) and edited manually. Phylogenetic hypotheses were inferred using Bayesian inference (BI), maximum-likelihood (ML) and maximum-parsimony (MP) analyses as described previously (Vershinina and Lukhtanov 2010, Talavera et al. 2013a). Briefly, Bayesian analyses were performed using the program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) with default settings as suggested by Mesquite (Maddison and Maddison 2015): burn-in=0.25, nst=6 (GTR + I + G). Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed. Chains were sampled every 10,000 generations. The average value of the Potential Scale Reduction Factor (PSRF) was 1.002 and average standard deviation of split frequencies was 0.01492, to the end of the analysis indicating that convergence was achieved, and a good sample from the posterior probability distribution was obtained.

The ML trees were inferred by using MEGA6 (Tamura et al. 2013) with the nucleotide substitution model T92 (Tamura 1992) as suggested by jModelTest (Posada 2008).

MP analysis was performed using a heuristic search as implemented in MEGA6 (Tamura et al. 2013). A heuristic search was carried out using the close-neighbour-interchange algorithm with search level 3 (Nei and Kumar 2000) in which the initial trees were obtained with the random addition of sequences (100 replicates). We used
non-parametric bootstrap values (Felsenstein 1985) to estimate branch support on the reconstructed ML and MP tree. Branch support was assessed using 1000 bootstrap replicates.

**Results and discussion**

This analysis recovered the *M. didyma* group as a strongly supported monophyletic clade (Fig. 1). Within this group many clades were well supported, whereas some of the relationships were not fully resolved (Figs 2 and 3). Within the complex we identified 12 differentiated major COI haplogroups. All of them showed a strict attachment to the localities (Fig. 4). Therefore in order to designate these haplogroups, we chose the oldest available name that was described from the area of each haplogroup: *M. mauretanica* Oberthür, 1909, *M. occidentalis* Staudinger, 1861, *M. didyma* Esper, 1779, *M. neera* Fischer de Waldheim, 1840, *M. interrupta* Colenati, 1846, *M. liliputana* Oberthür,

![Figure 1](image)

*Figure 1.* The Bayesian tree of *Melitaea* based on analysis of the cytochrome oxidase subunit I (COI) gene. Numbers at nodes indicate Bayesian posterior probability/ML bootstrap/MP bootstrap values. Scale bar = 0.1 substitutions per position.
DNA barcoding reveals twelve lineages with properties of phylogenetic...

**Figure 2.** Fragment of the Bayesian tree of *Melitaea didyma* complex (haplogroups *neera*, *liliputana*, *occidentalis*, *interrupta*, *latonigena*, *sutscha* and *didymoides*) based on analysis of the cytochrome oxidase subunit I (COI) gene. Numbers at nodes indicate Bayesian posterior probability/ML bootstrap/MP bootstrap values, with nonmatching clades using different analyses indicated by ‘-’. Scale bar = 0.1 substitutions per position.

1909, *M. turkestanica* Sheljuzhko, 1929, *M. mixta* Evans, 1912, *M. chitralensis* Moore, 1901, *M. latonigena* Eversmann, 1847, *M. didymoides* Eversmann, 1847 and *M. sutscha* Staudinger, 1892 (Figs 2 and 3). The name *M. liliputana* was selected for the Middle...
Figure 3. Fragment of the Bayesian tree of *Melitaea didyma* complex (haplogroups *turkestanica*, *mixta*, *chitralensis*, *mauretanica* and *didyma*) based on analysis of the cytochrome oxidase subunit I (*COI*) gene. Numbers at nodes indicate Bayesian posterior probability/ML bootstrap/MP bootstrap values, with non-matching clades using different analyses indicated by ‘-’. Scale bar = 0.1 substitutions per position.

East populations of the *M. didyma* complex. These populations have been known under the name *libanotica* Belter, 1934 in the literature (Larsen 1974, Benyamini 2002, Tshikolovets 2011). However, the name *liliputana* was preferred since ICZN states priority of the oldest available name (article 23, Principle of Priority).

