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Detecting cryptic species in sympatry and allopatry: analysis of hidden diversity in *Polyommatus* (*Agrodiaetus*) butterflies (Lepidoptera: Lycaenidae)

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Modern multilocus molecular techniques are a powerful tool in the detection and analysis of cryptic taxa. However, its shortcoming is that with allopatric populations it reveals phylogenetic lineages, not biological species. The increasing power of coalescent multilocus analysis leads to the situation in which nearly every geographically isolated or semi-isolated population can be identified as a lineage and therefore raised to species rank. It leads to artificial taxonomic inflation and as a consequence creates an unnecessary burden on the conservation of biodiversity. To solve this problem, we suggest combining modern lineage delimitation techniques with the biological species concept. We discuss several explicit principles on how genetic markers can be used to detect cryptic entities that have properties of biological species (i.e. of actually or potentially reproductively isolated taxa). Using these principles we rearranged the taxonomy of the butterfly species close to *Polyommatus* (*Agrodiaetus*) *ripartii*. The subgenus *Agrodiaetus* is a model system in evolutionary research, but its taxonomy is poorly elaborated because, as a rule, most of its species are morphologically poorly differentiated. The taxon *P.* (*A.*) *valiabadi* has been supposed to be one of the few exceptions from this rule due to its accurately distinguishable wing pattern. We discovered that in fact traditionally recognized *P. valiabadi* is a triplet of cryptic species, strongly differentiated by their karyotypes and mitochondrial haplotypes. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **116**, 468–485.

ADDITIONAL KEYWORDS: biological species concept – genetic lineages – karyotype – phylogenetic species concept – species delimitation – taxonomic inflation.

INTRODUCTION

Approaches to identification of cryptic species diversity

Cryptic species, morphologically indistinguishable or highly similar biological entities, seem to represent a substantial portion of plant and animal diversity (Beheregaray & Caccone, 2007; Pfenninger & Schwenk, 2007; Marchio & Piller, 2013; Marin *et al.*, 2013; Andrews *et al.*, 2014; Glazier & Etter, 2014; Nygren, 2014; Santos *et al.*, 2014), and therefore the search for these species is an urgent task of current biology. An estimation of cryptic species diversity is important for a better understanding of evolutionary processes and patterns of ecosystem functioning, and also has deep implications for nature conservation (Bickford *et al.*, 2006; Esteban & Finlay, 2010). Despite these facts, until recently, cryptic species detection was often not a result of particular biodiversity studies, but a by-product of biochemical, cytogenetic, ethological, physiological

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and other investigations (Lukhtanov & Shapoval, 2008; Lukhtanov, 2014; Miraldo *et al.*, 2014; Nygren, 2014).

A recently elaborated, more accurate approach to identification of cryptic species is based on analysis of genetic markers (Schlick-Steiner *et al.*, 2007; Dinca *et al.*, 2011). The recent increase in the number of reported cryptic species results from a growing number of studies including DNA-based techniques, especially large-scale approaches such as DNA barcoding, which often provide resolution beyond the boundaries of morphological information (Hebert *et al.*, 2003). DNA barcoding is becoming universally accepted as a useful tool for identification of known species and for detecting cryptic, previously unrecognized taxa (Hebert *et al.*, 2004; Barrett & Hebert, 2005; Janzen, Hajibabaei & Burns, 2005; Ward *et al.*, 2005; Hajibabaei *et al.*, 2006; Smith *et al.*, 2007; Lukhtanov *et al.*, 2009; Velzen, Larsen & Bakker, 2009; Vershinina & Lukhtanov, 2010; Sourakov & Zakharov, 2011; Lukhtanov, Shapoval & Dantchenko, 2014; but see Brower, 2006, 2010). It is particularly useful as an efficient start for taxonomic workflows (Kekkonen & Hebert, 2014). However, documenting cryptic diversity based on the DNA barcoding approach is generally not sufficient. Two groups of individuals may not belong to two different species even if there is a clear distinction between them in a single molecular marker. Not only interspecific differences, but also intrapopulation variability can result in such a bimodal distribution of a single molecular marker. Using mitochondrial molecular markers (e.g. the mitochondrial gene cytochrome c oxidase subunit I, COI) is especially risky for drawing conclusions about the presence of cryptic species (Donnelly *et al.*, 2013). Phenomena such as mitochondrial introgression (Zakharov *et al.*, 2009) and *Wolbachia* infection (Ritter *et al.*, 2013) can significantly increase the level of within-species genetic variation of mitochondrial markers, confusing impression of cryptic speciation (Ritter *et al.*, 2013). Generally, any conclusion based on a single gene (or a single morphological trait) can be misleading.

A more appropriate approach to species delimitation and the detection of cryptic taxa is based on the analysis of multiple markers (multilocus molecular data analysis in particular) (Tobias *et al.*, 2010; Yang & Rannala, 2010; Schwentner, Timms & Richter, 2011; Bacon *et al.*, 2012). The multilocus approach is especially effective when combined with Bayesian statistics and coalescence analysis, for example as implemented in the *BEAST 1.7.2 program (Heled & Drummond, 2010). However, in our opinion a serious shortcoming of this approach is that it, at least in cases of allopatric taxa, reveals phylogenetic lineages, but not biological (*sensu* Mayr, 1963) species. In other words, this species delimitation technique is based on the phylogenetic species concept (Cracraft, 1989) and inherited all its problems (Coyne & Orr, 2004), including a tendency for species oversplitting (Isaac, Mallet & Mace, 2004). The increasing power of coalescent multilocus analysis has led to the situation where nearly every geographical isolated or semi-isolated population can be identified as a lineage and therefore raised to species rank.

We believe that the next step in the development of better methods of cryptic species identification is a combination of modern lineage delimitation techniques with the biological species concept (BSC). The BSC (Poulton, 1904; Mayr, 1963) suggests that populations should be classified as separate species if they represent actually or potentially reproductively isolated entities. That isolation does not have to be complete, but should be strong enough to prevent taxa from blending together when they become sympatric (Mayr, 1963; Coyne & Orr, 2004). The biological species can be polytypic (Poulton, 1904; Mayr, 1963), i.e. can consist of morphologically and molecularly differentiated groups of populations. Under the polytypic species concept diagnosable allopatric entities can be classified as subspecies within the same species, unless there is evidence for reproductive isolation, or the taxa would be unlikely to fuse when they occur in sympatry. The polytypic species concept differs from the phylogenetic species concept (PSC) (Cracraft, 1989; Coyne & Orr, 2004), in which diagnosable entities can be classified as species regardless of whether they blend together in zones of overlap. In spite of this difference, these two concepts are compatible, because subspecies under the BSC can be often classified as species under the PSC. In our opinion, the polytypic BSC is preferable because in taxonomic practice, application of the PSC often results in proliferation of multiple weakly justified species names (Isaac *et al.*, 2004). Uncritical use of the PSC (Cracraft, 1989) leads to artificial taxonomic inflation (Isaac *et al.*, 2004) and as a consequence creates an unnecessary burden on the conservation of biodiversity (Zachos *et al.*, 2013).

AGRODIAETUS BUTTERFLIES

The subgenus *Agrodiaetus* is a monophyletic group within the genus *Polyommatus* (Talavera *et al.*, 2013). Adults of *Agrodiaetus* have a wingspan of only 2–4 cm, and the sexes are often dimorphic, with females typically brown and males blue on the upper surface of their wings. From the point of view of taxonomy, *Agrodiaetus* appears to be one of the most complicated groups of butterflies (Vila *et al.*, 2010). It displays one of the highest known rates of species

diversification (Coyne & Orr, 2004; Kandul *et al.*, 2004) and karyotype evolution (Lukhtanov *et al.*, 2005; Kandul, Lukhtanov & Pierce, 2007; Vershinina, Anokhin & Lukhtanov, 2015). The subgenus includes numerous morphologically indistinguishable cryptic species (Hesselbarth, Oorchot & Wagener, 1995; Lukhtanov, Shapoval & Dantchenko, 2008). The majority of these species are concentrated in the so-called 'brown' species complex, or *P. ripartii*-complex, which unites two sister clades (Wiemers, 2003; Kandul *et al.*, 2004, 2007; Lukhtanov *et al.*, 2005; Vila *et al.*, 2010; Dinca *et al.*, 2013; Przybyłowicz, Lukhtanov & Lachowska-Cierlik, 2014): the *dolus* group and the *admetus* group. With few exceptions, the species of these two groups have a characteristic feature: both males and females have similar brown coloration of the upperside of the wings. As a rule, the species of these groups are also similar in genitalia structure (Kolev, 2005) and wing pattern (Hesselbarth *et al.*, 1995). The species *P. valiabadi sensu* auctorum has been supposed to be one of the few exceptions from this rule due to exaggerated spots on the hind wing underside.

