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Hybridisation in *Colias* butterflies (Lepidoptera, Pieridae): identification of natural hybrids using unlinked molecular markers

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Abstract

In this report, we analyze a wild-caught *Colias* specimen from Kyrgyzstan with unusual wing pattern, which was provisionally identified as a natural hybrid between *Colias christophi helialaica* Schulte, 1988 and *Colias cocandica cocandica* Erschoff, 1874 due to its intermediate phenotype. Using one mitochondrial (*COI* barcode) and three nuclear (*Ca-ATPase*, *H3* and *CAD* gene fragments) markers, we confirm its hybrid origin and demonstrate that this specimen is a hybrid between a female of *Colias christophi helialaica* and a male of *Colias cocandica cocandica*. Our study shows that an analysis of mitochondrial and nuclear molecular markers can successfully discriminate natural hybrids in such a taxonomically challenging group of butterflies as the genus *Colias*.

Keywords

Central Asia, DNA barcodes, interspecific hybridisation, molecular markers

Introduction

The genus *Colias* (Pieridae: Coliadinae) is a large taxonomically challenging group of butterflies with approximately 90 described species distributed throughout most of the world (Verhulst 2000; Greishuber and Lamas 2007; Greishuber et al. 2012; Greishuber 2014). In contrast to many other groups of Lepidoptera, species of the genus are highly conservative with respect to the male genitalia structures, which often lack reliable diagnostic characters (Greishuber 2014). The differences in the wing pattern between *Colias* species are also often very subtle or even vanishing (Hammond and McCorkle 2003). Furthermore, many taxa exhibit significant intraspecific variability in the wing colouration and pattern (Ellers and Boggs 2002; Hammond and McCorkle 2008). The wide colour polymorphism and the limited applicability of external and internal diagnostic features make reliable identification and species-level boundaries definition very challenging by morphological grounds.

Although natural hybridisation is thought to be somewhat uncommon in animals, individuals with exceptional phenotypical traits, morphological characteristics at the intersection of different species, or of "intermediate" phenotypes regularly occur in nature (Uthicke et al. 2005; Lutterschmidt et al. 2007; Galindo-Ruiz et al. 2019; Eshenroder et al. 2021; Liberman et al. 2021; Gaudiano et al. 2022). Such individuals are often considered as putative hybrids. In entomological literature, numerous publications are regarded to possible cases of interspecific hybridisation between different species of Lepidoptera (Sperling 1990; Warren and Robbins 1993; ten Hagen 2003; Gil-T 2007; Mullen 2008; Jasso-Martínez et al. 2018; Shapoval et al. 2021), including the genus *Colias* (Ae 1959; Jahner et al. 2012; Dwyer et al. 2015; Dzurinka et al. 2021, 2022).

A number of *Colias* specimens, which are thought to represent natural hybrids, are known from private and museum collections (Schurian and Rose 1978; Schulte 1988). However, given the limited number of reliable diagnostic characters coupled with broad intraspecific variability common for many taxa of the genus, a caution is required in the interpretation of such individuals as hybrids. Recent reports have shown that the assumptions on the hybrid origin of phenotypic intermediates based on the analysis of external morphology alone can be misleading (Qin et al. 2023). In such cases, combined analysis of morphological and molecular data can serve as a powerful approach for both species identification and delimitation, and for natural hybrids discrimination (Shapoval et al. 2021).

At the beginning of July 2022, a putative hybrid specimen sharing the external characters of *Colias christophi helialaica* Schulte, 1988 and *Colias cocandica cocandica* Erschoff, 1874 was collected by Alexander Baryshev (Penza, Russia) in the Alay Mountains (Kyrgyzstan). In this study, we employ the combined approach (morphological and multigene data analysis) to confirm the hybrid origin of this specimen, ascertain parental species and identify the direction of the hybridisation.