The discovered haplogroups correspond to two traditionally recognized species (*M. didymoides* and *M. sutschana*) (Higgins 1941), to four taxa that were recognized as species relatively recently (*M. latonigena*, *M. interrupta*, *M. chitralensis* and *M. mixta*) (Lukhtanov and Kuznetsova 1989, Hesselbarth et al. 1995, Kolesnichenko 1999, Kolesnichenko 2002, Kolesnichenko and Kuznetsova 2003).
DNA barcoding reveals twelve lineages with properties of phylogenetic...

Figure 4. Distribution ranges of haplogroups *didyma* (■), *didymoides* (Δ), *interrupta* (●), *latonigena* (○), *liliputana* (◊), *mauretanica* (⃰), *mixta* (▼), *neera* (▲), *occidentalis* (#), *sutschana* (●), *turkestanica* (□) and *chitralensis* (►).

Table 1. Minimal uncorrected COI p-distances between 12 major haplogroups of the *M. didyma* species complex (%).

|          | 1. | 2. | 3. | 4. | 5. | 6. | 7. | 8. | 9. | 10. | 11. |
|----------|----|----|----|----|----|----|----|----|----|-----|-----|
| 1. *neera* |    |    |    |    |    |    |    |    |    |     |     |
| 2. *liliputana* | 1.3 |    |    |    |    |    |    |    |    |     |     |
| 3. *occidentalis* | 2.7 | 3.9 |    |    |    |    |    |    |    |     |     |
| 4. *interrupta* | 1.8 | 3.0 | 1.9 |    |    |    |    |    |    |     |     |
| 5. *latonigena* | 1.9 | 3.2 | 3.6 | 3.26 |    |    |    |    |    |     |     |
| 6. *sutschana* | 2.2 | 3.6 | 3. | 3.28 | 1.89 |    |    |    |    |     |     |
| 7. *didymoides* | 3.8 | 4.8 | 4.4 | 3 | 3.6 | 3.29 |    |    |    |     |     |
| 8. *turkestanica* | 1.6 | 2.7 | 2.4 | 2.43 | 2.16 | 2.73 | 3.89 |    |    |     |     |
| 9. *mixta* | 2.7 | 3.6 | 3 | 3.2 | 3.86 | 3.87 | 4.77 | 1.89 |    |     |     |
| 10. *chitralensis* | 4.3 | 4.7 | 4.6 | 4.1 | 4.3 | 4.3 | 5.2 | 3.2 | 2.4 |     |     |
| 11. *mauretanica* | 1.6 | 2.9 | 2.16 | 1.9 | 2.16 | 3 | 3.88 | 1.6 | 2.18 | 3.8 |     |
| 12. *didyma* | 1.9 | 3 | 2.73 | 2.4 | 2.44 | 3 | 4.48 | 1.6 | 3.3 | 1.61 |     |

Sympatry (or at least parapatry) (shown by green color) was demonstrated for the following taxa pairs: *mixta* and *turkestanica* (Kolesnichenko et al. 2011), *mixta* and *chitralensis* (Higgins 1941), *didymoides* and *sutschana* (Gorbunov 2001), *didymoides* and *latonigena* (Gorbunov 2001), *sutschana* and *latonigena* (Gorbunov 2001), *latonigena* and *neera* (Lukhtanov et al. 2007), *interrupta* and *neera* (parapatry in the North Caucasus, Tuzov and Churkin 2000) and *interrupta* and *liliputana* (parapatry in Armenia and Turkey, Hesselbarth et al. 1995).

Here we also report an observation of parapatry between *neera* and *turkestanica* in South Altai and Zaisan valley in East Kazakhstan (shown by green color). In this area the distribution ranges of these taxa overlap, however, the taxa are separated ecologically: *M. neera* is associated with the steppe biotopes and *M. turkestanica* is associated with deserts.