Here we use a combination of chromosomal and DNA mitochondrial markers to demonstrate that *P. valiabadi sensu* auctorum is a triplet of cryptic species, strongly differentiated by their karyotypes and mitochondrial haplotypes.

MATERIAL AND METHODS

Insects

Population samples of different taxa of the subgenus *Polyommatus* (*Agrodiaetus*) were collected during the period 2002–2007 in Iran and Azerbaijan (Table 1). We paid particluar attention to visiting the type-localities of described taxa. In particular, we visited the vicinity of Valiabad village in northern Iran (the type locality of *P. valiabadi*) and Zuvand plateau in south-east Azerbaijan (the type locality of *P. rjabovianus*) (Fig. 1).

After capturing a butterfly in the field, we placed it in a glassine envelope for 1–2 h to keep it alive until we processed it. Testes were removed from the abdomen and placed in a small 0.5-mL vial with a freshly prepared fixative (ethanol and glacial acetic acid, 3:1). Then each wing was carefully removed from the body using forceps. The wingless body was placed in a plastic 2-mL vial with pure 96% ethanol. Thus, after fixation we had three components collected for each butterfly: (1) a vial containing the butterfly testes (for karyotype analysis), (2) a vial containing the butterfly wingless body (for DNA analysis) and (3) a glassine envelope containing the wings. The set specimens of the donor butterflies are kept in the Zoological Institute of the Russian Academy of Science (St. Petersburg).

Chromosome preparation and karyotyping

Testes were stored in the fixative for 1–12 months at 4 °C. Then the gonads were stained in 2% acetic orcein for 30–60 days at 18–20 °C. Different stages of male meiosis were examined by using a light microscope (Amplival, Carl Zeiss). We used an original two-phase method of chromosome analysis (Lukhtanov & Dantchenko, 2002; Lukhtanov, Vila & Kandul, 2006).

DNA extraction and sequencing preparation

For analysis we used a 653-bp fragment within the mitochondrial COI gene, and 572-bp fragment within a nuclear marker, internal transcribed spacer 2 (ITS2). DNA extraction, amplification and further preparations for sequencing were held on the base of 'Laboratory of Animal Genetics' of Saint-Petersburg State University and Chromas Core Facility, Saint-Petersburg State University Research Park.

The first two abdominal segments were used for DNA extraction. The segments were homogenized in lysis buffer [25 mmol L^{-1} EDTA, 75 mmol L^{-1} NaCl, 10 mmol L^{-1} Tris (pH 7.5)]. After adding 10% SDS and proteinase K (20 mg μL^{-1}) the samples were incubated for 2 h at 60 °C. For DNA extraction from lysate we used a standard method for nucleic acid purification by extraction first with phenol/chloroform (1:1) and then with chloroform to remove any remaining phenol (Sambrook & Russell, 2006). This method was chosen instead of the commonly used CTAB method because the latter gave some by-products. DNA was precipitated with isopropyl alcohol in the presence of 0.1 mol L^{-1} NaCl and pelleted by centrifugation. The pellets were washed with 70% ethanol and dissolved in ddH_2O. Extracted DNA samples were stored at −20 °C.

For DNA amplification for COI we used primers *COI*-F1 5′-CCACAAATCATAAAGATATTGGAC-3′ and *COI*-R1 5′-TGATGAGCTCATACAATAAATCC TA-3′, and for *ITS2* we used primers ITS2F 5′-CATA TGCCACACTGTTCGTCTG-3′ and ITS2R 5′-GATAT CCGTCAGCGCAACG-3′.

The polymerase chain reaction (PCR) was carried out with Taq-polymerase (Sileks) in 20 μL of PCR buffer containing $MgCl_2$ [2.5 mmol L^{-1}], dNTP [200 μmol L^{-1} each] and forward and reverse primer [20 pmol each]. Amplification for COI was carried out with the following conditions: initial denaturation at 94 °C for 3 min, then 30 cycles of 30 s at 94 °C, 30 s at 51 °C (the annealing temperature) and

Table 1. List of specimens included in this study

Sample and GenBank codes	Species	Chromosome number	COI haplogroup	Locality, date and collector(s)
1996A06	*pseudorjabovi*	*n* = *c*. 79		Azerbaijan, Talysh, Zuvand, Goveri, 20 June 1995, A. Dantchenko leg.
2003F818 KR265490	*pseudorjabovi*	*n* = *c*. 79	p	Azerbaijan, Talysh, Mistan, 38.656330°N, 48.414607°E, 1700 m alt., 28 July 2003, A. Dantchenko & V. Lukhtanov leg.
2003F819 KR265491	*pseudorjabovi*	*n* = 79	p	As above
2003F820 AY954020 Holotype	*pseudorjabovi*	*n* = 79	p	As above
2003F821 KR265487	*pseudorjabovi*	*n* = 79	p	As above
2003F822 KR265488	*pseudorjabovi*	*n* = *c*. 79		As above
2003F838 KR265496	*pseudorjabovi*	*n* = 79	p	As above
2014A01 KR265480	*pseudorjabovi*		p	As above
2014A01 KR265481	*pseudorjabovi*		p	As above
2014A03 KR265482	*pseudorjabovi*		p	As above
2014A04 KR265483	*pseudorjabovi*		p	As above
2014A05 KR265484	*pseudorjabovi*		p	As above
2014A09 KR265500	*pseudorjabovi*		p	As above
2014A11 KR265489	*pseudorjabovi*		p	As above
2003F816	*rjabovianus rjabovianus*	*n* = 49	r	As above
2003F817 KR265477	*rjabovianus rjabovianus*	2*n* = 98	r	As above
2003F824 KR265478	*rjabovianus rjabovianus*	*n* = 49	r	As above
2003F830 KR265475	*rjabovianus rjabovianus*	*n* = 49	r	As above
2003F836 KR265476	*rjabovianus rjabovianus*	*n* = 49	r	As above
2014A10 KR265479	*rjabovianus rjabovianus*		r	As above
2002Mas3 KR265497	*rjabovianus masul*	*n* = 43	r	Iran, Gilan, vicinity of Masuleh, 37.185°N, 048.906°E, 2200 m alt., 3 August 2002, A. Dantchenko & V. Lukhtanov leg.
2002Mas4	*rjabovianus masul*	*n* = 43		As above
2002VL474 Holotype	*rjabovianus masul*	*n* = 43	r	As above
2002VL476	*rjabovianus masul*	2*n* = *c*. 86		As above
2002VL477	*rjabovianus masul*	*n* = *c*. 43		As above
2014A06 KR265485	*rjabovianus masul*		r	As above
2014A07 KR265498	*rjabovianus masul*		r	As above
2014A08 KR265499	*rjabovianus masul*		r	As above
2007Z860	*valiabadi*	*n* = 23		Iran, Mazandaran, Valiabad, 36°15′N; 051°18′E, 2100 m alt., 3 August 2007, V. Lukhtanov & N. Shapoval leg.
2007Z861	*valiabadi*	2*n* = 46		As above
2007Z863	*valiabadi*	*n* = 23		As above
2007Z864	*valiabadi*	2*n* = 46		As above
2007Z865	*valiabadi*	*n* = 23		As above
2007val_01 KR265495	*valiabadi*	*n* = 23	v	As above
2007val_02 KR265486	*valiabadi*	*n* = 23	v	As above