Materials and methods

The putative hybrid and specimens of the putative parental species (Fig. 1) were collected in the Alay Mountains (Kyrgyzstan) by Alexander Baryshev (Penza, Russia) during the field studies in 2022. One mitochondrial (cytochrome oxidase subunit I (*COI*)) and three nuclear (*Ca-ATPase* (sarco/endoplasmic reticulum calcium ATPase), *H3* (Histone h3) and *CAD* (Carbamoyl-Phosphate Synthetase 2, Aspartate Transcarbamylase, and Dihydroorotase)) genes were used as molecular markers. One or two legs from each specimen were taken for DNA extraction using the CTAB-based method (Murray and Thompson 1980) with some modifications (Lukhtanov and Shapoval 2008; Lukhtanov et al. 2008). Mitochondrial DNA barcode (a 658 bp fragment of the *COI* gene) was amplified using LCO1490/HC2198 primer pair (Folmer et al. 1994). A 445 bp fragment of the *Ca-ATPase*, a 328 bp fragment of the *H3* and a 847 bp fragment of the *CAD* genes were amplified using Ca-ATPase_F/Ca-ATPase_R (Wahlberg et al. 2016), H3aF/H3aR (Colgan 1998), CAD743nF/CADmidR CADmidF/CAD1028R (Wahlberg and Wheat 2008) primer pairs, respectively. The PCR amplifications were performed in a 25 μL reaction volume per sample. Each reaction contained 1 μL template DNA (ca. 10–50 ng genomic DNA), 1.3 μL of both forward and reverse primers aliquoted to a standard concentration of 10 μM, 5 μL of 5× ScreenMix (Evrogen, Moscow, Russia) and 16.4 μL of ddH_2O. The temperature profiles for *COI*, *Ca-ATPase*, *H3* and *CAD* genes were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C (*COI*)/55 °C (*Ca-ATPase*, *CAD*)/60 °C (*H3*) for 30 s and extension at 72 °C for 1 min 30 s, with a final extension at 72 °C for 10 min. Amplified fragments were purified using QIAquick PCR Purfication Kit (Qiagen, USA). Purification was carried out according to the manufacturer's protocol. Purified PCR products were used for direct sequencing or subsequent cloning. We cloned the nuclear *CAD* gene fragments for the putative hybrid specimen for which standard sequencing revealed intra-individual heterogeneities in the form of single nucleotide polymorphism. The cloning procedure was performed following previously described protocols (Lukhtanov et al. 2015; Shapoval and Lukhtanov 2015). 20 clones for each *CAD* fragment were sequenced. Sequencing of the double-stranded product was carried out at the Research Resource Center for Molecular and Cell Technologies (St. Petersburg State University, St. Petersburg, Russia) using ABI 3500xL analyzer (Applied Biosystems, Waltham, MA, USA).

The sequences were checked, edited and aligned using CHROMAS 2.6.6 (http://www.technelysium.com.au/), Geneious v.8.1.6 (Kearse et al. 2012) and BioEdit v.7.0.3 (Hall 2011) software. Primer sequences were cropped. Three nuclear genes were concatenated resulting in the final dataset comprising a total of 1620 bp (445 bp of the *Ca-ATPase*, 328 bp of the *H3* and 847 bp of the *CAD*). The mitochondrial *COI* gene was analysed independently. Since *Colias croceus* (Geoffroy, 1785) was earlier inferred as one of the basal clades within the genus *Colias*, we used previously obtained mitochondrial and nuclear sequences of this species as outgroup to root

the mitochondrial and nuclear phylograms. The *COI* dataset of the phylogenetic analysis included in total sequences of 11 specimens: an outgroup, a putative hybrid, three *C. cocandica cocandica* samples and six *C. christophi helialaica* sequences (five of them were sequenced during this study and one more sequence was obtained from BOLD). The list of the specimens used for the molecular analysis with the full collection data and GenBank/BOLD Accession Numbers are given in the Table 1. The best-fitting substitution models for the *COI* dataset and for the concatenated *Ca-ATPase+H3+CAD* nuclear genes fragments were estimated based on the Bayesian information criterion (BIC) using jModelTest v.2.1.10 (Darriba et al. 2012). The analyses were performed using the MrBayes v3.2.7a software (Ronquist et al. 2012) with the nucleotide substitution model HKY + I for *COI* dataset and F81 for the concatenated nuclear fragments as suggested by jModelTest v2.1.10. Two independent MCMC runs of 10 million generations, with four simultaneous chains (one cold and three heated) for each analysis, were performed. The sampling of trees and parameters were set to every 1000 generations. TRACER, v1.7.1 was used for summarising the results of the Bayesian phylogenetic analysis (http: //beast.bio.ed.ac.uk/Tracer). The first 10% of trees were discarded as burn-in prior to computing a consensus phylogeny and posterior probabilities. The consensus of the obtained trees was visualised using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Genetic distances among *COI* barcodes were calculated using MEGA v.7.0.14 (Kumar et al. 2016).

