
ORIGINAL ARTICLE

Notes on the molecular taxonomy of the *Proclossiana eunomia* complex (Lepidoptera, Nymphalidae: Argynnini): analysis of DNA barcodes

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Submitted: 24.11.2017. Accepted: 15.01.2018

The *Proclossiana eunomia* (Esper, 1799) complex is currently composed of the several subspecies distributed throughout Palaearctic region and North America. Despite the fact that some of the taxa have differences in wing pattern and body size, previous assumptions on taxonomy not supported by molecular data. Therefore, the identity of certain populations of this complex has remained unclear and the taxonomic status of several recently described taxa is debated. Here, we provide insights into systematics of some Palaearctic members of this group using molecular approach, based on the analysis of the barcoding fragment of the *COI* gene taking into account known morphological differences.

Key words: mitochondrial DNA; haplotype mitochondrial network; molecular systematics; phylogeography; subspecies; taxonomic status.

Introduction

The Bog Fritillary, *Proclossiana eunomia* (Esper, 1799) was originally described from "Ostpreussen, Königsberg" (present day the Kaliningrad region). *P. eunomia* is a widespread boreo-alpine butterfly species, occupying wetland habitats. The species is local in temperate regions of western and eastern Europe (ranging from central France across southern Belgium, Germany, Poland, Austria, Czech Republic, Belarus, northern Ukraine to European part of Russia), very local in the Balkans (Serbia, Bulgaria), the Caucasus and Transcaucasia, northeastern Turkey (Kars), the Volga region, the Urals and the lowlands of Western Siberia. The butterfly is more common in Fennoscandia, Baltic countries and northwestern Russia, northern and eastern Siberia, the mountains of southern Siberia, southeastern Kazakhstan, northern and northwestern Mongolia, southeastern China (Heilongjiang province) and Sakhalin (Lukhtanov & Lukhtanov, 1994; Kudrna, 2002; Tuzov & Bozano, 2006; Lukhtanov et al., 2007; Lvovsky & Morgun, 2007; Tshikolovets et al., 2009a, b; Tshikolovets, 2011; Lang, 2012; Yakovlev, 2012; Toropov & Zhdanko, 2013), as well as in the Nearctic, ranging from Alaska and Labrador to Wisconsin and across the mountains to Colorado (Scott, 1986; Pyle, 2006). The taxonomic structure of this widespread species requires revision.

A number of taxa were described from Siberia: *acidalia* Böber, 1809 (type locality – "de la Sibérie"); *asiatica* Staudinger, 1901 (type locality – "Sib.[erie] m.[eridionale] (Alt.[ai], Saj.[an], Kent.[ei], Amur)"); *riamia* Korshunov et Ivonin In Korshunov, 1998 (type locality – "Kamennyi ryam, near Kuznetskoe village, Chulym district, Novosibirsk region"); *stromi* Korshunov, 1998 (type locality – "W. Sayan, Pazaryk-Ergak-Tayga Range") and *yakovlevi* Korb, 1999 (type locality – "Altai, Ukok, Kudabaj river"). Recently, three subspecies of *P. eunomia* were described: *P. eunomia itelmena* Gorbunov, 2007 In Gorbunov & Kosterin, 2007 from Kamchatka Peninsula; *P. eunomia exspectata* Morgun, 2011 and *P. eunomia tenera* Morgun, 2011 from the mountains of the Greater Caucasus and Armenia, respectively.

In their book V. and A. Lukhtanov (Lukhtanov & Lukhtanov, 1994) noted three subspecies for Siberia and east of Eurasia: ssp. *eunomia* (Middle and South Ural), ssp. *ossianus* Herbst, 1800 (Arctic and North Siberia) and ssp. *asiatica* Staudinger, 1901 (Middle and South Siberia, West Siberian Plain, Altai). Korshunov also noted these three subspecies for the region (Korshunov & Gorbunov, 1995) and additionally described two new subspecies: *P. eunomia stromi* and *P. eunomia riamia* (Korshunov, 1998). His interpretation of the distribution of *P. eunomia* subspecies is as follows: ssp. *eunomia* for the east Siberia to the Middle and Southern Trans-Urals; ssp. *ossianus* for the forest-tundra and the taiga zone of Asia, reaching north to Taimyr; ssp. *acidalia* (= *asiatica*) for the mountains of southern Siberia. According to Korshunov this taxon distributed "further in the mountains of South Siberia, Yakutia and the north of the Far East" (Korshunov, 1998, p. 20). In this context it should be understood as east of the distribution area of ssp. *stromi*, and judging by the type locality – east of Todzha Village [Eastern Tuva]. Bogdanov generally follows the point of view proposed by Korshunov and Gorbunov (1995) specifying the distribution of ssp. *ossiana* as western Chukotka, Kamchatka, and the northern part of the Khabarovsk region. About the subspecies *riamina* (sic!) Bogdanov stated that: "its status requires a revision" (Bogdanov in Tuzov et al., 2000, p. 43). Gorbunov (2001) mentioned three subspecies for Siberia (ssp. *eunomia*; ssp. *ossianus*, ssp. *acidalia*) and also established a new synonymy, but without providing any rationale: *ossianus* = *riamina* and *acidalia* = *asiatica* = *stromi* = *yakovlevi*. Korb (2005) specifying the distribution of *P. eunomia* subspecies: ssp. *acidalia* (mountains of South Siberia), ssp. *asiatica* (South and Middle Siberia, Amur, Ussuri regions and Sakhalin), ssp. *eunomia* (Central

area of European part, Caucasus) and ssp. *ossianus* (North of Eurasia, Far East and Kamchatka). Dubatolov et al. (2005) recognizes two subspecies for eastern Eurasia: ssp. *ossianus* (Chukotka, Magadan, Kamchatka, Khabarovsk, Jewish Autonomous region, Amur, Sakhalin, Yakutia, northern Transbaikal regions, ?NE China) and ssp. *acidalia* (= *asiatica*) (Transbaikal, Sayans and Altai). Tuzov and Bozano (2006) accepted the following taxonomic structure of *P. eunomia* (here we omit western European and North American taxa): ssp. *eunomia* (central and southern Europe, Caucasus), ssp. *ossianus* (= *riamia*) (from northern Europe to western Chukotka, Kamchatka, Sakhalin and the Okhotsk Sea coast), ssp. *acidalia* (= *asiatica* = *stromi* = *yakovlevi*) (from Altai and Sayan Mountains, southern Siberia to Amur and Ussuri regions). Korb and Bolshakov noted ssp. *itelmena* for Kamchatka and Chukotka; ssp. *ossianus* for the northern regions of Eurasia and partly middle part of European Russia (except for the distribution of ssp. *itelmena*); ssp. *eunomia* for Central Europe, Middle and Southern Ural and Caucasus; ssp. *acidalia* for Southern Siberia, Amur, Primorsky krai and Sakhalin (Korb & Bolshakov, 2011); and indicated the subspecies described by Morgun (2011) from the Caucasus and Transcaucasia as valid (Korb & Bolshakov, 2016).