Sympatry was also found between haplogroups *occidentalis* and *didyma* sensu stricto in Spain (shown by yellow, Dincă et al. 2015). However, morphology and ecology of the bearers of these haplogroups were not analyzed in the contact zone. Therefore, evolutionary and taxonomic interpretation of this case of sympatry is difficult. It may represent sympatric distribution of two different species or may be a consequence of mitochondrial introgression between the allopatric pair *occidentalis-didyma*.
snichenko et al. 2011), to five recognized subspecies (*M. didyma occidentalis*, *M. didyma didyma*, *M. didyma neera*, *M. didyma liliputana* and *M. didyma turkestanica*) (Higgins 1941, Larsen 1974, Benyamini 2002, Tshikolovets 2011) and to one form (*M. mauritanica*) whose status (subspecies or individual variations) is unclear (Higgins 1941).

There is good evidence based on analysis of morphology and observations of taxa in sympathy that *M. didymoides*, *M. sutschana*, *M. latonigena*, *M. interrupta*, *M. chitratalensis* and *M. mixta* represent true biological species (Higgins 1941, Lukhtanov and Kuznetsova 1989, Hesselbarth et al. 1995, Kolesnichenko 1999, Kolesnichenko et al. 2011). Theoretically, the remainder of the *M. didyma* complex can be interpreted as a single species *M. didyma*. However, such an interpretation meets two difficulties. Firstly, such a lumping would result in a polyphyletic assemblage. Monophyly is the basic principle of phylogenetics and taxonomy. The majority of taxonomists currently believe that monophyly, in the narrow sense used by Hennig (Hennig 1966, Envall 2008, Hörandl and Stuessy 2010) is mandatory. Thus avoiding non-monophyletic groups and focusing on monophyletic entities is the preferable option in practical terms (Talavera et al. 2013b). The COI barcodes alone can provide weak evidence for monophyly of taxa since trees inferred from single markers sometimes display relationships that reflect the evolutionary histories of individual genes rather than the species being studied. Mitochondrial introgression (Zakharov et al. 2009) and *Wolbachia* infection (Ritter et al. 2013) can lead to additional bias in inferring phylogenetic relationships. Despite these limitations, we argue that, until not falsified, clusters based on DNA barcode monophyly represent preferable primary taxonomic hypotheses than the clusters based on para- or polyphyletic DNA barcode assemblages.

Secondly, the uncorrected p-distances between these taxa are high (from 1.3% between *neera* and *liliputana* to 3.9% between *liliputana* and *occidentalis*). Although some of them are lower than the ‘standard’ 2.7–3.0% DNA-barcoding threshold usually used for allopatric taxa as an indicator for their species distinctness (Lambert et al. 2005, Lukhtanov et al. 2015), even the lowest distances are comparable with those found between other six well recognized species. For example, distances between *interrupta*, *latonigena* and *mixta* and their sympatric/parapatric non-conspecifics are 1.6–1.9% (Table 1).

Finally, five of the six remaining haplogroups (*occidentalis*, *didyma* sensu stricto, *neera*, *liliputana* and *turkestanica*) are morphologically distinct and have been considered as separate taxonomic entities (subspecies) (Higgins 1941, Larsen 1974, Benyamini 2002, Tshikolovets 2011). Their monophyly with respect to the COI gene reinforces the conclusion that they represent independent lineages of evolution.