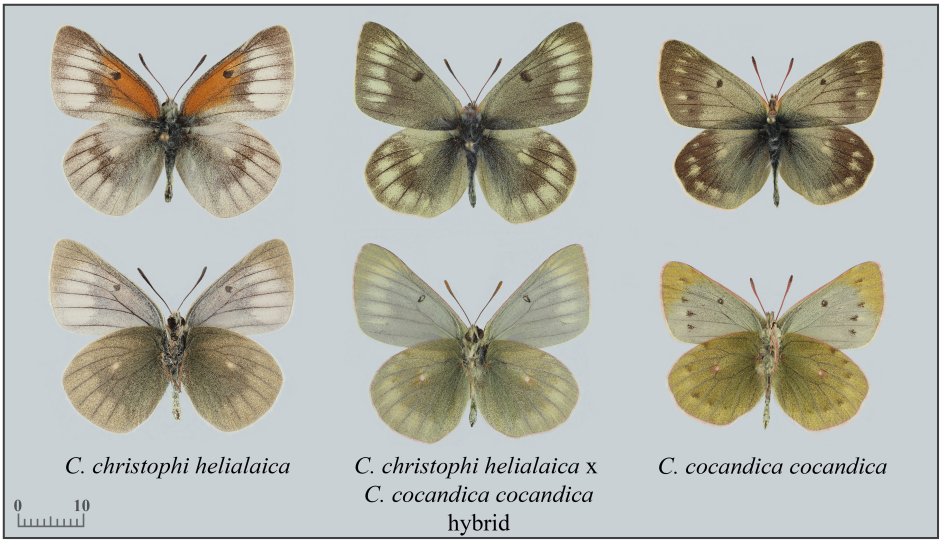

Figure 1. Putative hybrid specimen (center) and its putative parental species, female of *C. christophi helialaica* (left) and male of *Colias cocandica cocandica* (right) [Kyrgyzstan, Alay Range, Tengiz-Bay Pass, h = 3680 m, 39°40'17.2" N; 72°08'06.9" E, 08–10.VII.2022, A. Baryshev leg.].

Table 1. List of studied specimens

Taxon	Sample ID	GenBank/BOLD Accession Number				Locality
		COI	*H3*	*Ca-ATPase*	*CAD*	
C. cocandica × *C. christophi*	BR01	OR364041	OR539248 OR539249	OR539259 OR539260	OR539271 OR539272	Kyrgyzstan*
C. christophi	BR02	OR364042	OR539252	OR539264	OR539276	Kyrgyzstan*
C. christophi	BR03	OR364043	OR539253	OR539265	OR539277	Kyrgyzstan*
C. christophi	BR04	OR364044	OR539254	OR539266	OR539278	Kyrgyzstan*
C. christophi	BR05	OR364045	OR539255	OR539267	OR539279	Kyrgyzstan*
C. christophi	BR06	OR364046	OR539256	OR539268	OR539280	Kyrgyzstan*
C. christophi	–	SACOL191-18	–	–	–	Kyrgyzstan*
C. cocandica	BR08	OR364047	OR539250	OR539261	OR539273	Kyrgyzstan*
C. cocandica	BR09	OR364048	OR539251	OR539262	OR539274	Kyrgyzstan*
C. cocandica	BRX10	OR364049	OR539257	OR539263	OR539275	Kyrgyzstan*
C. croceus	CL151	OR178497	OR539252	OR539269	OR539270	Russia, Kaliningrad**

* – Kyrgyzstan, Alay Range, Tengiz-Bay Pass, h = 3680 m, 39°40'17.2'' N; 72°08'06.9'' E, 08–10. VII.2022, A. Baryshev leg.; ** – Russia, Kaliningrad Oblast, Zelenogradsky District, Courish Spit, "Fringilla" field station 55°05'17" N; 20°44'04" E, 28.VIII.2016, A. Shapoval leg.

Results

The phylogenetic analysis based on the mitochondrial *COI* marker recovered *C. christophi helialaica* and *C. cocandica cocanidca* as two strongly supported clades (Fig. 2) separated by at least 17 fixed nucleotide substitutions (mean uncorrected p-distance is 2.66%). The analysed specimens of *C. cocandica cocandica* formed a single haplotype, while *C. christophi helialaica* specimens constituted two haplotypes, differing in one nucleotide substitution (A => G) in the position 508. The putative hybrid specimen shared the mitochondrial DNA barcode with *C. christophi helialaica* (specimens SACOL191-18, BR02 (OR364042), BR03 (OR364043), BR05 (OR364044)).