When examining large series of *P. eunomia* from different parts of southern Siberia, we have established that the distribution of this butterfly is essentially uninterrupted and all the characters indicated by various authors (small size, thin contrast pattern) fit within the limits of population variability.

Thus, contradictory taxonomic interpretations of intraspecific structure of *P. eunomia* exist in the literature. It should be noted, that correct taxonomic identification is often not possible based primarily on external morphological characters (Hajibabaei et al., 2006; Dincă et al., 2013; Shapoval & Lukhtanov, 2016). In contrast, the use of molecular markers (e.g. DNA barcodes) is becoming universally accepted as a useful and efficient tool for species identification, detecting previously unrecognized taxa and assisting in the resolution of taxonomic and phylogenetic problems (Hebert et al., 2004; Barrett & Hebert, 2005; Janzen et al., 2005; Hajibabaei et al., 2006; Smith et al., 2007; Velzen, Larsen & Bakker, 2009; Sourakov & Zakharov, 2011; Lukhtanov, Shapoval & Dantchenko, 2014), but see Brower (2006, 2010). It is particularly useful as an efficient start for taxonomic workflow (Kekkonen & Hebert, 2014). Yet, detailed phylogenetic studies are unavailable for *P. eunomia*. This paper addresses a DNA-based analysis of this taxon.

Material and methods

Specimens

Butterflies used for this analysis were collected in different regions of Russia, Belarus, Ukraine and Lithuania (Fig. 1). List of collected specimens with information including sampling localities is given in Table 1. DNA barcodes from a total of 37 individuals of *P. eunomia* were sequenced for this study. Additionally, 27 sequences of *P. eunomia* available from the GenBank and BOLD databases were included in our analysis. We used sequence of *Coenonympha tullia elwesi* (Davenport, 1941) from Altai (GenBank accession number MG757148) as an outgroup to root the phylogram. Representatives of all principal members of the genus *Boloria* available from the GenBank database were included in our analysis to test the monophyly of *P. eunomia* and clarify phylogenetic relationships. The barcode analysis of *P. eunomia* complex involved 539 sequences.



Fig. 1. Map showing sampling localities of the analyzed specimens.

DNA processing and sequencing

DNA extraction, amplification and further preparations for sequencing were held on the base of "Taxon" Research Resource Center and Department of Karyosystematics of the Zoological Institute of the Russian Academy of Sciences (St. Petersburg). DNA extraction from a single leg removed from the specimen was accomplished using the QIAamp DNA Investigator Kit (Qiagen, Netherlands) following the manufacturer's protocol. A fragment of the mitochondrial *cytochrome oxidase I* gene (*COI*) was used as a mitochondrial molecular marker. Standard lepidopteran barcode primers (Hebert et al., 2004) were used for DNA amplification and resulted in a 658 bp fragment of the *COI* gene. The PCR amplifications were performed in a 50 µl reaction volume containing ca. 10-20 ng genomic DNA and 0.5 mM each of forward and reverse primer, 1 mM dNTPs, 10x PCR Buffer (0.01 mM Tris-HCl, 0.05 M KCl, 0.1% Triton X-100: pH 9.0), 1 unit Taq DNA Polymerase (Thermo Fisher Scientific, Lithuania), 5 mM MgCl₂. Amplification for *COI* gene fragment was carried out with the following conditions: initial denaturation at 94° C for 1 min, followed by 30 cycles of denaturation at 94° C for 45 s, annealing at 50° C for 45 s, and extension at 72° C for 1 min with a final extension at 72° C for 10 min. Amplified fragments were purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Lithuania). Purification was carried out according to the manufacturer's protocol. Purified PCR product was used for direct sequencing.

Table 1. List of studied material (64 specimens). (*) – sequences obtained in the present study. (**) – sequence obtained in the present study and used as an outgroup.