Table 1. *Continued*

Sample and GenBank codes	Species	Chromosome number	COI haplogroup	Locality, date and collector(s)
2003F482	*khorasanensis*	$n = 84$		Iran, Razavi Khorasan, Kuh-e-Sorkh Mts, S of Fariman, 35°28′N; 059°52′E, 1800 m alt., 8 July 2003, A. Dantchenko & V. Lukhtanov leg.
2003F484	*khorasanensis*	$n = 84$		As above
2003F486	*khorasanensis*	$n = 84$		As above
2003F511	*khorasanensis*	$n = 84$		As above
2003F526 AY954013	*khorasanensis*	$n = 84$	k	Iran, Razavi Khorasan, Kopetdagh Mts, 15 km N Quchan, 37°15′N; 058°34′E, 10 July 2003, A. Dantchenko & V. Lukhtanov leg.
2003F527	*khorasanensis*	$n = 84$		As above
2003F528	*khorasanensis*	$n = 84$		As above
2003F538	*khorasanensis*	$n = 84$		As above
2003F858	*admetus malievi*	$n = 78$		Azerbaijan, Talysh, Zuvand, Gasmolyan, 38.656°N; 048.415°E, 1700 m alt., 29 July 2003, A. Dantchenko & V. Lukhtanov leg.
2003F902 KJ906515	*admetus malievi*	$n = c.$ 70–80	a	Azerbaijan, Talysh, Mistan, 38.656330°N, 48.414607°E, 1700 m alt., 30 July 2003, A. Dantchenko & V. Lukhtanov leg.
2003F903 EF104617	*admetus malievi*	$n = 79$	a	As above
2003F905	*admetus malievi*			As above
2003F944	*admetus malievi*	$2n = c.$ 158–160		Azerbaijan, Talysh, Mistan, 38.656330°N, 48.414607°E, 1700 m alt., 31 July 2003, A. Dantchenko & V. Lukhtanov leg.
2003F947	*admetus malievi*	$n = 79$		As above
admetus_1	*admetus* ssp.		a	Turkey, Gümüshane Prov., Kilkit, July 2001, A. Dantchenko & V. Lukhtanov leg.

Sample ID, collection data, GenBank accession codes and chromosome number for specimens are shown.

30 s at 72 °C. Amplification for ITS2 was carried out with the following conditions: initial denaturation at 94 °C for 2 min, then 30 cycles of 30 s at 94 °C, 30 s at 60 °C (the annealing temperature) and 30 s at 72 °C.

After amplification, PCR mix was loaded in 1% agarose and specific product was separated by gel electrophoresis. Pieces of gel, containing the required DNA fragment, were cut out, mixed with 3× excess of 7 mol L^{-1} NaJ solution and incubated under vortex conditions until the gel completely dissolved. Then, double-stranded DNA was purified using the method of 'DNA purification from agarose gels with MP@SiO_2 magnetic particles' according with the manufacturer's protocol (Sileks). Purified DNA fragments were extracted with ddH_2O from magnetic particles pelleted with magnetic rack and collected in a fresh tube. The concentration of purified DNA was analysed via gel electrophoresis.

Sequencing of double-stranded product was carried out at the Research Resource Center for Molecular and Cell Technologies.

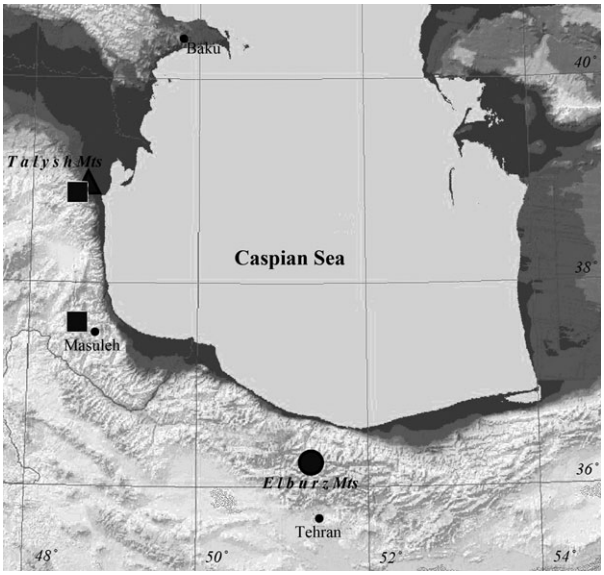

Figure 1. Distribution ranges of *P. pseudorjabovi* (▲), *P. rjabovianus* (■) and *P. valiabadi* (●).

Sequences and phylogeny analyses

The analysis involved 112 COI sequences (including outgroup). Among them there were 88 GenBank sequences and 24 new sequences. These samples represent all the 21 known species of the *P. ripartii* complex (Figs 3, 4). We used all the COI sequences available from GenBank (Wiemers, 2003; Kandul *et al.*, 2004, 2007; Vila *et al.*, 2010) except for the samples that had low overlap with our target COI region and few samples of unclear identity for which the chromosome data were not available. The length of GenBank sequences varied from 665 to 2286 bp. Sequences were aligned using BioEdit software (Hall, 1999) and edited manually. The final data set alignment contained overlapping sequences of different length. It included 2286 sites, with 377 variable sites and 268 parsimony-informative sites. The nucleotide frequencies were A = 32.90%, T/U = 36.75%, C = 16.66% and G = 13.69%. The transition/transversion rate ratios were k1 = 6.014 (purines) and k2 = 7.02 (pyrimidines). The overall transition/transversion bias was $R = 2.816$, where $R = [A*G*k1 + T*C*k2]/[(A + G)*(T + C)]$. The homogeneity test of substitutional patterns did not reject the null hypothesis that sequences have evolved with the same pattern of substitution. The disparity index indicated no larger differences in base composition biases than expected based on evolutionary divergence between the sequences and by chance alone.

The ITS2 analysis involved 49 sequences. Among them there were 43 GenBank sequences (including two specimens of our target species *P. valiabadi*) (Wiemers, 2003) and six new sequences (three specimens of *P. pseudorjabovi* and three specimens of *P. rjabovianus masul*). The length of GenBank sequences varied from 540 to 700 bp. Sequences were aligned using BioEdit software (Hall, 1999) and edited manually. The final data set alignment contained overlapping sequences of different length and included 702 sites, with 45 variable sites and 18 parsimony-informative sites. The nucleotide frequencies were A = 25.00%, T/U = 25.00%, C = 25.00% and G = 25.00%. The estimated transition/transversion bias (R) was 3.55.

Phylogenetic hypotheses were inferred using Bayesian inference (BI), maximum-likelihood (ML) and maximum-parsimony (MP) analyses. jModelTest was used to determine optimal substitution models for BI and ML analyses (Posada, 2008).

Bayesian analyses were performed using the program MrBayes 3.1.2. The program ran 10 000 000 generations with default settings. Two runs of 10 000 000 generations with four chains (one cold and three heated) were performed. Chains were sampled every 1000 generations, and burn-in was determined based on inspection of log likelihood over time plots using TRACER, version 1.4 (available from http://beast.bio.ed.ac.uk/Tracer).

The ML trees were inferred by using MEGA5 (Tamura *et al.*, 2011).

MP analysis was performed using a heuristic search as implemented in MEGA5 (Tamura *et al.*, 2011). A heuristic search was carried out using the close-neighbour-interchange algorithm with search level 3 (Nei & 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.

Museum work

The main zoological collections containing specimens of the *P.* (*A.*) *valiabadi*-complex from Iran and Transcaucasus and type series of *Agrodiaetus rjabovi* Forster, 1960 and *Agrodiaetus valiabadi* Rose et Schurian, 1977 were investigated: Zoologische Staatssammlung, Munich, Germany (ZSSM), Staatliches Museum für Naturkunde, Karlsruhe, Germany (SMNK), Zoological Museum of the Moscow State University, Moscow, Russia (ZMMU) and State Darwin Museum, Moscow, Russia (SDM).