With respect to the nuclear *Ca-ATPase* and *H3* genes, all the studied specimens displayed no intra-individual polymorphism (usually represented by dual peaks of similar height in the electropherograms), and no fixed differences between *C. christophi helialaica* and *C. cocandica cocandica* were found. The analysis of the nuclear *CAD* gene fragments revealed a relatively low level of intra-individual heterozygosity for the species analysed. Aside from heterozygous sites, the *CAD* sequences possessed several fixed nucleotide substitutions separating *C. christophi helialaica* and *C. cocandica cocandica* (namely, T=>A in the position 298, A=>T in the position 579 and T=>C in the position 730). The *CAD* fragments of the putative hy-

brid specimen were heterozygous in these positions, bearing nucleotides of both *C. christophi helialaica* and *C. cocandica cocandica* (Fig. 3).

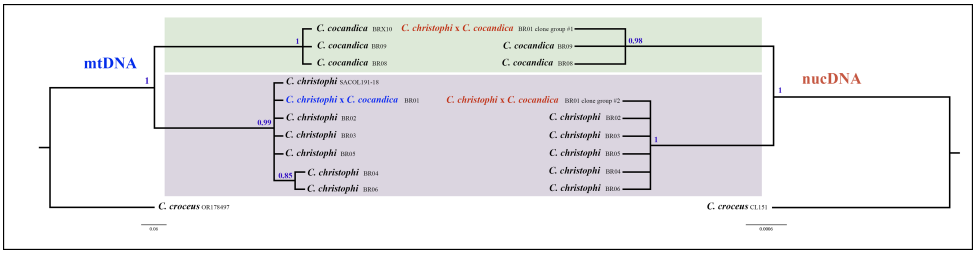

Figure 2. The Bayesian consensus trees of the analysed specimens of *C. christophi helialaica*, *C. cocandica cocandica* and the putative hybrid, inferred from *COI* sequences (left) and concatenated alignment of three nuclear markers (*Ca-ATPase*, *H3*, *CAD*) (right). Numbers at nodes indicate Bayesian posterior probabilities. Scale bar = 0.06 and 0.0006 substitutions per position for mtDNA tree and nucDNA tree, respectively.

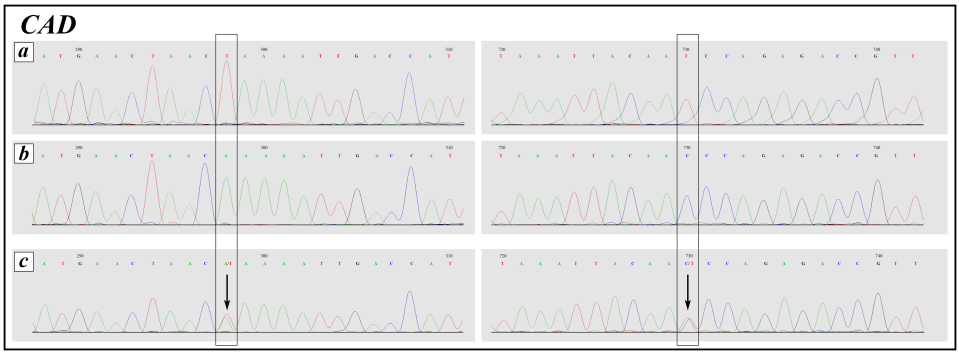

Figure 3. Electropherograms of two *CAD* nuclear gene fragments of parental species (a,b) and putative hybrid (c). Fixed interspecific nucleotide differences between *C. christophi helialaica* and *C. cocandica cocandica* are boxed. Arrows indicate mixed signals in putative hybrid.

To elucidate the visible heterogeneities, *CAD* fragments of the putative hybrid specimen were cloned and 20 clones per fragment were sequenced. Since the all cloned sequences formed two distinct variants, the identical clones were merged and included in the concatenated molecular matrix as two independent samples, "clone group #1" and "clone group #2". Bayesian analysis based on the concatenated alignment of the nuclear markers recovered two highly supported clades, corresponding to *C. christophi helialaica* and *C. cocandica cocandica*. "Clone group #1" and "clone group #2" clustered within the *C. cocandica cocandica* and *C. christophi helialaica* clades, respectively, confirmed our suggestion that the heterozygosity of the studied specimens revealed by the direct sequencing of the nuclear genes is a result of shared combination of the both lineages, *C. christophi helialaica* and *C. cocandica cocandica*.