GenBank/ BOLD number	TAXON	COI haplotype	Locality	Altitude	Date	Collectors/ References
MG757148**	<i>Coenonympha tullia elwesi</i>	–	Russia, Altai Rep., Kosh-Agach distr., vicinity of Tabozhok vill.	2000 m	03.08.2016	R. Yakovlev
MG735633*	<i>Proclossiana eunomia ossiana</i>	Eu_07	Russia, Moscow reg., Sergiev Posad distr., SE Maloye Lake, Bat'kovskoye raised bog	135 m	08.06.2013	S. Kovalev
MG735634*	<i>Proclossiana eunomia ossiana</i>	Eu_01	Russia, Moscow reg., Sergiev Posad distr., vicinity of Bol'shoye Lake, Bat'kovskoye raised bog	130 m	28.05.2016	S. Kovalev
MG735635*	<i>Proclossiana eunomia ossiana</i>	Eu_01	Russia, Moscow reg., Shatursky distr., 1.7 km NW Tugolesy railway station	125 m	10.06.2016	S. Kovalev
MG735636*	<i>Proclossiana eunomia ossiana</i>	Eu_01	Russia, Moscow reg., Shatursky distr., 1.7 km NW Tugolesy railway station	125 m	10.06.2016	S. Kovalev
MG735637*	<i>Proclossiana eunomia ossiana</i>	Eu_04	Russia, Yaroslavl reg., Pereslavsky distr., 1.1 km NE Zhupeev villa, Polovetsko-Kupanskoe (Bol'shoye) raised bog	170 m	04.06.2016	S. Kovalev
MG735638*	<i>Proclossiana eunomia ossiana</i>	Eu_07	Russia, Yaroslavl reg., Pereslavsky distr., 1.1 km NE Zhupeev villa, Polovetsko-Kupanskoe (Bol'shoye) raised bog	170 m	04.06.2016	S. Kovalev
MG735639*	<i>Proclossiana eunomia eunomia</i>	Eu_01	Russia, Yaroslavl reg., Pereslavsky distr., 2.6 km SE Kupanskoye villa, National Park «Plesheevo Lake»	140 m	13.06.2016	S. Kovalev
MG735640*	<i>Proclossiana eunomia eunomia</i>	Eu_01	Russia, Yaroslavl reg., Pereslavsky distr., 2.6 km SE Kupanskoye villa, National Park «Plesheevo Lake»	140 m	13.06.2016	S. Kovalev
MG735641*	<i>Proclossiana eunomia eunomia</i>	Eu_02	Russia, Moscow reg., Ruzsky distr., 3.6 km NE Novogorbovo village, State Reserve «Glubokoye Lake»	210 m	07.06.2015	S. Kovalev
MG735642*	<i>Proclossiana eunomia eunomia</i>	Eu_02	Russia, Moscow reg., Ruzsky distr., 3.6 km NE Novogorbovo village, State Reserve «Glubokoye Lake»	210 m	07.06.2015	S. Kovalev
MG735643*	<i>Proclossiana exspectata</i>	eunomia	Russia, North Caucasus, Karachay-Cherkess Republic, Karachayevsky distr., Nature Reserve «Teberda», Nazylkol river valley	2450 m	23.07.2011	D. Morgun
MG735644*	<i>Proclossiana exspectata</i>	eunomia	Russia, North Caucasus, Karachay-Cherkess Republic, Karachayevsky distr., Nature Reserve «Teberda», Nazylkol river valley	2450 m	23.07.2011	D. Morgun
MG735645*	<i>Proclossiana exspectata</i>	eunomia	Russia, North Caucasus, Karachay-Cherkess Republic, Karachayevsky distr., Nature Reserve «Teberda», Nazylkol river valley	2450 m	23.07.2011	D. Morgun
MG735646*	<i>Proclossiana exspectata</i>	eunomia	Russia, North Caucasus, Karachay-Cherkess Republic, Karachayevsky distr., Nature Reserve «Teberda», Nazylkol river valley	2450 m	23.07.2011	D. Morgun
MG735651*	<i>Proclossiana exspectata</i>	eunomia	Russia, North Caucasus, Karachay-Cherkess Republic, Karachayevsky distr., Nature Reserve «Teberda», Nazylkol river valley	2450 m	21.07.2011	D. Morgun
MG735647*	<i>Proclossiana eunomia acidalia</i>	Eu_22	Russia, Buryatia, Tunkinsky distr., Mondy villa, Huluigasha Mts.	1800 m	24- 30.06.2016	A. Kosarev
MG735648*	<i>Proclossiana eunomia acidalia</i>	Eu_24	Russia, Buryatia, Tunkinsky distr., Mondy villa, Huluigasha Mts.	1800 m	24- 30.06.2016	A. Kosarev
MG735649*	<i>Proclossiana eunomia riamia</i>	Eu_21	Russia, Novosibirsk reg., Chulym distr., Kamennyi ryam		16.06.2005	A. Kareev
MG735650*	<i>Proclossiana eunomia riamia</i>	Eu_19	Russia, Novosibirsk reg., Chulym distr., Kamennyi ryam		16.06.2005	A. Kareev
MG735652*	<i>Proclossiana eunomia riamia</i>	Eu_20	Russia, Omsk reg., Krutinsky distr., 44 km NW Krutinka villa, 5 km SW Gulyai-Pole villa		07- 08.06.2016	S. Knyazev
MG735653*	<i>Proclossiana eunomia riamia</i>	Eu_20	Russia, Omsk reg., Krutinsky distr., 44 km NW Krutinka villa, 5 km SW Gulyai-Pole villa		07- 08.06.2016	S. Knyazev
MG735654*	<i>Proclossiana eunomia ossiana</i>	Eu_01	Russia, Karelia, Kondopoga reg., Nature Reserve «Kivach», bog Blizkoe		31.05.2016	V. Gorbach
MG735655*	<i>Proclossiana eunomia ossiana</i>	Eu_01	Russia, Karelia, Kondopoga reg., Nature Reserve «Kivach», bog Blizkoe		31.05.2016	V. Gorbach
MG735656*	<i>Proclossiana eunomia</i>	Eu_06	Ukraine, near Kiev, Belichi villa, tract Lyubka		02.06.2011	I. Plyusch
MG735657*	<i>Proclossiana eunomia</i>	Eu_06	Ukraine, near Kiev, Belichi villa, tract Lyubka		02.06.2011	I. Plyusch
MG735658*	<i>Proclossiana eunomia eunomia</i>	Eu_01	Ukraine, Rivne reg., Nature Reserve «Rivne», near villa Bel'sk		22.06.2005	I. Plyusch
MG735659*	<i>Proclossiana eunomia eunomia</i>	Eu_05	Russia, South Ural, Chelyabinsk reg., near Ozersk		15.06.2015	S. Rybalkin
MG735660*	<i>Proclossiana eunomia eunomia</i>	Eu_05	Russia, South Ural, Chelyabinsk reg., near Ozersk		15.06.2015	S. Rybalkin
MG735661*	<i>Proclossiana eunomia eunomia</i>	Eu_01	Lithuania, Vilnius reg., Daubeni villa.		06.06.2015	D. Mikalauskas
MG735662*	<i>Proclossiana eunomia eunomia</i>	Eu_01	Lithuania, Vilnius reg., Daubeni villa.		06.06.2015	D. Mikalauskas
MG735663*	<i>Proclossiana eunomia eunomia</i>	Eu_01	Belarus, Minsk reg., Nature reserve «Talka»		18.06.2016	A. Kulak
MG735664*	<i>Proclossiana eunomia eunomia</i>	Eu_03	Belarus, Minsk reg., Nature reserve «Talka»		18.06.2016	A. Kulak
MG735665*	<i>Proclossiana eunomia eunomia</i>	Eu_03	Belarus, on the border of Minsk and Vitebsk reg., Biosphere Reserve «Berezinsky»		20.06.2016	A. Kulak
MG735666*	<i>Proclossiana eunomia eunomia</i>	Eu_01	Belarus, on the border of Minsk and Vitebsk reg., Biosphere Reserve «Berezinsky»		20.06.2016	A. Kulak
MG735667*	<i>Proclossiana eunomia itelmena</i>	Eu_18	Russia, Kamchatka p-la, Bistrinskiy distr., Esso villa, Sredinniy r., Oligende mts.	1100 m	09- 20.07.2005	D. Goshko
MG735668*	<i>Proclossiana eunomia itelmena</i>	Eu_17	Russia, Kamchatka p-la, Bistrinskiy distr., Esso villa, Sredinniy r., Oligende mts.	1100 m	09- 20.07.2005	D. Goshko
MG735669*	<i>Proclossiana eunomia itelmena</i>	Eu_17	Russia, Kamchatka p-la, Bistrinskiy distr., Esso villa, Sredinniy r., Oligende mts.	1100 m	09- 20.07.2005	D. Goshko
JF415682	<i>Proclossiana eunomia</i>	Eu_10	Germany, Bavaria, Oberbayern reg., Koenigsdorf, Weitfilz	600 m	03.06.2005	Huasmann et al., 2011
JF415681	<i>Proclossiana eunomia</i>	Eu_11	Germany, Bavaria, Oberbayern reg., Bad Toelz-Wolfratshausen, Zellwies	600 m	21.05.2009	Huasmann et al., 2011
JX034677	<i>Proclossiana eunomia</i>	Eu_10	Germany, Bavaria, Schwaben reg., Ostallgaeu, Lamerdingen, Kleinkitzighofen	600 m	10.06.2004	Mutanen et al., 2012
GU655024	<i>Proclossiana eunomia</i>	Eu_11	Germany, Bavaria, Oberbayern reg., Landsberg am Lech,	708 m	14.06.2009	Huasmann