RESULTS

Karyotypes (Table 1, Fig. 2)

Polyommatus pseudorjabovi

The haploid chromosome number $n = 79$ was found in meiotic metaphase I (MI) and meiotic metaphase

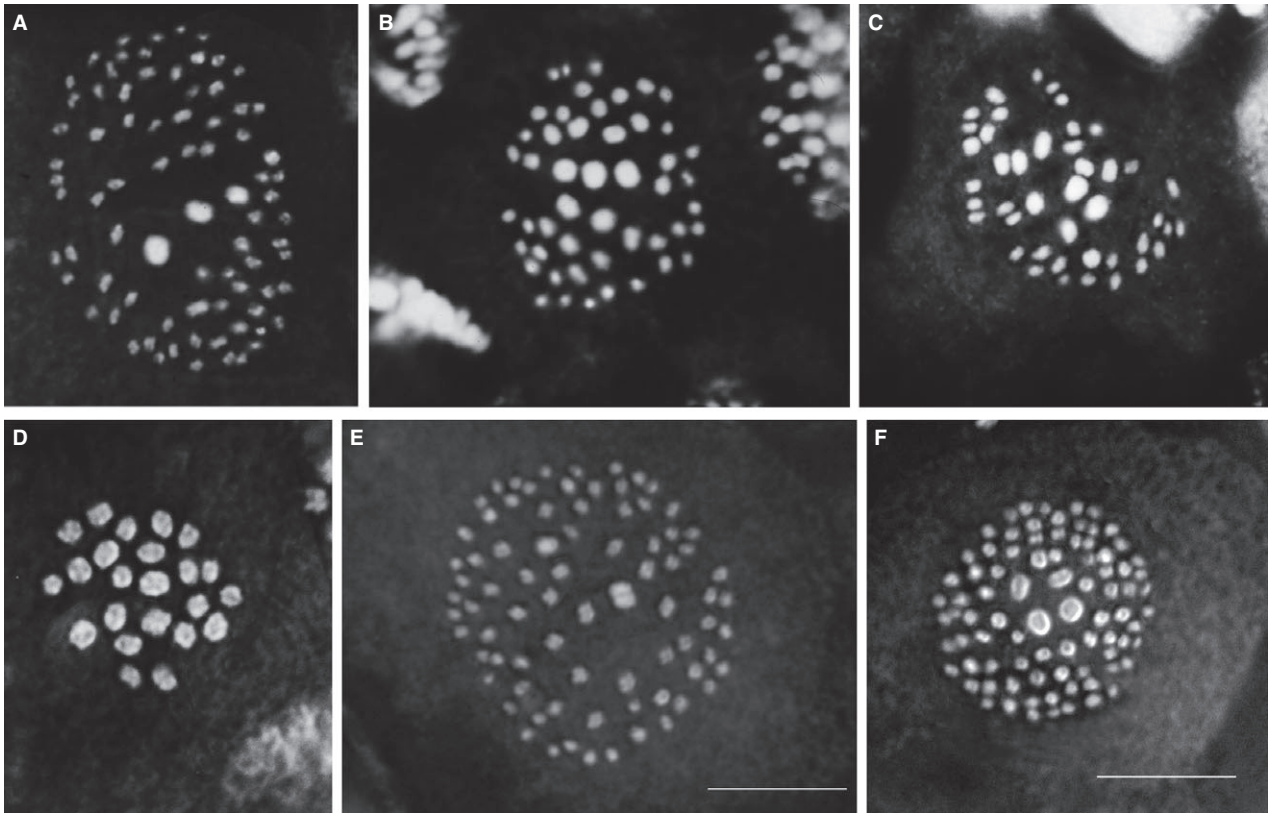

Figure 2. Male meiosis I karyotypes of: *P. pseudorjabovi*, $N = 79$ (A); *P. rjabovianus rjabovianus*, $N = 49$ (B); *P. rjabovianus masul*, $N = 43$ (C); *P. valiabadi*, $N = 23$ (D); *P. admetus malievi*, $N = 79$ (E); and *P. khorasanensis*, $N = 84$ (F). Scale bar = 10 μm in all figures.

II (MII) cells of four studied individuals. In three specimens, we counted approximately $n = 79$ in meiotic MI. The last count was done with an approximation due to the overlapping of some bivalents. The karyotype displayed three large bivalents in the centre of metaphase plates. Bivalent 1 was 1.2–1.5 times larger than bivalent 2, and the latter was 1.2–1.5 times larger than bivalent 3.

Polyommatus rjabovianus rjabovianus

The haploid chromosome number $n = 49$ was found in meiotic MI and MII cells of four studied individuals. In one specimen, the diploid chromosome number $2n = 98$ was observed in male asynaptic meiosis. The meiotic karyotype was strongly asymmetric with 6–10 larger bivalents in the centre of the metaphase plate and 39–43 smaller bivalents in the periphery. However, the larger and smaller bivalents do not form two distinct size groups, but a gradient size row in which the largest bivalents are approximately 10 times larger than the smallest bivalents.

Polyommatus rjabovianus masul

The haploid chromosome number $n = 43$ was found in MI and MII cells of three studied individuals. In one specimen, we counted approximately $n = 43$ in MI. The last count was an approximation due to the overlapping of some bivalents. In one specimen, the diploid chromosome number was estimated as $2n = 86$ in male asynaptic meiosis. The meiotic karyotype was strongly asymmetric with 6–8 larger bivalents in the centre of the metaphase plate and 35–37 smaller bivalents in the periphery. However, the larger and smaller bivalents do not form two distinct size groups, but a gradient size row in which the largest bivalents are approximately 10 times larger than the smallest bivalent.

Polyommatus valiabadi

The haploid chromosome number $n = 23$ was found MI and MII cells of five studied individuals. In two specimens, the diploid chromosome number was estimated as $2n = 46$ in male asynaptic meiosis. In MI cells, all bivalents formed a gradient size row. The karyotype contained no exceptionally large or small bivalents.

Polyommatus admetus malievi

The haploid chromosome number $n = 79$ was found in MI and MII cells of two studied individuals. The haploid chromosome number $n = 78$ was found in MI

cells of one studied individual. In one specimen, we counted approximately $n = 78–80$ in MI. The last count was an approximation due to the overlapping of some bivalents. In one specimen, the diploid chromosome number $2n = 158–160$ was estimated in male asynaptic meiosis. The karyotype displayed one larger bivalent in the centre of the metaphase plate.

Polyommatus khorasanensis

The haploid chromosome number $n = 84$ was found in MI and MII cells of eight studied individuals. In the karyotype, four larger bivalents and 80 smaller bivalents were observed. The two largest bivalents (1 and 2) were almost equal in size, and bivalents 3 and 4 were 1.4–1.5 times smaller than bivalents 1 and 2. As in other *Agrodiaetus* species (Lukhtanov & Dantchenko, 2002), the small bivalents formed a regular circle, whereas the four large bivalents occupied a position in the middle of the circle.

Analysis of COI clusters

Our analysis recovered the *dolus* (Fig. 3) and *admetus* (Fig. 4) species groups as strongly supported monophyletic clades. This agrees with results of other studies (Wiemers, 2003; Kandul *et al.*, 2004, 2007; Lukhtanov *et al.*, 2005; Vershinina & Lukhtanov, 2010; Vila *et al.*, 2010). Within each of these two main groups, many clades are well supported, whereas some of the relationships are not fully resolved.

The most intriguing result of the COI analysis is that the specimens that were collected in Talysh (Azerbaijan) and were without identified in the field as *rjabovianus* were divided into two only distantly related clusters: one cluster within the *dolus* group (*rjabovianus*) and the other within the *admetus* group (described as a novel species *P. pseudorjabovi* sp. nov. in the Appendix).

Within the *dolus* group the first cluster (Fig. 3) unites four specimens of *P. valiabadi* collected in northern Iran. The second cluster unites representatives of several taxa inhabiting Europe, Turkey and Iran. The third cluster unites specimens of *P. rjabovianus* collected in Azerbaijan and Iran.

Within the *admetus* group, one of the clusters comprises specimens of *P. ripartii* from Europe and Siberia and *P. nephohiptamenos*. The next cluster comprises *P. demavendi* and *P. ripartii* from Turkey, Iran and Armenia. The third weakly supported cluster unites three well-supported lineages: *P. khorasanensis*, *P. pseudorjabovi* and *P. admetus*.