Discussion

The hybrid specimen was found flying together with other *Colias* species, namely *C. eogene* C. Felder & R. Felder, 1865, *C. alpherakii* Staudinger, 1882, *C. christophi helialaica* and *C. cocandica cocandica*. Externally, the hybrid specimen resembles the two distantly related taxa, *C. cocandica cocandica* and *C. christophi helialaica*, combining the morphological characters of both species. Greenish colouration of the dorsal side of the wings and the hindwing with angled outer margin are the characters of *C. cocandica cocandica*; well-developed white submarginal band and rounded outer margin of the hindwing are the characters of *C. christophi helialaica*. Interestingly, the left and right sides of the hybrid specimen significantly differ in size and wing shape (Fig. 4). The left forewing and the hindwing to some extent resemble those of *C. cocandica cocandica* (smaller size and angled outer margin of the hindwing), while the right forewing and the hindwing similar to those of *C. christophi helialaica* (larger size and rounded outer margins).

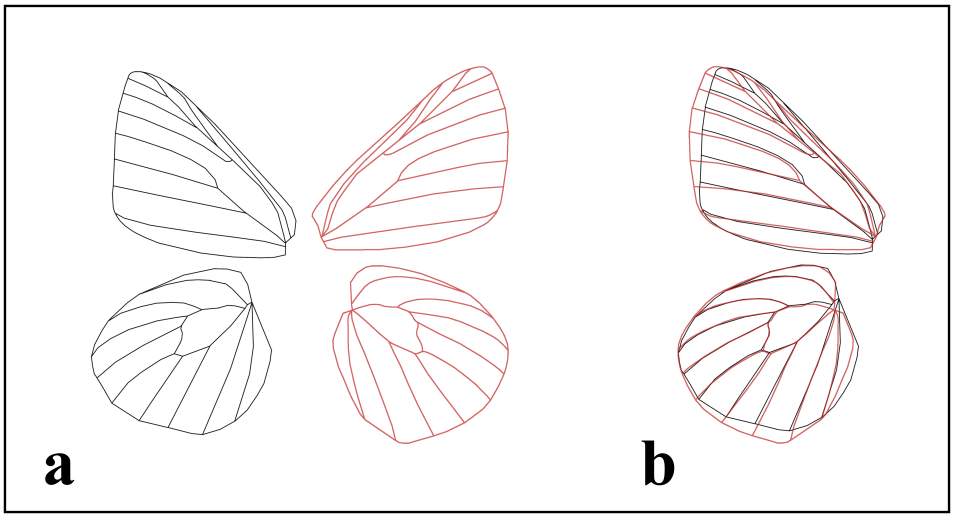

Figure 4. Wireframe representation of the forewings and hindwings of the *Colias cocandica cocandica* x *C. christophi helialaica* hybrid (a). Left side (black) and mirrored right side (red), showing the differences in wing shape and wing size (b).

Ongoing interspecific hybridisation has been suggested for several *Colias* taxa. One of the examples is North American sister taxa *C. eurytheme* Boisduval, 1852 and *C. eriphyle* Edwards, 1876. The latter taxon has often been considered as a subspecies of *C. philodice* Godart, 1819, a close relative of *C. eurytheme* widely distributed throughout the eastern USA. The phylogenetic relationships among *C. eurytheme*, *C. eriphyle* and *C. philodice* remain poorly understood, and the taxonomic status of these taxa are a matter of debates (Layberry et al. 1998; Wheat and Whatt 2008). Notwithstanding, regularly occurring individuals of an intermediate phenotype ob-

served in the areas of sympatry of *C. eurytheme* and *C. eriphyle* have been regarded in the literature as hybrids of the two taxa, although no molecular analysis confirming hybrid origin of such specimens has been performed (Gerould 1946; Taylor 1972; Jahner et al. 2012). Recent molecular studies confirmed continuous gene flow and introgression between *C. eurytheme* and *C. eriphyle*, however, the specimens of the intermediate phenotype were found to be genetically indistinguishable from *C. eriphyl*e, being a "colour morphs" of *C. eriphyle*, not genetic hybrids (Dwyer 2015).