Therefore, we hypothesize that the *M. didyma* complex is represented by the following 12 species that can be recognized by a phylogenetic species concept (Cracraft 1989, Coyne and Orr 2004) (taxa 1–5) and by both phylogenetic and biological species concepts (taxa 6–12):

1) *M. liliputana* Oberthür, 1909 (Armenia, Turkey, Syria, Israel)
2) *M. occidentalis* Staudinger, 1961 (Spain)
3) *M. didyma* Esper, 1779 (west Europe)

4) *M. neera* Fischer de Waldheim, 1840 (east Europe, north Caucasus, west Siberia, north Kazakhstan)

5) *M. mauretanica* Oberthür, 1909 (north Africa, south Spain)

6) *M. interrupta* Colenati, 1846 (Caucasus, Turkey, Iran)

7) *M. turkestanica* Sheljuzhko, 1929 (Kazakhstan, Kyrgyzstan, Uzbekistan, Tajikistan, west China)

8) *M. mixta* Evans, 1912 (Tajikistan, Pakistan, Afghanistan)

9) *M. chitralensis* Moore, 1901 (north Pakistan)

10) *M. latonigena* Eversmann, 1847 (Asian Russia, north-east Kazakhstan, Mongolia, north-west China)

11) *M. didymoides* Eversmann, 1847 (Asian Russia, Mongolia, North China)

12) *M. sutschana* Staudinger, 1892 (Far East Russia, Korea, North-East China)

**Acknowledgements**

The financial support for this study was provided by the grant N 14-14-00541 from the Russian Science Foundation to the Zoological Institute of the Russian Academy of Sciences. We thank Andrei Sourakov and Andrew Warren (University of Florida) for their help in work with Lepidoptera collection in McGuire Center for Lepidoptera and Biodiversity. We are grateful to V.V.Tikhonov for samples from Caucasus. We thank A.V.Novikova, N.A.Shapoval and A.O.Vershinina for help in collecting material in Israel. The work was partially performed using equipment of the ‘Chromas’ Core Facility and Centre for Molecular and Cell Technologies of St. Petersburg State University.

**References**

Ashfaq M, Akhtar S, Khan AM, Adamowicz SJ, Hebert PDN (2013) DNA barcode analysis of butterfly species from Pakistan points towards regional endemism. Molecular Ecology Resources 13: 832–843. doi: 10.1111/1755-0998.12131

Benyamini D (2002) A Field Guide to the Butterflies of Israel. Keter Publishing House, Jerusalem, Israel, 248 pp.

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland, Ma, USA, 545 pp.

Cracraft J (1989) Speciation and its ontology: the empirical consequences of alternative species concepts for understanding patterns and processes of differentiation. In: Otte D, Endler JA (Eds) Speciation and its Consequences. Sinauer Associates, Sunderland, Massachusetts, USA, 28–59.

deWaard JR, Ivanova NV, Hajibabaei M, Hebert PDN (2008) Assembling DNA barcodes: analytical protocols. In: Martin CC (Ed.) Environmental Genomics, Methods in Molecular Biology. Humana Press, Totowa, New Jersey, 410: 275–283. doi: 10.1007/978-1-59745-548-0_15
Dincă V, Montagud S, Talavera G, Hernández-Roldán H, Munguira ML, García-Barros E, Hebert PDN, Vila R (2015) DNA barcode reference library for Iberian butterflies enables a continental-scale preview of potential cryptic diversity. Scientific Reports 5: 12395. doi: 10.1038/srep12395

Envall M (2008) On the difference between mono-, holo-, and paraphyletic groups: a consistent distinction of process and pattern. Biological Journal of the Linnean Society 94: 217–220. doi: 10.1111/j.1095-8312.2008.00984.x

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783–791.

Gorbunov P (2001) The Butterflies of Russia: Classification, Genitalia, Keys for Identification (Lepidoptera: Hesperioidea and Papilionoidea). Thesis, Ekaterinburg, Russia, 320 pp.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95‒98.

Hennig W (1966) Phylogenetic Systematics. University of Illinois Press, Urbana, IL, 263 pp.

Hesselbarth G, Oorchot H, Wagener S (1995) Die Tagfalter der Türkei unter Berücksichtigung der angrenzenden Länder. Selbstverlag Siegbert Wagener, Bocholt, Vol. 1‒3, 1354 pp.