KX044586	<i>Proclossiana eunomia</i>	Eu_10	Diessen am Ammersee, Vilgertshofener Forst Germany, Bavaria, FFB, Maisach	14.05.2011	et al., 2011 Mutanen et al., 2016
FJ664012	<i>Proclossiana eunomia</i>	Eu_01	Russia, Saint-Petersburg reg., Oranienbaum, Tamengont	22.06.2001	Lukhtanov et al., 2009
FJ664014	<i>Proclossiana eunomia</i>	Eu_01	Russia, Pskovskaya obl., Sebezh distr., Osyno	01.07.2001	Lukhtanov et al., 2009
FJ664013	<i>Proclossiana eunomia</i>	Eu_01	Russia, Pskovskaya obl., Sebezh distr., Osyno	01.07.2001	Lukhtanov et al., 2009
GU676265	<i>Proclossiana eunomia</i>	Eu_16	Spain, Cantabria, Brana Vieja	1408 m	Dincă et al., 2015
GU676266	<i>Proclossiana eunomia</i>	Eu_16	Spain, Cantabria, Brana Vieja	1408 m	Dincă et al., 2015
GU676267	<i>Proclossiana eunomia</i>	Eu_16	Spain, Cantabria, Brana Vieja	1408 m	Dincă et al., 2015
GU676268	<i>Proclossiana eunomia</i>	Eu_16	Spain, Cantabria, Brana Vieja	1408 m	Dincă et al., 2015
HM901618	<i>Proclossiana eunomia</i>	Eu_14	Spain, Catalonia, Girona reg., Meranges	1555m	Dincă et al., 2015
HM901612	<i>Proclossiana eunomia</i>	Eu_14	Spain, Catalonia, Girona reg., Meranges	1555 m	Dincă et al., 2015
GU677019	<i>Proclossiana eunomia</i>	Eu_14	Spain, Catalonia, Girona reg., Meranges	1555 m	Dincă et al., 2015
JF853660	<i>Proclossiana eunomia</i>	Eu_22	Finland, Ostrobotnia borealis pars australis, Kiiminki		Mutanen et al., 2012
HM875824	<i>Proclossiana eunomia</i>	Eu_22	Finland, Ostrobotnia borealis pars australis, Kempele		Mutanen et al., 2012
HM875823	<i>Proclossiana eunomia</i>	Eu_23	Finland, Ostrobotnia borealis pars australis, Kempele		Mutanen et al., 2012
PHLAI555-13	<i>Proclossiana eunomia</i>	Eu_08	Austria, Tirol, Nordtirol reg., Reither Moor/ Seefeld S	1178 m	Huemer & Hebert, 2016
PHLAI572-13	<i>Proclossiana eunomia</i>	Eu_10	Austria, Tirol, Nordtirol reg., Gasthaus Stefansbruecke - Innsbruck S	700 m	Huemer & Hebert, 2016
KM572223	<i>Proclossiana eunomia</i>	Eu_10	Austria, Vorarlberg, Im Moos/ Bizau W	650 m	Huemer et al., 2014
GU676336	<i>Proclossiana eunomia</i>	Eu_15	Andorra, Refugi de Riu dels Orris, per sota del (vall del Madriu), Escaldes-Engordany	2180 m	Dincă et al., 2015
PHLAI571-13	<i>Proclossiana eunomia</i>	Eu_09	Italy, South Tyrol, Suedtirol reg., Fischerwiesen, Sankt Valentin auf der Heide	1450 m	Huemer & Hebert, 2016
PHLAI570-13	<i>Proclossiana eunomia</i>	Eu_09	Italy, South Tyrol, Südtirol reg., Reschen N		Huemer & Hebert, 2016
KX047880	<i>Proclossiana eunomia</i>	Eu_22	Norway, Nord-Trondelag, Lierne reg., Jakthuset, Nordli		Mutanen et al., 2016

Phylogenetic reconstruction

The sequences were edited and aligned using CHROMAS 2.4.3 (<http://www.technelysium.com.au/>), Geneious 8.1.6 (Kearse et al., 2012), and BioEdit 7.0.3 (Hall, 2011) software. The alignment was unambiguous, as all the sequences were of equal length and included no insertions/deletions. Primer sequences were cropped. A Bayesian approach for estimating phylogeny was used. Bayesian analyses were performed using the program MrBayes 3.2 (Ronquist et al., 2012), with the nucleotide substitution model GTR+G+I as suggested by jModelTest (Posada, 2008). TRACER, v1.4 was used for summarizing the results of Bayesian phylogenetic analyses (<http://beast.bio.ed.ac.uk/Tracer>). A median-joining haplotype network was built using PopART (Clement et al., 2002). Genetic distances were calculated for *COI* gene using MEGA v7.0.14 (Kumar, Stecher & Tamura, 2016).