Another pair of taxa, *C. croceus* – *C. erate* (Esper, 1805), have been postulated to occasionally hybridise (Dinca et al. 2011; Dzurinka et al. 2021, 2022), given that numerous specimens with intermediate morphological features (e.g., with yellow-orange wing colouration) occur in nature. However, the lack of reliable morphological diagnostic characters and wide colour variation coupled with mitochondrial barcodes and nuclear DNA sharing among these two taxa (Dzurinka et al. 2022) make DNA-based and morphology-based identifications of their putative hybrids unreliable.

Finally, hybridisation has been asserted, without evidence, as a cause for poorly understood and unclear morphological variations in other *Colias* species (Hovanitz 1963). Thus, we can conclude that many cases of hybridisation and identification of the reported phenotypic intermediates as true hybrids are not firmly supported. Such conclusions require confirmation by a comprehensive analysis, involving both detailed morphological analysis and targeted gene/genome sequences.

In this study, we show that the analysis of the mitochondrial and nuclear DNA genes coupled with the analysis of the external morphology can successfully discriminate natural hybrids in such a taxonomically complicated group of butterflies as the genus *Colias*. We analyse a *Colias* specimen with unusual wing pattern, which was provisionally identified as a natural hybrid. Molecular analysis has shown that this specimen bears the mitochondrial haplotype of *C. christophi helialaica*, while the nuclear fragments are heterozygous, sharing a combination of *C. cocandica cocandica* and *C. christophi helialaica* lineages. The molecular results are confirmed by the intermediate morphological characters, clearly seen in the hybrid individual: it combine both the traits of *C. cocandica cocandica* and *C. christophi helialaica*. Our findings empirically demonstrate the possibility of genetic introgression between distantly related and morphologically very different species of the genus *Colias*.

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References

Ae SA (1959) A Study of Hybrids in *Colias* (Lepidoptera, Pieridae). Evolution 13(1): 64–88.

Colgan DJ, McLauchlan A, Wilson GDF, Livingston SP, Edgecombe GD, Macaranas J, Cassis G, Gray MR (1998) Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. Australian Journal of Zoology 46(5): 419–437.

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: More models, new heuristics and parallel computing. Nature Methods 9(8): 772. https://doi.org/10.1038/nmeth.2109

Dinca V, Zakharov EV, Hebert PD, Vila R (2011) Complete DNA barcode reference library for a country's butterfly fauna reveals high performance for temperate Europe. Proceedings of the Royal Society B 278(1704): 347–355. https://doi.org/10.1098/rspb.2010.1089

Dwyer HE, Jasieniuk M, Okada M, Shapiro AM (2015) Molecular evidence for hybridization in *Colias* (Lepidoptera: Pieridae): Are *Colias* hybrids really hybrids? Ecology and Evolution 5(14): 2865–2877. https://doi.org/10.1002/ece3.1574

Dzurinka M, Šemeláková M, Panigaj L (2022) Taxonomy of hybridizing *Colias croceus* (Geoffroy, 1785) and *Colias erate* (Esper, 1805) (Lepidoptera, Pieridae) in light of mitochondrial and nuclear DNA, with occurrence and effects of Wolbachia infection. Zoologischer Anzeiger 299: 73–81. https://doi.org/10.1016/j.jcz.2022.05.007

Dzurinka M, Mutanen M, Šemeláková M, Csanády M, Mikitová B, Panigaj L (2021) The intraspecific variability of *Colias croceus* (Geoffroy, 1785) and *C. erate* (Esper, 1805) (Lepidoptera, Pieridae) from the perspective of comparative morphology. Zoomorphology 140, 353–363. https://doi.org/10.1007/s00435-021-00535-3

Ellers J, Boggs CL (2002) The evolution of wing color in *Colias* butterflies: heritability, sex linkage, and population divergence. Evolution 56(4): 836–840. https://doi.org/10.1111/j.0014-3820.2002.tb01394.x

Eshenroder RL, Kao Yu-C, O'Brien TP, Olds CM, Davis CL, Duncan AT (2021) Replacement of the typical *artedi* form of Cisco *Coregonus artedi* in Lake Huron by endemic shallow-water Ciscoes, including putative hybrids. Transactions of the American Fisheries Society 150(6): 792–806. https://doi.org/10.1002/tafs.10328

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek RC (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3(5): 294–299.