Analysis of ITS2 clusters

The ITS2 sequences obtained in this study as well as the ITS2 sequences obtained from GenBank showed low variability between closely related taxa. As found previously (Vila *et al.*, 2010), the BI, ML and MP trees generated exclusively from ITS2 data (not shown) recover only the deepest nodes defining the *dolus* and the *admetus* species groups as distinct highly supported clades. *Polyommatus pseudorjabovi* was included in the *admetus* species group. The analysis also revealed *P. valiabadi* + *P. rjabovianus* as a distinct clade whose placement on the tree remained unresolved. Thus, the utility of ITS2 is limited, although, because it is a nuclear marker, it independently confirms that our target sympatric taxa *P. pseudorjabovi* and *P. rjabovianus* belong to two different clusters of individuals.

Museum work

We examined the holotype and paratypes of *P.* (*A.*) *rjabovianus* (Koçak, 1980) (= *Agrodiaetus rjabovi* Forster, 1960, the latter name is invalid as the secondary homonym of *Lycaena thersites rjabovi* Obraztsov, 1936, currently *Polyommatus thersites rjabovi*) preserved in ZSSM and revealed that all males with the karyotype $n = 49$ fit completely to the holotype specimen. These males are very close externally to males with $n = 79$ (including three large chromosome pairs) collected in the same biotope but differ from the latter in having barely perceptible, but nearly discrete features. The males with $n = 49$ have slightly darker brown colour, sharper submarginal lunulae on the underside of the hindwings and fine but clear pronounced submarginal lunulae on the underside of the forewings. These features are slight but allow us to regard the cluster with $n = 49$ and the holotype of *Agrodiaetus rjabovi* as representing the same species with certainty.

We also revealed that the holotype specimen of *P.* (*A.*) *valiabadi* (originally described as *Agrodiaetus rjabovi valiabadi*) preserved in the collection of K. Schurian, Germany, and all studied males with $n = 23$ [collected from the type locality of *P.* (*A.*) *valiabadi*] are externally undistinguishable and thus regarded as belonging to the same species with certainty.

DISCUSSION

Detecting cryptic species in sympatry: *P. rjabovianus* and *P. pseudorjabovi*

To distinguish between inter- and intraspecific variations, biologists often use a genotypic cluster approach (Mallet, 2001, 2006; Mallet & Willmott, 2003), in which a bimodal distribution of genetic and morphological characters of specimens in an area is considered evidence of the existence of two species.

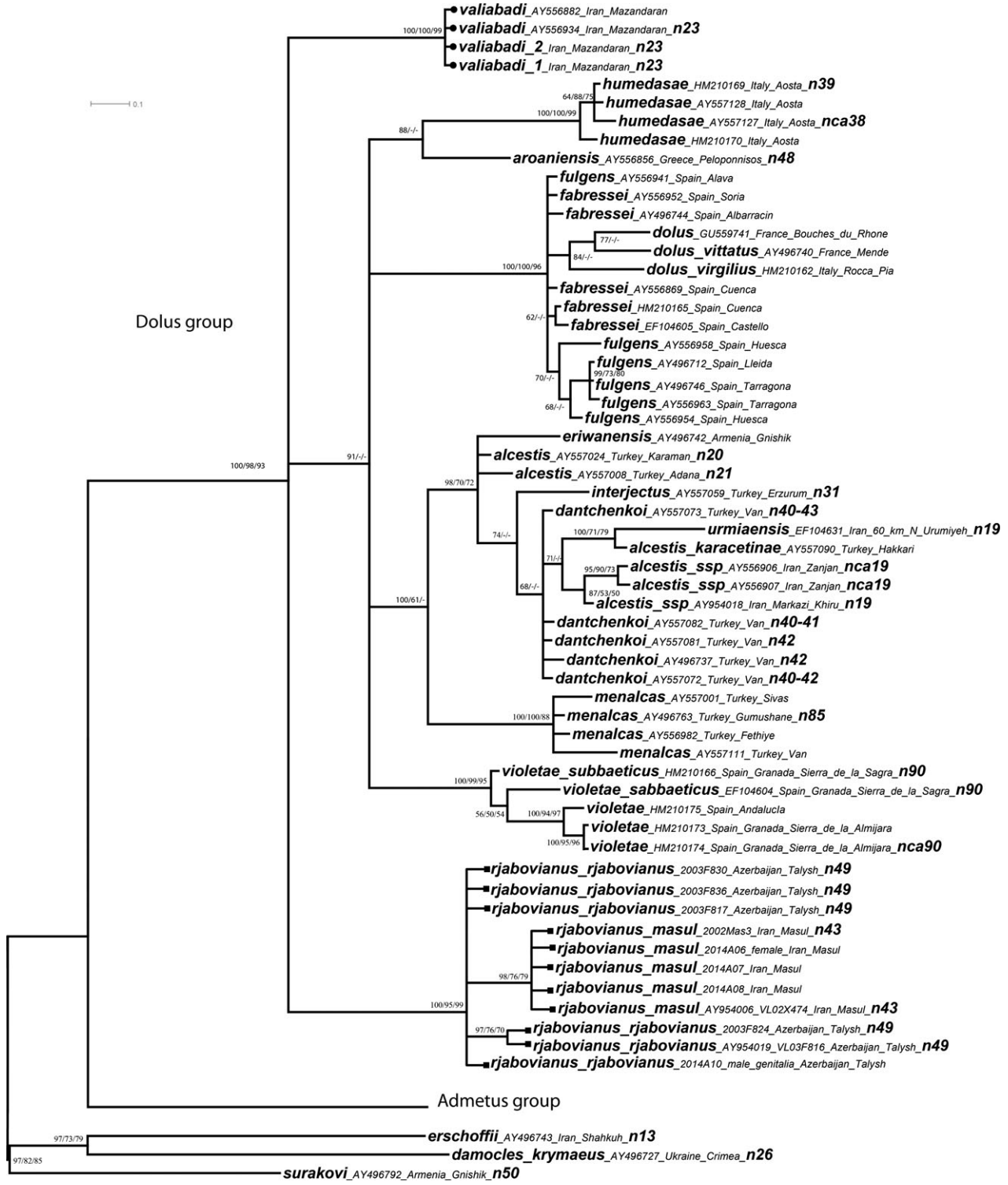

Figure 3. Fragment of the Bayesian tree of *Agrodiaetus* (*dolus* group), based on analysis of the cytochrome oxidase subunit I (COI) gene from 111 samples of *Agrodiaetus*. Haploid chromosome numbers (*n*) are indicated after specimen codes. Numbers at nodes indicate Bayesian posterior probability/ML bootstrap/MP bootstrap values, with non-matching clades using different analyses indicated by '–'. Scale bar = 0.1 substitutions per position.