Higgins LG (1941) An illustrated catalogue of the Palearctic Melitaea (Lep. Rhopalocera). Transactions of the Royal Entomological Society of London 91: 175–365. doi: 10.1111/j.1365-2311.1941.tb01045.x

Higgins LG (1955) A descriptive catalogue of the genus Mellicta Billberg (Lepidoptera: Nymphalidae) and its species, with supplementary notes on the genera Melitaea and Euphydryas. Transactions of the Royal Entomological Society of London 106: 1–131. doi: 10.1111/j.1365-2311.1955.tb01262.x

Hörandl E, Stuessy TF (2010) Paraphyletic groups as natural units of biological classification. Taxon 59: 1641–1653.

Huelsenbeck JP, Ronquist F (2001) MrBayes: Bayesian inference of phylogeny. Bioinformatics 17: 754–755. doi: 10.1093/bioinformatics/17.8.754

Kolesnichenko KA (1999) A review of the East Palaearctic taxa of the Melitaea didyma (Esper, [1779])–group. 1. The M. ala Staudinger, 1881 – M. chitralensis Moore, 1901 – complex. Atalanta 30: 87‒117, 388‒391.

Kolesnichenko KA, Churkin SV, Berdiev RK (2011) A review of the East Palaearctic taxa of the Melitaea didyma (Esper, [1779]) – group. Part II. Neue Entomologische Nachrichten 67: 257–278.

Lambert DM, Baker A, Huynen L, Haddrath O, Hebert PDN, Millar CD (2005) Is a large-scale DNA-based inventory of ancient life possible? Journal of Heredity 96(3): 279–284. doi: 10.1093/jhered/esi035

Larsen TB (1974) Butterflies of Lebanon. Classey Publications, Beirut, Lebanon, 256 pp.

Leneveu J, Chichvarkhin A, Wahlberg N (2009) Varying rates of diversification in the genus Melitaea (Lepidoptera: Nymphalidae) during the past 20 million years. Biological Journal of the Linnean Society 97: 346–361. doi: 10.1111/j.1095-8312.2009.01208.x

Lukhtanov VA, Kuznetsova VG (1989) The analysis of the karyotype variability in the butterflies of the Melitaea didyma group along with the evidence of the species distinctness of
**DNA barcoding reveals twelve lineages with properties of phylogenetic...**

*M. latonigena* (Lepidoptera, Nymphalidae). Zoologichesky Zhurnal 68(12): 38–46. [In Russian with English summary]

Lukhtanov VA, Sourakov A, Zakharov EV, Hebert PDN (2009) DNA barcoding Central Asian butterflies: increasing geographical dimension does not significantly reduce the success of species identification. Molecular Ecology Resources 9: 1302–1310. doi: 10.1111/j.1755-0998.2009.02577.x

Lukhtanov VA, Dantchenko AV, Vishnevskaya MS, Saifirdinova AF (2015) Detecting cryptic species in sympathy and allopatry: analysis of hidden diversity in *Polyommatus* (*Agrodiaetus*) butterflies (Lepidoptera: Lycaenidae). Biological Journal of the Linnean Society 116(2): 468–485. doi: 10.1111/bij.12596

Lukhtanov VA, Vishnevskaya MS, Volynkin AV, Yakovlev RV (2007) Butterflies (Lepidoptera, Rhopalocera) of West Altai. Entomologicheskoe Obozrenie 86(2): 347–369. doi: 10.1134/s001387380705003x [In Russian with English summary]

Lvovsky AL, Morgan DV (2007) Butterflies of the Eastern Europe. KMK Scientific Press, Ltd., Moscow, Russia, 443 pp. [In Russian]

Maddison WP, Maddison DR (2015) Mesquite: a modular system for evolutionary analysis. Version 3.04. http://mesquiteproject.org

Nei M, Kumar S (2000) Molecular Evolution and Phylogenetics. Oxford University Press, NY, USA, 333 pp.