Results and Discussion

The phylogenetic analysis based on the mitochondrial *COI* marker (Fig. 2) recovered all *P. eunomia* sequences in a highly supported clade (Bayesian posterior probability = 1). Within *P. eunomia* clade, no distinct intraspecific sublineages were recovered and monophly was not retrieved for any described subspecies, indicating a close evolutionary relationship among all the individuals of this species (Fig. 3). Specimens from Novosibirsk and Omsk region, identified as *P. eunomia riamia* was found to be paraphyletic with respect to other *P. eunomia* individuals. In general, *P. eunomia* appeared to be genetically homogenous with respect to *COI* (the average genetic distance among all studied specimens was $0.41\% \pm 0.11\%$), which is also reflected in the *COI* haplotype network (Fig. 4). Haplotype network analysis of a dataset of 64 specimens revealed 24 haplotypes, which however, cannot be

clustered in specific haplogroups. Each haplotype differs from neighboring haplotype not more than by two nucleotide substitutions. The analysis shows that there is no significant difference in the range of inter- and intrapopulation *COI* sequence divergence. Thus,

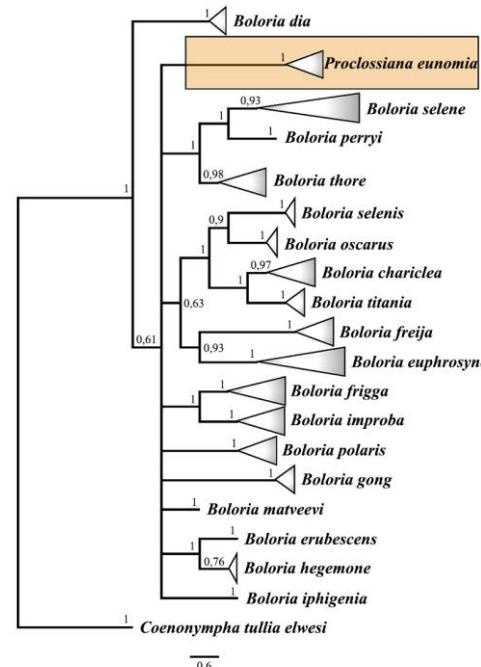


Fig. 2. The phylogenetic tree of the *Boloria* and *Proclossiana* species based on analysis of the *cytochrome oxidase subunit I* (*COI*) gene from 539 specimens. Numbers at nodes indicate Bayesian posterior probability. Branches with Bayesian posterior probability values >0.5 are shown. Scale bar = 0.6 substitutions per position. *Proclossiana eunomia* cluster highlighted in orange.

composition of each haplotype does not always reflect current taxonomic interpretations, the particular geographical areas and geographical distribution of butterflies. Unlike *COI* gene sequences, by which *P. eunomia* displayed remarkable genetic homogeneity given the geographical area covered, different populations do often demonstrate prominent morphological differences in wing pattern, wing shape and body size (Figs. 5–6).

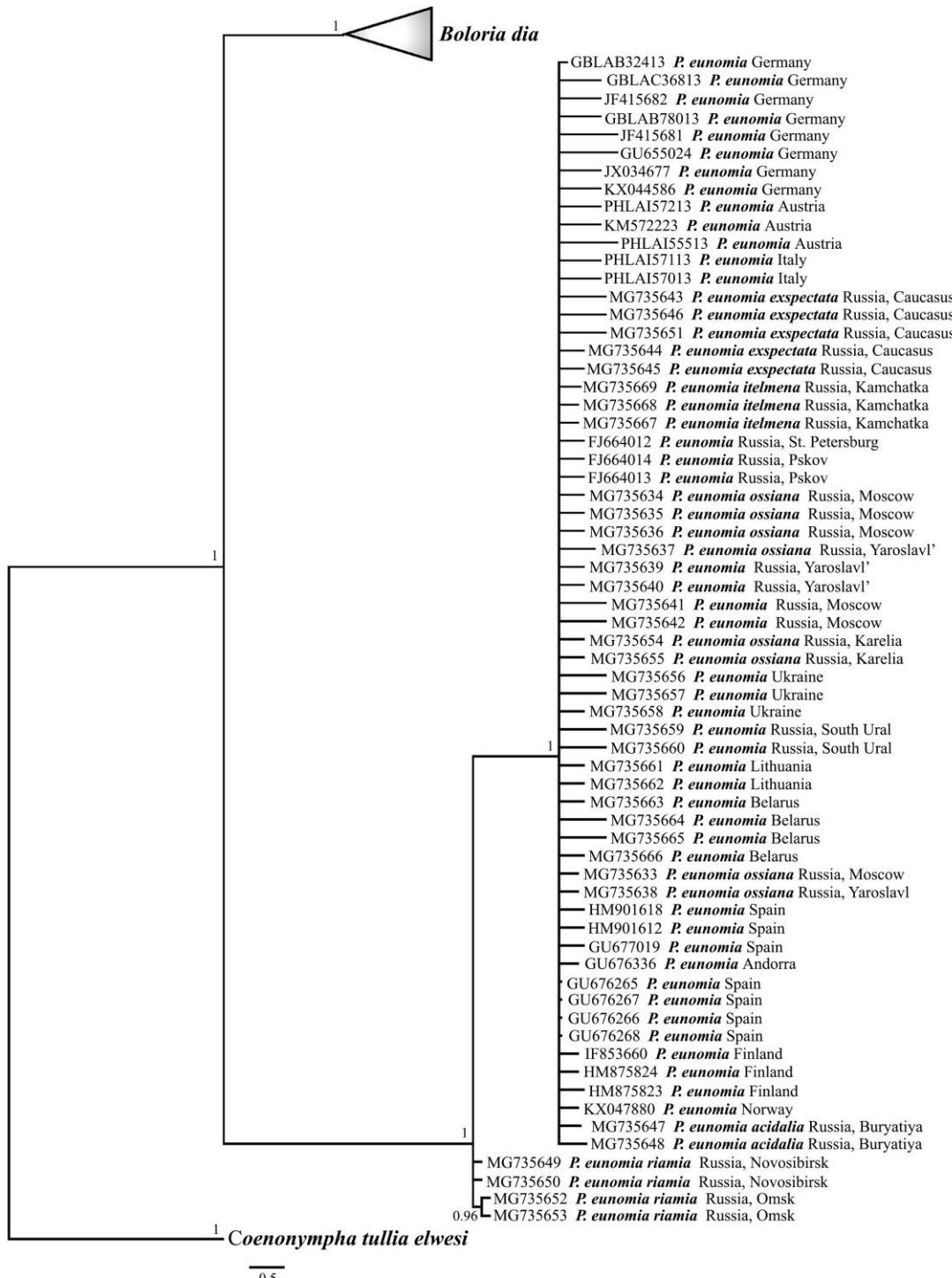


Fig. 3. Consensus Bayesian tree of the *Proclossiana eunomia* complex inferred from *COI* sequences. Numbers at nodes indicate Bayesian posterior probability. Branches with Bayesian posterior probability values >0,8 are shown. Scale bar = 0.5 substitutions per position

Proclossiana eunomia itelmena

Proclossiana eunomia itelmena was described on the basis of slight morphological differences in wing shape and wing coloration (the pattern on the upperside is thinner than in ssp. *acidalia*, the marginal spots on the hindwing underside are silver) (Gorbunov & Kosterin, 2007). Three specimens of *P. eunomia itelmena*, collected at the type locality (Kamchatka, Esso Village) form two haplotypes: **Eu_17** and **Eu_18** (Fig. 4). These haplotypes differ by one nucleotide substitution within the 628 bp region of the *COI* gene. At the same time, haplotype **Eu_17** of *P. eunomia itelmena* differs by only one nucleotide substitution from haplotypes **Eu_16**, which comprise specimens of *P. eunomia ossiana* from Spain (Cantabria) and **Eu_07**, which consists of two specimens collected in Moscow region and Yaroslavl region.