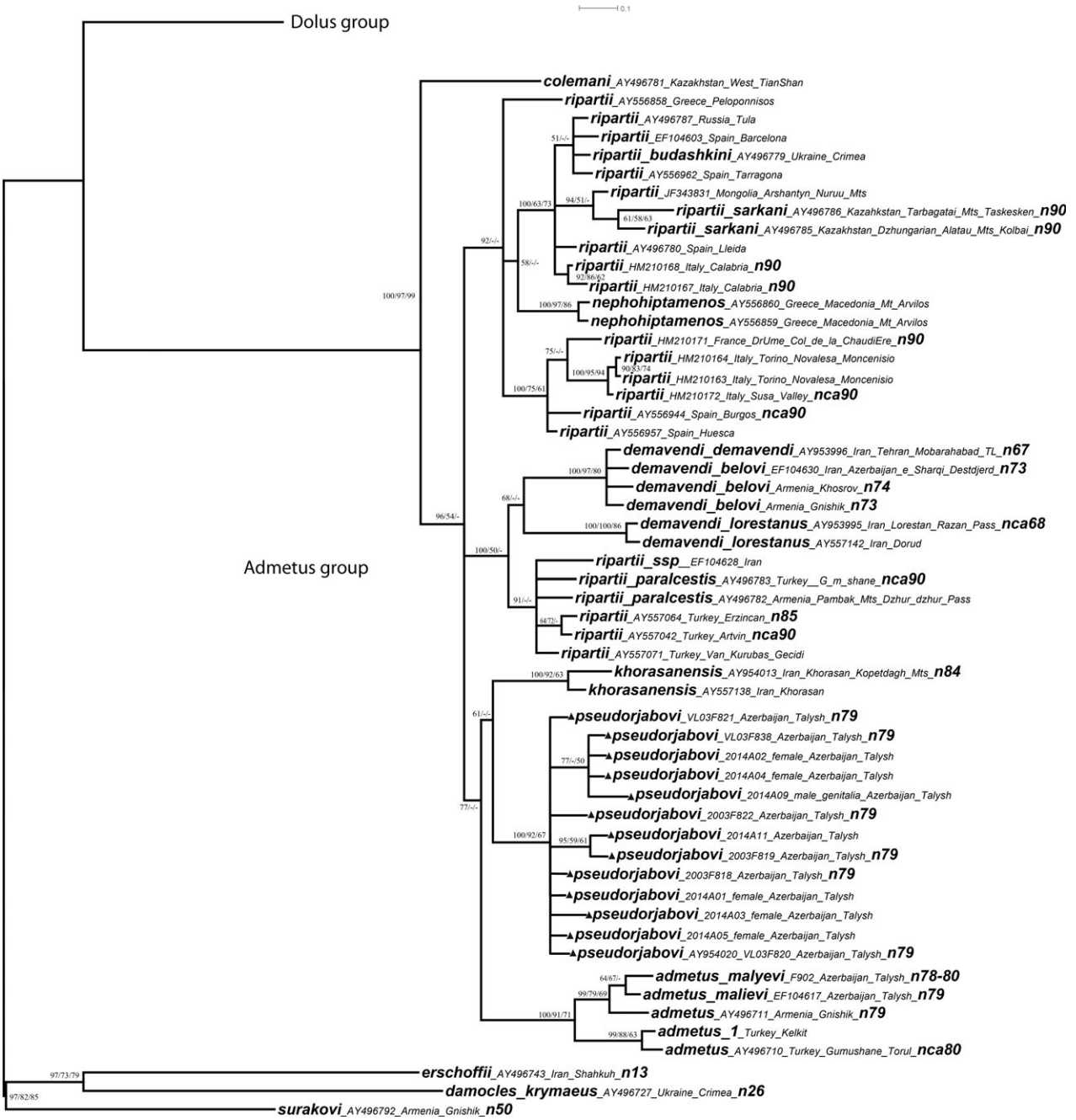

Figure 4. Fragment of the Bayesian tree of *Agrodiaetus* (*admetus* group), based on analysis of the cytochrome oxidase subunit I (COI) gene from 111 samples of *Agrodiaetus*. Haploid chromosome numbers (*n*) are indicated after specimen codes. Numbers at nodes indicate Bayesian posterior probability/ML bootstrap/MP bootstrap values, with non-matching clades using different analyses indicated by '–'. Scale bar = 0.1 substitutions per position.

Because gene flow between members of different biological species is normally either absent or weak (Coyne & Orr, 2004) even in their contact zone, a non-random association (i.e. linkage disequilibrium) of at least two unlinked alleles typical for each group is expected. Conversely, when two groups of individuals represent variants of intraspecific polymorphism, a random combination of these markers is expected, if these markers do not belong to the same linkage group. Here we argue for the use of a combination of mitochondrial molecular and nuclear chromosomal markers. The chromosome sets (karyotypes)

and the mitochondrial gene COI belong to two different, not physically linked parts of the genome, namely to nuclear (karyotypes) and mitochondrial (COI) markers. In the case of intra-population variability we should expect linkage equilibrium of nuclear and mitochondrial markers. In other words, we should expect the situation in which the markers combine at random and form all possible combinations of mitochondrial and chromosomal characters. Moreover, in the case of intra-population variability we should expect that heterozygotes will be found for nuclear markers.

In the case of two cryptic species there is no or very limited hybridization between clusters. Thus, we can expect strong linkage disequilibrium of nuclear and mitochondrial markers. In other words, we can expect the situation in which some variants of mitochondrial and chromosomal characters form stable, species-specific combinations, and other combinations of the markers do not exist at all. Furthermore, for two cryptic species, no or a very limited number of heterozygotes for nuclear markers are expected (Lukhtanov & Shapoval, 2008; Lukhtanov *et al.*, 2008).

In our research we found that a taxon usually identified by taxonomists as *P. rjabovianus* (Dantchenko, 2000; Tshikolovets, Naderi & Eckweiler, 2014) is represented in Talysh, south-east Azerbaijan, by two groups of individuals. The representatives of these groups have different chromosome numbers, $n = 49$ and 79. They also have fixed differences in 38 nucleotide positions within the studied 653-bp fragment of the mitochondrial COI gene (barcoding gap = 5.8%) and form discrete clusters in BI, ML and MP trees. Moreover, these two clusters belong to different species groups within the '*riparitii*-complex'. At the same time the representatives of these groups are similar in male wing upperside and underside (Fig. 5) and genitalia structure (Fig. 6).

Analysis of our data shows that the case we found in Talysh corresponds exactly to the situation expected for two sympatric cryptic species, not for intra-population variability. First, the karyotype $n = 49$ is always accompanied by the mitochondrial haplogroup *r*, and the karyotype $n = 79$ is always accompanied by the mitochondrial haplogroup *p*. Thus, there is a linkage disequilibrium of these physically unlinked markers. Second, no evidence for chromosomal heterozygotes was found. In the case of hybridization between $n = 49$ and 79 forms, we should expect that (1) multiple multi- or/and univalents will be observed in the MI stage of meiosis, and (2) intermediate chromosome numbers between 49 and 79 will be found in diploid cells. Such karyotypes were not observed in individuals studied from Talysh, indicating absence or at least rarity of hybridization between $n = 49$ and 79 forms.

Thus, both data types – the conjugacy of chromosomal marker $n = 49$ with mitochondrial marker *r* and the conjugacy of chromosomal marker $n = 79$ with mitochondrial marker *p*, as well as the absence of chromosomal heterozygotes – support the conclusion that these clusters represent two different species, not a case of intra-population variability. One of these entities is represented by *P. rjabovianus*. The second entity is represented by a novel species, *P. pseudorjabovi* sp. nov., which is formally described in the Appendix. This conclusion is also supported by analysis of nuclear molecular marker ITS2, which independently confirmed that our target taxa *P. pseudorjabovi* and *P. rjabovianus* belonged to two different clusters of individuals.

Detecting cryptic species in allopatry: *P. valiabadi* and *P. rjabovianus*

When taxa are allopatric, the direct application of the BSC is more difficult (Mayr, 1963; Coyne & Orr, 2004), and we consider two or more allopatric taxa as separate species if they are genetically distant and their pooling would result in a non-monophyletic 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, 1950, 1966; Envall, 2008; Hörandl & Stuessy, 2010) (= holophyly *sensu* Ashlock, 1971) is mandatory. Thus, avoiding non-monophyletic groups and focusing on monophyletic entities *sensu* Hennig is the preferable option in practical terms (Talavera *et al.*, 2013). The mitochondrial COI gene itself can provide weak evidence for monophyly of taxa as trees inferred from single markers sometimes display relationships that reflect the evolutionary history 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. In other words, the combination of any groups of individuals and or populations resulting in a non-monophyletic DNA-barcode assemblage should be avoided.