Galindo-Ruiz N, Velasquez-Velez MI, Cano-Cobos Y, Sanchéz-Guillén RA, Realpe E (2019) Description of a putative hybrid between *Ischnura cyane* and *I. capreolus* from Colombia (Odonata: Coenagrionidae). Notulae odonatologicae 9(4): 144–151. https://doi.org/10.5281/zenodo.3539752

Gaudiano L, Corriero G, Villani M, Anile S (2022) Gone before it's known? Camera-trapping shows alarming levels of putative hybrids in the wildcat (*Felis silvestris*) population of the Gargano National Park (Southern Italy). Mammalia 86(6): 581–584. https://doi.org/10.1515/mammalia-2021-0125

Gil-T F (2007) A natural hybrid of *Polyommatus bellargus* (Rottemburg, 1775) x *P. albicans* (Herrich-Schäffer, 1852) and notes about probable hybrid of *P. punctifera* (Oberthür,

1876) x *P. albicans* (Lepidoptera, Lycaenidae). Nachrichten des Entomologischen Vereins Apollo 28(1/2): 11–13.

Greishuber J (2014) Guide to the butterflies of the Palearctic region. Pieridae part II. Omnes Artes, Milano, 86 pp.

Greishuber J, Lamas G (2007) A synonymic list of the genus *Colias* Fabricius, 1807 (Lepidoptera: Pieridae). Mitteilungen der Münchner Entomologischen Gesellschaft 97: 131–171.

Greishuber J, Worthy B, Lamas G (2012) The genus *Colias* Fabricius, 1807. Jan Haugum's annotated catalogue of the Old World Colias (Lepidoptera, Pieridae). Tshikolovets Publications, Pardubice, 432 pp.

ten Hagen W (2003) Freilandhybriden bei Bläulingen aus Ostanatolien und Iran (Lepidoptera: Lycaenidae). Nachrichten des Entomologischen Vereins Apollo 23(4): 199–203.

Hall T (2011) BioEdit: An important software for molecular biology. GERF Bulletin of Biosciences 2(1): 60–61.

Hammond PC, McCorkle DV (2003) A new desert subspecies of *Colias occidentalis* (Pieridae) from southeastern Oregon. Journal of the Lepidopterists' Society 57(4): 274–278.

Hammond PC, McCorkle DV (2008) A review of geographic variation and possible evolutionary relationships in the *Colias scudderii-gigantea* complex of North America (Pieridae). Journal of the Lepidopterists' Society 62(4): 201–215.

Hovanitz W (1963) The origin of a sympatric species in *Colias* through the aid of natural hybridization. Journal of Research on the Lepidoptera 1(4): 261–274; 2(3): 205–223.

Jahner JP, Shapiro AM, Forister ML (2012) Drivers of hybridization in a 66-generation record of *Colias* butterflies. Evolution 66(3): 818–830. https://doi.org/10.1111/j.1558-5646.2011.01481.x

Jasso-Martínez JM, Machkour-M'Rabet S, Vila R, Rodríguez-Arnaiz R, Castañeda-Sortibrán AN (2018) Molecular evidence of hybridization in sympatric populations of the *Enantia jethys* complex (Lepidoptera: Pieridae). PLoS ONE 13(5): e0197116. https://doi.org/10.1371/journal.pone.0197116

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12): 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Kumar S, Stecher G, Tamura K (2016) MEGA 7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054

Layberry RA, Hall PW, LaFontaine JD (1998) The Butterflies of Canada. University of Toronto Press, Toronto, 354 pp.

Liberman E, Chemagin A, Volosnikov G, Zhigileva O (2021) Identification and characterization of hybrids of *Acipenser ruthenus* and *Acipenser baerii* (Actinopterygii, Acipenseriformes)from the Irtysh River. Acta Biologica Sibirica 7: 261–282. https://doi.org/10.3897/abs.7.e67157

Lukhtanov VA, Shapoval NA (2008) Detection of cryptic species in sympatry using population analysis of unlinked genetic markers: A study of the *Agrodiaetus kendevani* species

complex (Lepidoptera: Lycaenidae). Doklady Biological Sciences 423: 432–436. https://doi.org/10.1134/S0012496608060197

Lukhtanov VA, Shapoval NA, Dantchenko AV (2008) *Agrodiaetus shahkuhensis* sp. n. (Lepidoptera, Lycaenidae), a cryptic species from Iran discovered by using molecular and chromosomal markers. Comparative Cytogenetics 2(2): 99–114.