Proclossiana eunomia exspectata

Proclossiana eunomia exspectata was described as a separate subspecies on the basis of male genitalia characters (structure and shape of the valva and its costal process), the external morphological characters and the ecological preferences (Morgun, 2011). Five specimens of *P. eunomia exspectata*, collected at the type locality (Russia, North Caucasus, vicinity of Teberda, Nazylkol River valley) form two haplotypes: **Eu_12** and **Eu_13** (Fig. 4). These haplotypes differ by one nucleotide substitution within the 628 bp region of the *COI* gene. Haplotype **Eu_13** of *P. eunomia exspectata* differs by two nucleotide substitutions from haplotypes **Eu_16**, which comprise specimens of *P. eunomia ossiana* from Spain (Cantabria) and **Eu_07**, which consists of two specimens collected in Moscow region and Yaroslavl region.

Proclossiana eunomia riamia

Of the Asian range of the distribution, the most externally distinguished subspecies is *Proclossiana eunomia riamia*. *P. eunomia riamia* was described as a separate subspecies on the basis of larger wingspan and wing coloration (very dark and contrast background of the upperside of the wings, in particular, totally black marginal area on the hindwings; brick-red background of the hindwing underside; silver pale elements on the hindwing underside) (Korshunov, 1998). *P. eunomia riamia* found only associated with raised bogs (the so-called, ryams) (Fig. 7) in the south of West-Siberian Plain. Raised bogs, isolated from other mesophilic communities, significantly differ from the surrounding forest-steppe in flora (Valutskiy, 2011) and fauna of Papilionoidea (Knyazev, 2009; Ivonin et al., 2013; Yakovlev & Kareev, 2013). It was of special interest to study *P. eunomia riamia*, since it represents a remote population, separated by more than 300-350 km from the closest known locality of *P. eunomia* in Altai Republic. Four specimens of *P. eunomia riamia* collected at the type locality and Omsk region form three haplotypes: **Eu_19**, **Eu_20**, **Eu_21**. These haplotypes differ by 1-2 nucleotide substitutions within the 628 bp region of the *COI* gene. Haplotypes of *P. eunomia riamia* differs by at least two nucleotide substitutions from neighboring haplotype **Eu_16** (Fig. 4). Furthermore, *P. eunomia riamia* specimens have single fixed substitution (C→T) at position 42 of the *COI* gene, which never occurred in other studied representatives of *P. eunomia* and form a separated paraphyletic lineage in Bayesian inference tree.

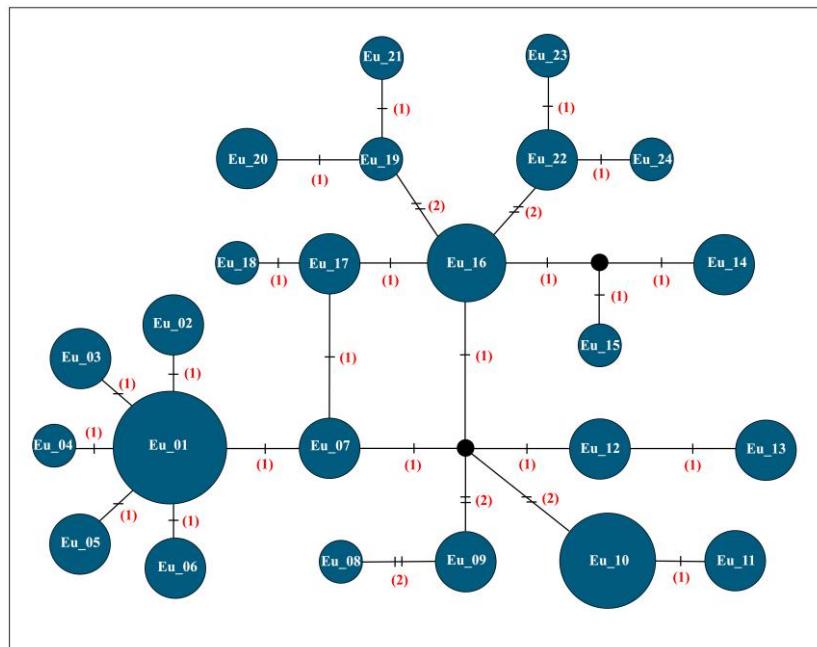


Fig. 4. Haplotype network based on *COI* barcodes. Every mutation is marked with a bar; number of mutations separating haplotypes is given in parentheses. The circle size is proportional to the number of samples represented.

Proclossiana eunomia acidalia

Proclossiana eunomia acidalia inhabits the taiga zone of the Urals and Siberia (up to western Transbaikal). The subspecies can be distinguished by thin strokes on the upperside of the wings, and light yellow (without silvery and pearly elements) pattern of the underside of the wings. Two specimens from Buryatia, identified as *P. eunomia acidalia* form two haplotypes (**Eu_24** and **Eu_22**), separated by one nucleotide substitution. One of these specimens (GenBank accession number MG735647) share *COI* haplotype with the samples of *P. eunomia* from Norway and Finland (Fig. 4, haplotype **Eu_22**).

To conclude, our results reveals little congruence between previous interpretations of the variously recognized subspecies of *P. eunomia* based mainly on geographical distribution and/or relatively minor morphological characters with molecular data (*COI* gene) obtained. We revealed high level of similarity in *COI* barcodes between all studied populations of *P. eunomia*. Thus, molecular data provide evidence to support a monotypic species hypothesis rather than the recognition of several subspecific forms, with the exception of *P. eunomia riamia*. *P. eunomia riamia* has noticeable morphological differences in wing pattern, accompanied by distinct position on the phylogenetic tree and presence of fixed nucleotide substitution, which does not allow us to synonymize this butterfly to any other subspecies of *P. eunomia*.