Huge genetic distance can provide additional evidence for non-conspecifity. Two allopatric taxa can be considered as different species if DNA distance between them is comparable or exceeds those found in other species pairs in the group under study, or if the COI distance exceeds the 'standard' 2.7–3.0% DNA-barcoding threshold (Lambert *et al.*, 2005). It

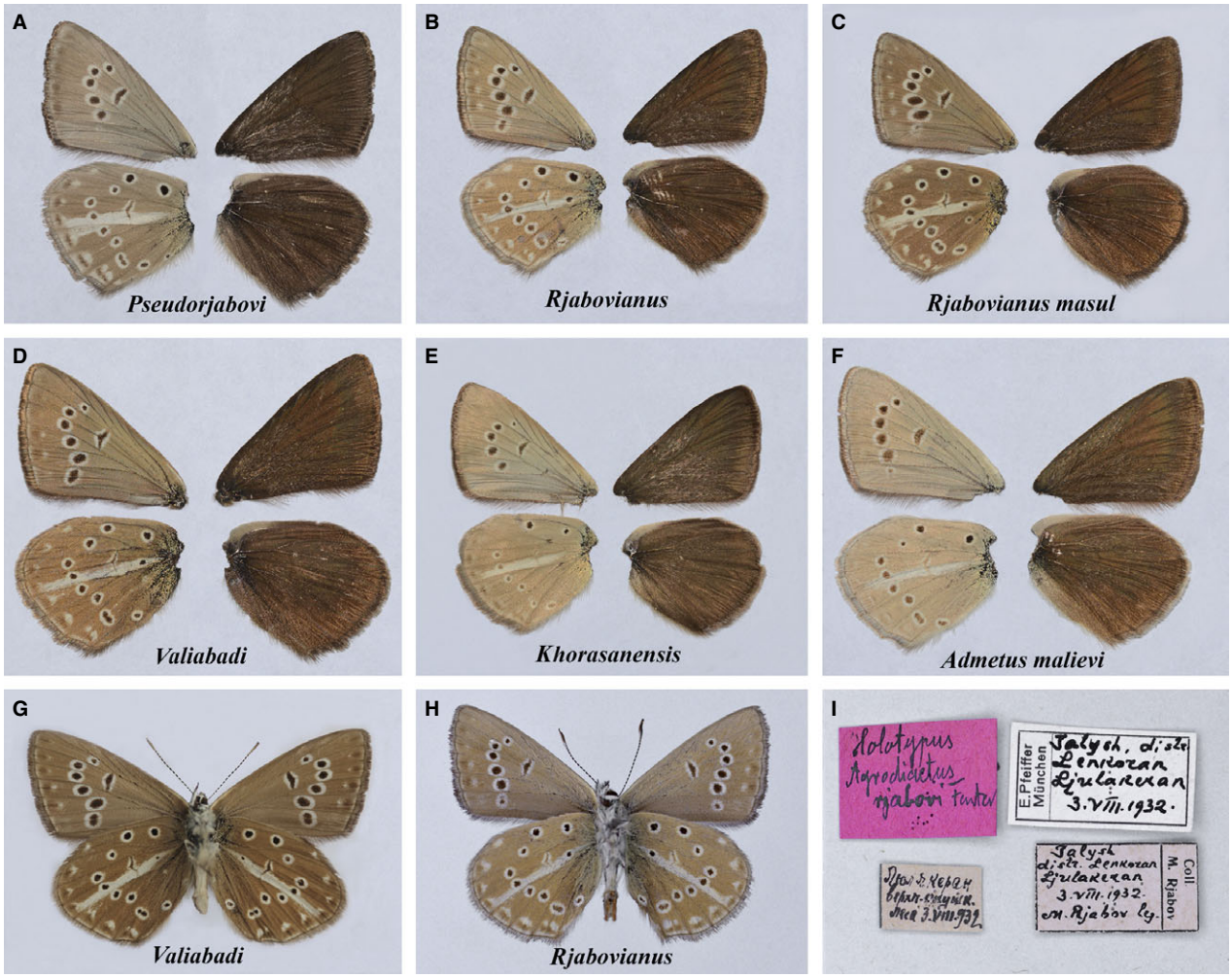

Figure 5. Underside (left) and upperside (right) of the male wings. A, ***P. pseudorjabovi* sp. nov.**, holotype, sample 2003F820. B, *P. rjabovianus rjabovianus*, sample 2003F830. C, ***P. rjabovianus masul* ssp. nov.**, holotype, sample 2002VL474. D, *P. valiabadi*, sample Z864. E, *P. khorasanensis*, sample 2003F526 AY954013. F, *P. admetus malievi*, holotype, sample 2003F903 EF104617. G, holotype of *P. valiabadi*, Elburs-Nordseite (Chalus-Tal), Umgebung Vali-Abad, 1900–2100 m NN, 25 km nördlich Kandevantunnel 10.VII.1975, K. Schurian leg. (in collection of K. Schurian). H, holotype of *P. rjabovianus* (Koçak, 1980) (= *Agrodiaetus rjabovi* Forster, 1960). I, holotype of *P. rjabovianus* (Koçak, 1980), labels, 'Talysh, distr. Lenkoran, Ljulakeran, 3.VIII.1932. M. Rjabov leg'.

was demonstrated that such a deep level of uncorrected *p*-distance between COI barcodes is practically always associated with species level of the taxa compared (Hebert *et al.*, 2003; but see Zakharov *et al.*, 2009). Although this level is not an absolute threshold to distinguish between species, it can be used as a useful criterion while deciding on the taxonomic status of a group under analysis and inferring hypotheses about species borders.

Gross chromosome differences can also be considered indirect evidence for reproductive incompatibility of allopatric taxa and thus for their nonconspecifity (King, 1993). Indeed, it is well known that in some cases even relatively small differences in chromosome structure can result in post-zygotic isolation (Ferree & Barbash, 2009). The latter phenomenon is not universal, and in other cases heterozygosity for multiple chromosome rearrangements does not result in sterility (Nagaraju & Jolly, 1986). In Lepidoptera, extreme cases of intraspecific variation in chromosome numbers (Lukhtanov *et al.*, 2011) and fertile crosses between chromosomally divergent races (Nagaraju & Jolly, 1986) seem to be inconsistent with strong selection against chromosomal heterozygotes. However, this does not mean that chromosome differences are irrelevant to reproductive isolation, because chromosomally divergent taxa can be protected from merging through a

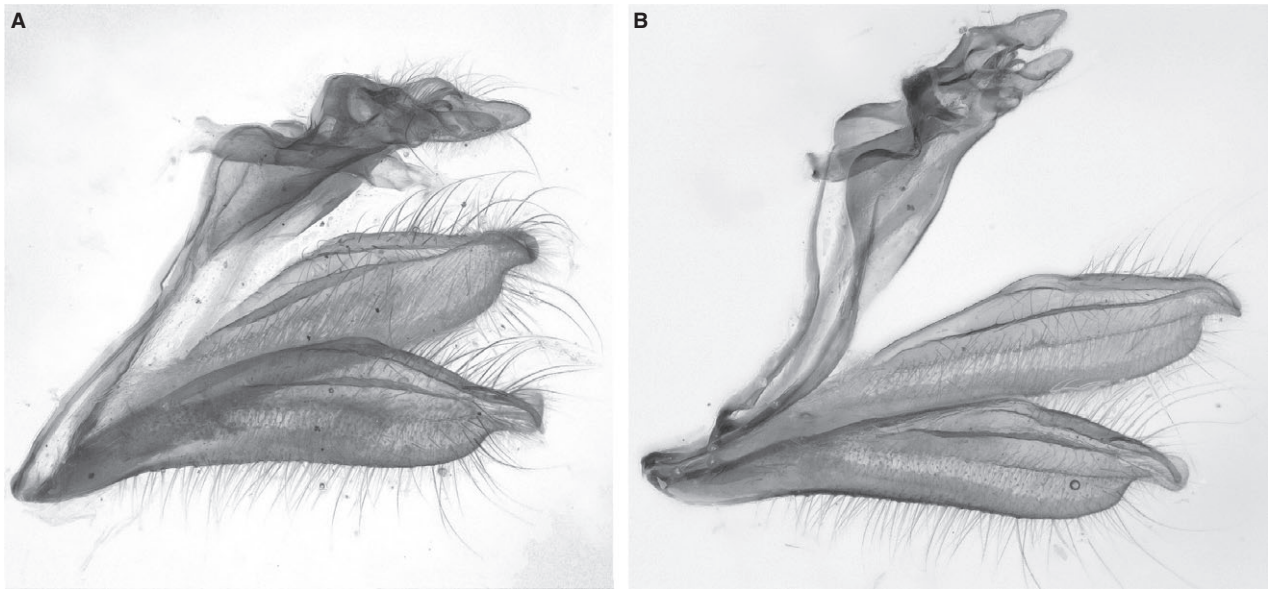

Figure 6. Male genitalia of *P. pseudorjabovi* (A, specimen 2014A09) and *P. rjabovianus* (B, 2014A10). Lateral view.

suppressed-recombination mechanism (Faria & Navarro, 2010; Lukhtanov *et al.*, 2015).