Oorschot H van, Coutsis J (2014) The Genus *Melitaea* Fabricius, 1807 (Lepidoptera: Nymphalidae, Nymphalinae). Taxonomy and systematics with special reference to the male genitalia. Tshikolovets Publications, Pardubice, Czech Republic, 360 pp.

Posada D (2008) jModel Test: phylogenetic model averaging. Molecular Biology and Evolution 25(7): 1253–1256. doi: 10.1093/molbev/msn083

Ritter S, Michalski SG, Settele J, Wiemers M, Fric ZF, Sielezniew M, Šašić M, Rozier Y, Durka W (2013) *Wolbachia* infections mimic cryptic speciation in two parasitic butterfly species, *Phengaris teleius* and *P. nausithous* (Lepidoptera: Lycaenidae). PLoS ONE 8(11): 1–13. doi: 10.1371/journal.pone.0078107

Talavera G, Lukhtanov VA, Rieppel L, Pierce NE, Vila R (2013a) In the shadow of phylogenetic uncertainty: the recent diversification of *Lysandra* butterflies through chromosomal change. Molecular Phylogenetics and Evolution 69: 469–478. doi: 10.1016/j.ympev.2013.08.004

Talavera G, Lukhtanov VA, Pierce NE, Vila R (2013b) Establishing criteria for higher-level classification using molecular data: the systematics of *Polyommatus* blue butterflies (Lepidoptera, Lycaenidae). Cladistics 29: 166–192. doi: 10.1111/j.1096-0031.2012.00421.x

Tamura K (1992) Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C content biases. Molecular Biology and Evolution 9(4): 678–687.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30: 2725–2729. doi: 10.1093/molbev/mst197

Tshikolovets VV (2011) Butterflies of Europe and the Mediterranean Area. Tshikolovets Publications, Pardubice, Czech Republic, [i–v]+544 pp.
Tuzov VK, Churkin S (2000) Genus Melitaea. In: Tuzov VK (Ed.) Guide to the Butterflies of Russia and Adjacent Territories (Lepidoptera, Rhopalocera). Volume 2, Pensoft Publishers, Sofia-Moscow, 59–76.

Vershinina AO, Lukhtanov VA (2010) Geographical distribution of the cryptic species Agrodiaetus alcestis alcestis, A. alcestis karacetinae and A. demavendi (Lepidoptera, Lycaenidae) revealed by cytogenetic analysis. Comparative Cytogenetics 4(1): 1–11. doi: 10.3897/compytogen.v4i1.21

Vila R, Bjorklund M (2004) The utility of the neglected mitochondrial control region for evolutionary studies in Lepidoptera (Insecta). Journal of Molecular Evolution 58(3): 280–290. doi: 10.1007/s00239-003-2550-2

Wahlberg N, Zimmermann M (2000) Pattern of phylogenetic relationships among members of the tribe Melitaeini (Lepidoptera: Nymphalidae) inferred from mitochondrial DNA sequences. Cladistics 16: 347–363. doi: 10.1006/clad.2000.0136

Zakharov EV, Lobo NF, Nowak C, Hellma JJ (2009) Introgression as a likely cause of mtDNA paraphyly in two allopatric skippers (Lepidoptera: Hesperiidae). Heredity 102: 590–599. doi: 10.1038/hdy.2009.26

**Supplementary material 1**

Table S1
Authors: Elena A. Pazhenkova, Evgeny V. Zakharov, Vladimir A. Lukhtanov
Data type: Excel table.
Explanation note: Collection data of the samples sequenced in this study.
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

**Supplementary material 2**

Table S2
Authors: Elena A. Pazhenkova, Evgeny V. Zakharov, Vladimir A. Lukhtanov
Data type: Excel table.
Explanation note: List of the samples used in this study
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.