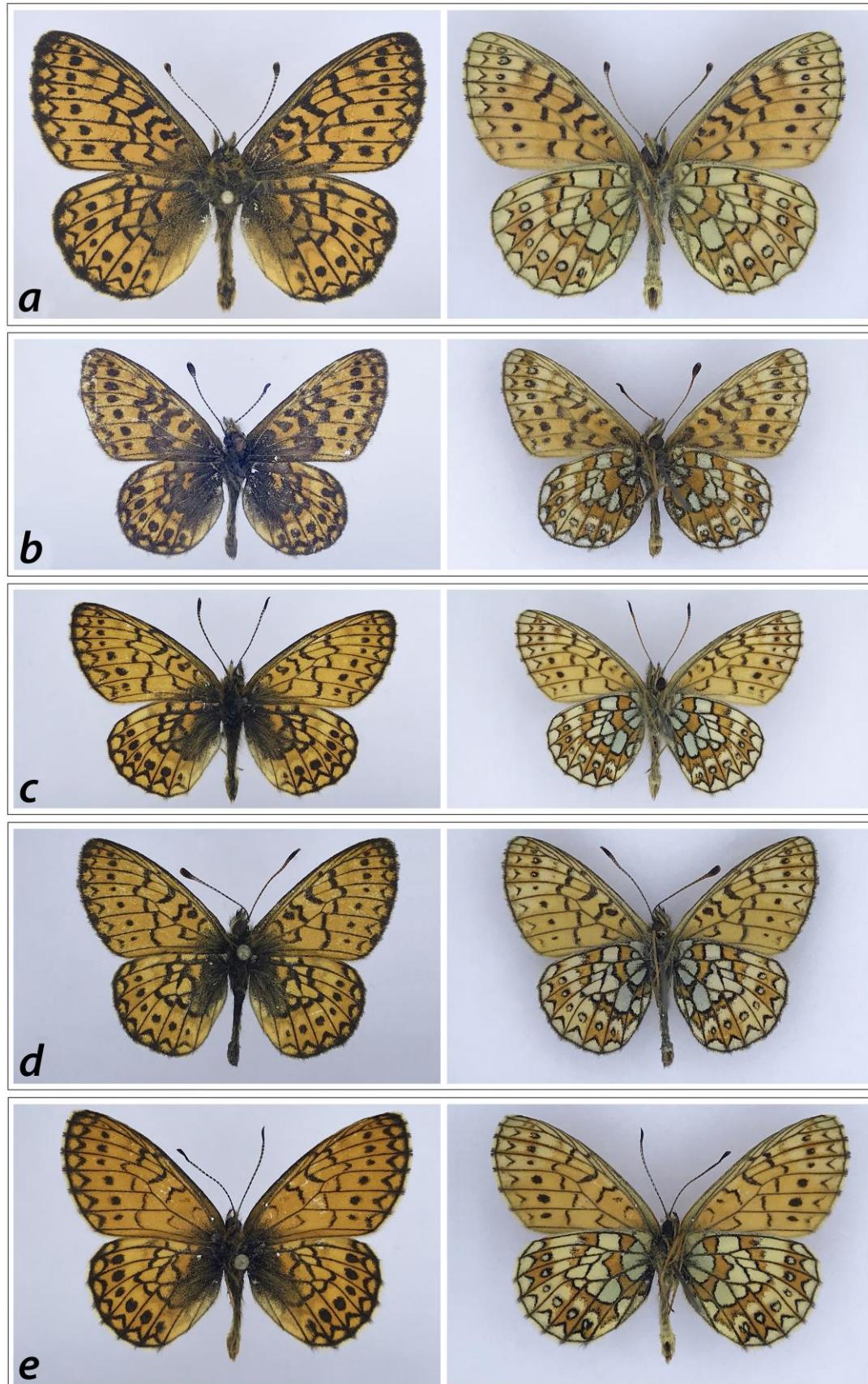


Fig. 5. *Proclassiana eunomia*, adult male specimens: upperside (right) and underside (left). **a)** *P. e. eunomia*, Ukraine, near Kiev, Belichi vill., tract Lyubka, 2.06.2011, leg. I. Pljutsch; **b)** *P. e. ossiana*, Norway, Finnmark, Gorgia, 16.06.1992, leg. I. Pljutsch; **c)** *P. e. acidalia* (topotype of *yakovlevi*), Russia, Altai, Ukok plateau, Mai-Pak, 2300 m, 29.06.1997, leg. R. Yakovlev; **d)** *P. e. acidalia*, Mongolia, Hovd aimak, Bulgan-gol basin, middle stream of Ulyasutaj-Gol river, 2500–3000 m, 25–26.06.2004, leg. R. Yakovlev & D. Ryzhkov; **e)** *P. e. acidalia* (topotype of *stromi*), Russia, S. Tuva, 20 km N Samagaltaj, 1200 m, 17.06.2001, leg. R. Yakovlev.