Lukhtanov VA, Shapoval NA, Anokhin BA, Saifitdinova AF, Kuznetsova VG (2015) Homoploid hybrid speciation and genome evolution via chromosome sorting. Proceedings of the Royal Society B 282: 20150157. http://dx.doi.org/10.1098/rspb.2015.0157

Lutterschmidt WI, Escobar SA, Wilson ED (2007) Multivariate analyses of shell morphology of putative hybrid box turtles. Southeastern Naturalist 6(4): 571–576.

Mullen SP, Dopman EB, Harrison RG (2008) Hybrid zone origins, species boundaries, and the evolution of wing-pattern diversity in a polytypic species complex of North American admiral butterflies (Nymphalidae: *Limenitis*). Evolution 62(6): 1400–1417. https://doi.org/10.1111/j.1558-5646.2008.00366.x

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8(19): 4321–4325.

Qin H, Xu H, Capron A, Porth A, Cui M, Keena MA, Deng X, Shi J, Hamelin RC (2023) Is there hybridization between 2 species of the same genus in sympatry?—The genetic relationships between *Anoplophora glabripennis*, *Anoplophora chinensis*, and putative hybrids. Insect Science. https://doi.org/10.1111/1744-7917.13256

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Schulte A (1988) Bemerkungen über Colias-Arten aus dem Alai: 1. *Colias christophi helialaica* n. ssp., eine neue Pieriden-Unterart aus dem östlichen Alai (UdSSR); 2. Gibt es Freiland-Hybriden zwischen *Colias eogene* Fldr. 1865 und *Colias cocandica* Ersch. 1874? Nachrichten des Entomologischen Vereins Apollo 9(3): 149–158.

Schurian KG, Rose K (1978) Bemerkungen zur Hybridisierung zwischen *Colias aurorina* H.S. und *Colias sagartia* Led. (Lepidoptera, Pieridae). Nachrichtenblatt der Bayerischen Entomologen 27: 129–131.

Shapoval NA, Lukhtanov VA (2015) Intragenomic variations of multicopy *ITS2* marker in *Agrodiaetus* blue butterflies (Lepidoptera, Lycaenidae). Comparative Cytogenetics 9(4): 483–497. https://doi.org/10.3897/CompCytogen.v9i4.5429

Shapoval NA, Yakovlev RV, Kuftina GN, Lukhtanov VA, Knyazev SA, Romanovich AE, Krupitsky AV (2021) Identification of natural hybrids between *Ahlbergia frivaldszkyi* (Lederer, 1853) and *Callophrys rubi* (Linnaeus, 1758) (Lepidoptera, Lycaenidae) using mitochondrial and nuclear markers. Insects 2021(12): 1124. https://doi.org/10.3390/insects12121124

Sperling FAH (1990) Natural hybrids of *Papilio* (Insecta: Lepidoptera): poor taxonomy or interesting evolutionary problem? Canadian Journal of Zoology 68(8): 1790-1799.

Taylor OR (1972) Random vs. non-random mating in the sulfur butterflies *Colias eurytheme* and *Colias philodice*. Evolution 26(3): 344–356.

Uthicke S, Purcell S, Blockmans B (2005) Natural hybridization does not dissolve species boundaries in commercially important sea cucumbers. Biological Journal of the Linnean Society 85(3): 261–270. https://doi.org/10.1111/j.1095-8312.2005.00489.x

Verhulst J (2000) Les *Colias* du Globe. Monograph of the genus *Colias*. Goecke & Evers, Keltern, 571 pp.

Wahlberg N, Wheat CW (2008) Genomic outposts serve the phylogenomic pioneers: Designing novel nuclear markers for genomic DNA extractions of Lepidoptera. Systematic Biology 57(2): 231–242. https://doi.org/10.1080/10635150802033006

Wahlberg N, Peña C, Ahola M, Wheat CW, Rota J (2016) PCR primers for 30 novel gene regions in the nuclear genomes of Lepidoptera. ZooKeys 596: 129–141. https://doi.org/10.3897/zookeys. 596.8399

Warren AD, Robbins RK (1993) A natural hybrid between *Callophrys* (*Callophrys*) *sheridanii* and *C.* (*Incisalia*) *augustinus* (Lycaenidae). Journal of the Lepidopterists' Society 47(3): 236–240.

Wheat CW, Watt WB (2008) A mitochondrial-DNA-based phylogeny for some evolutionary-genetic model species of *Colias* butterflies (Lepidoptera, Pieridae). Molecular Phylogenetics and Evolution 47(3): 893–902. https://doi.org/10.1016/j.ympev.2008.03.013