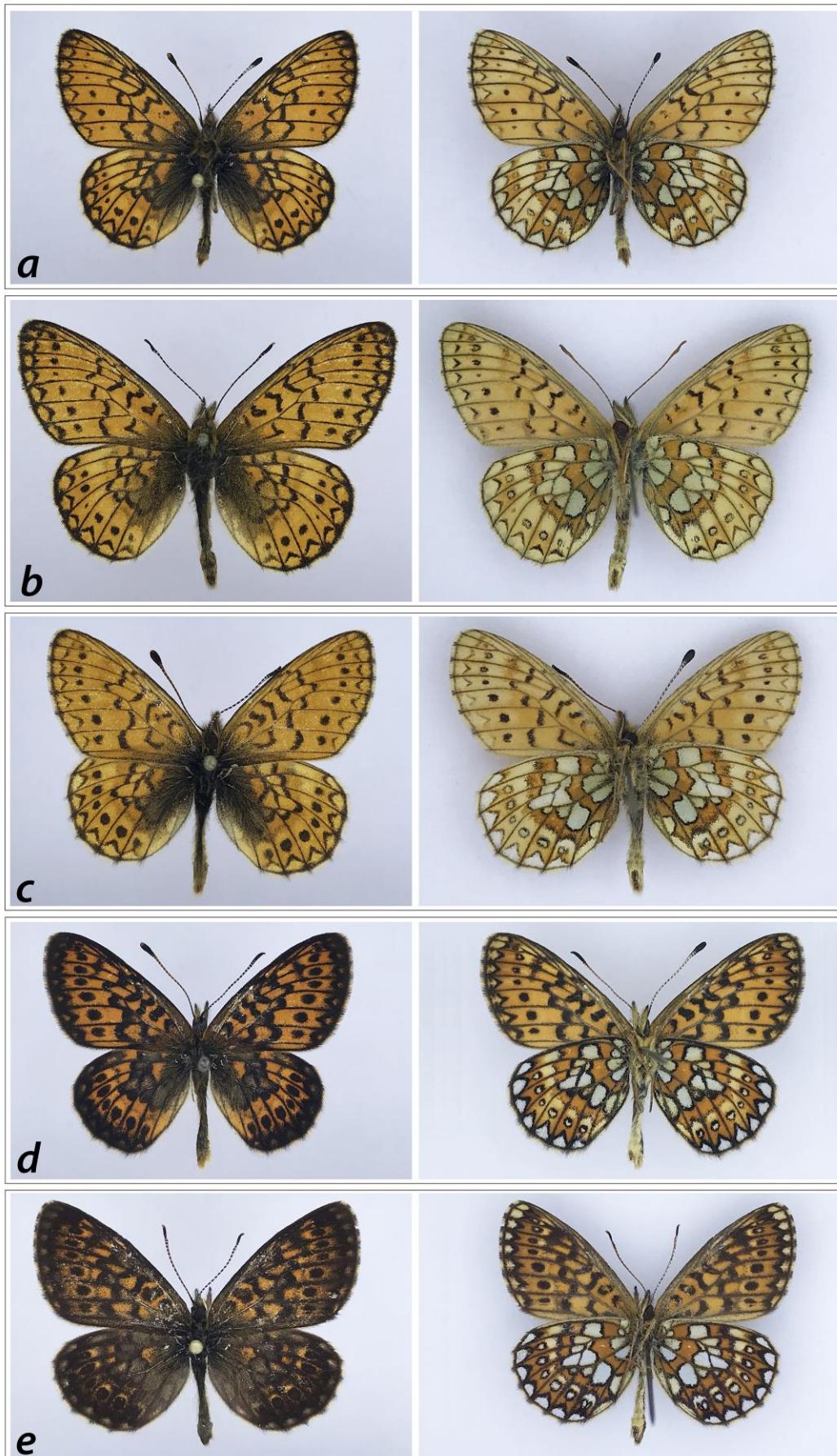


Fig. 6. *Proclossiana eunomia*, adult male specimens: upperside (right) and underside (left). **a)** *P. e. acidalia*, Russia, Buryatia, Tunkinski distr., Mondy vill., Khulugaisha Mt., 1800 m, 24–30.06.2016, leg. A. Kosarev; **b)** *P. e. acidalia*, Russia, Chelyabinsk reg., Katav-Ivanovsk env., 12.06.2007, leg. V. Zurilina; **c)** *P. e. itelmena*, Russia, Kamchatka, Esso vill., 600 m, 12.07.2003, ex coll. D. Goshko; **d)** *P. e. riamia*, Russia, Omsk reg., Krutinsky distr., 44 km NW Krutinka vill., 5 km SW Gulyai-Pole vill., 7.06.2016, leg. S. Knyazev; **e)** *P. e. riamia*, Russia, Novosibirsk reg., Chulym distr., near Kuznetsky vill., Kamenny Ryam, 19.06.2006, leg. V. Ivonin.

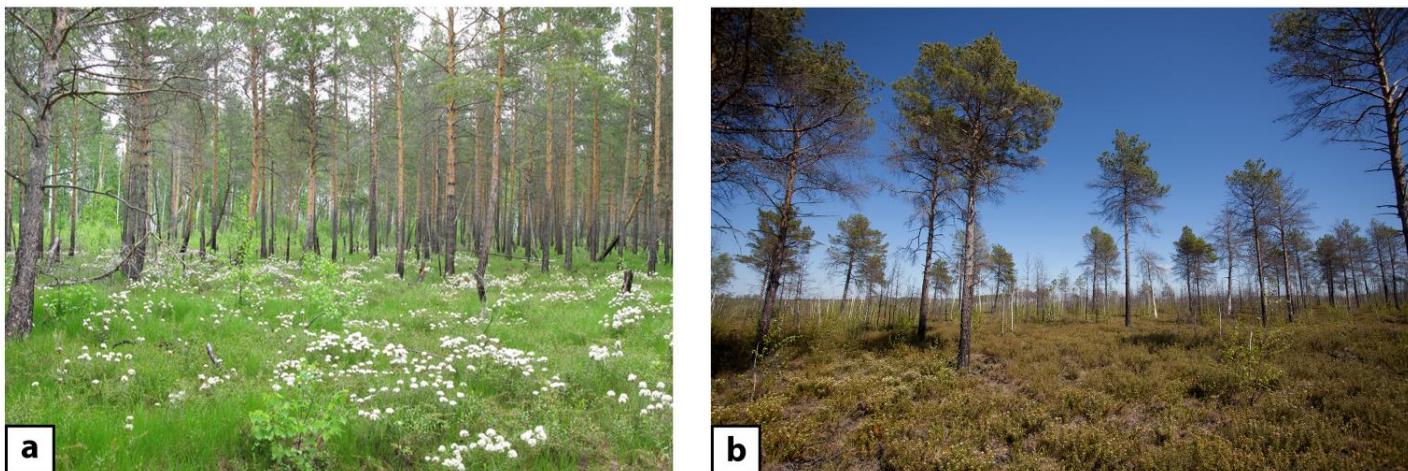


Fig. 7. Habitat of *P. eunomia riamia*. **a)** Novosibirsk reg., Gus'kovsky Ryam (photo by V. Ivonin); **b)** Omsk reg., "Orlovskoe zajmistiche" Ryam, 5 km SW Gulyaj Pole village (photo by S. Knyazev)

Acknowledgements

The authors are grateful to Prof. Boris Kondratieff (Fort Collins) for language editing. We thank S. Knyazev (Omsk), V. Dubatolov, V. Ivonin, A. Kareev (Novosibirsk), V. Gorbach (Petrozavodsk), A. Kosarev (Irkutsk), I. Pljutsch (Kiev) for providing samples of specimens, important for this study. The financial support for this study was provided by the grant No. 16-54-00118 Бел_а from the Russian Foundation for Basic Research and grant No. Б16Р-159 from Belorussian Foundation for Basic Research. The work was performed using equipment of the "Taxon" Research Resource Center (http://www.ckp-rf.ru/ckp/3038/?phrase_id=8879024) of the Zoological Institute of the Russian Academy of Sciences (St. Petersburg).

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Citation:

Yakovlev, R. V., Shapoval, N. A., Kuftina, G. N., Kulak, A.V., Kovalev, S.V. (2018). Notes on the molecular taxonomy of the *Proclossiana eunomia* complex (Lepidoptera, Nymphalidae: Argynnini): analysis of DNA barcodes. *Ukrainian Journal of Ecology*, 8(1), 222–232.



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