Polyommatus rjabovianus was considered as a subspecies of *P. valiabadi* (Rose & Schurian, 1977; Eckweiler & Häuser, 1997; Dantchenko, 2000; Tshikolovets *et al.*, 2014) due to their phenotypic similarity and allopatry. However, our karyological research shows that chromosome numbers of these taxa differ dramatically, $n = 23$ for *P. valiabadi* instead of $n = 49$ and 43 for *P. rjabovianus* and *P. rjabovianus masul*, respectively. There is no well-established general rule to determine how many or what types of chromosome rearrangements can be tolerated before the merging of taxa is impossible. To be conservative, we consider *P. rjabovianus rjabovianus* ($n = 49$) and *P. rjabovianus masul* ($n = 43$) as subspecies differentiated by at least six fixed chromosome fusions/fissions. However, the difference between *P. rjabovianus* ($n = 43$, 49) and *P. valiabadi* ($n = 23$) is obviously too high to interpret them as conspecific populations. Additionally, *P. rjabovianus* and *P. valiabadi* did not appear on our reconstruction as a clearly monophyletic group, and the uncorrected *p*-distance between their COI barcodes (3.17–3.52%) exceeds the 2.7–3.0% DNA-barcoding threshold. Thus, in accordance with the criteria mentioned above they should be considered as different species.

CONCLUSION

For studying the biological diversity within the blue butterflies and for making taxonomic decisions, we have used DNA-barcode-based lineage delimitation together with simple methods which have been widely and traditionally used by taxonomists who accept the biological and polytypic species concepts. In combination with several additional criteria such as monophyly, fixed threshold of a single marker and using a character (chromosome number) that suggests that the species potentially may be postzygotically isolated, this allowed us to detect three previously unknown taxonomic entities. Two of them possess properties of biological species, and the third lineage can be treated a subspecies if the polytypic species concept is applied, or a species in the frame of PSC. Although the discussed principles can be considered as a 'general' approach to delimitation of cryptic species in sympatry and allopatry, we note that the cases reported here are quite extreme in the sense that, despite morphological similarity, genetic and chromosomal differentiation between discovered species is high. We also note that a very wide 'grey zone' still remains for cases in allopatry and even in sympatry (low level of genetic differentiation, no fixed genetic markers, only slightly reduced fertility, presence of hybrids), and it is not easy to reach a clear conclusion in many cases. This 'grey zone' arises not because of a weakness of the discussed species delimitation criteria, but rather because of continuous character evolution which has resulted in a number of so-called 'bad species', young entities that demonstrate non-concordance between criteria conventionally used for species delimitation; some criteria argue for distinction at the species level, while others argue against it (Descimon & Mallet, 2009).

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APPENDIX

Descriptions of novel taxa

Polyommatus (Agrodiaetus) pseudorjabovi sp. nov

HOLOTYPE. Male. Forewing length 17.0 mm. Field code 2003F820, GenBank accession no. AY954020. Azerbaijan, Talysh mnt., Zuvand plateau, vicinity of Mistan village, 38.656330°N, 48.414607°E, 1700 m alt., 28.07.2003, A. Dantchenko & V. Lukhtanov leg. In Zoological Institute of the Russian Academy of Science (St. Petersburg).

PARATYPES. Eight males. Forewing length 16.5–17.5 mm. Field codes 2003F818, 2003F819, 2003F821, 2003F822, 2003F838, 2014A09 and 2014A11, the same data as holotype. Field code 1996A06, Azerbaijan, Talysh, Zuvand, Goveri, 20 June 1995, A. Dantchenko leg. five females. Forewing length 15.0–16.0 mm. Field codes A01, A02, A03, A04, A05, the same data as holotype.

Males

Upperside: ground colour brown with slightly darker veins. Discoidal, submarginal and antemarginal marking absent on both fore- and hindwings. Forewings with a good developed sex brand and scale-tuft. Fringe brown as ground colour.

Underside: ground colour light brown with coffee-milk tint. Greenish blue basal suffusion slightly visible. Basal black spots present only on hindwings. Discoidal black spots present on fore- and hindwings. Postdiscal black marking large and wide circled with white on both fore- and hindwings. Submarginal marking well pronounced on hindwing, antemarginal markings are presented by fuzzy lunulae. The spots of E^3 stria between sub- and antemarginal lunulae darker than ground colour. On the forewings sub- and antemarginal marking strongly reduced and pronounced as brown diffused spots. White streak on hindwings underside clearly visible, enlarged distally. In a single specimen the white stroke is reduced. Fringe light brown, slightly darker than underside ground colour.

Genitalia: the male genitalia have a structure typical for other species of the subgenus *Agrodiaetus* (Coutsis, 1986). Uncus divided into two sclerotized lobes. Gnatos situated at their bases, in the form of sclerotized hooks. Juxta with two long narrow branches. Aedeagus straight and relatively short. Valvae very narrow, more than four times as long as wide, with a strongly convex and setose longitudinal membranous fold on the ventral wall. The costal margin of the valvae is bent medially, so that a membranous subcostal groove is formed between this margin and the longitudinal fold. Sacculus extends along the entire ventral margin of the valvae.

Females

Upperside: ground colour as in males, but with small discoidal spote and with clear visible marginal spots on hindwings. Fringe greyish brown.

Underside: ground colour and general design as in males but submarginal markings less pronounced. Greenish blue basal suffusion near invisible. White streak on hindwings underside enlarged distally in three of four specimens. Fringe greyish brown.

Diagnosis

Genetically *P. pseudorjabovi* and *P. rjabovianus* are not closely related and differ drastically in karyotype and COI haplotypes. They have different chromosome numbers, $n = 79$ (*pseudorjabovi*) and $n = 49$ (*rjabovianus*). They also have fixed differences in 38 nucleotide positions within the studied 653-bp fragment of the mitochondrial COI gene (barcoding gap = 5.8%) and form discrete clusters in BI, ML and MP trees.

Phenotypically the new taxon is extremely similar to sympatric and often syntopic *P. rjabovianus rjabovianus* Koçak, 1980, but on average differs by slightly lighter ground colour and by slightly fuzzier shape of antemarginal lunulae on the underside of hindwings (in *P. rjabovianus* these lunulae are usually smaller and sharper). Additionally, in the new species the marginal lunulae on the underside of forewings are usually reduced whereas in *P. rjabovianus* they are visible as white fuzzy spots. In the new species, the spots of E^3 striae between sub- and antemarginal lunulae are slightly darker than the ground colour whereas in *P. rjabovianus* they have nearly the same intensity. Valve in male genitalia of the new species (Fig. 6A) is slightly wider than in *P. rjabovianus* (Fig. 6B).

From sympatric and syntopic *P. admetus malievi* Dantchenko & Lukhtanov (2004) the new species can be easily distinguished by well-pronounced submarginal design on the hindwing underside. Submarginal spots on the underside of *P. admetus malievi* are bordered from inner part by fine but clear visible lunulae of E^3 striae. In the new species spots of the postdiscal row on the underside of forewings are more shifted basally in the cell between veins Cu1 and Cu2. *Polyommatus pseudorjabovi* and *P. admetus malievi* are distinct with respect to COI haplotypes (Fig. 4). They share the same chromosome number $n = 79$, but their karyotypes are different: in *P. pseudorjabovi* MI karyotype displays three large bivalents in the centre of the metaphase plate, whereas in *P. admetus malievi* MI karyotype displays only one large bivalent in the centre of the metaphase plate.

On the COI tree the new species appears as a sister taxon to allopatric *P. khorasanensis* (Carbonell, 2001) but differs strongly in morphology: it has larger size, darker ground colour and strongly pronounced pattern on underside of fore- and hindwings. *Polyommatus pseudorjabovi* and *P. khorasanensis* are distinct with respect to *COI* haplotypes (Fig. 4) and their karyotypes (Fig. 2).

Bionomy

Polyommatus pseudorjabovi sp. nov. inhabits tragacanth steppes and dry meadows on upper border of oak forest from 1350 to 2000 m altitude. It was found in complete syntopy with *P. rjabovianus* although the peaks of the flight are different in these two species with *P. rjabovianus* flying approximately 2 weeks later (from beginning of July to end of August). Egg-laying was observed on undetermined plant species of the genus *Onobrychis* (Fabaceae).

Polyommatus (*Agrodiaetus*) *rjabovianus masul* ssp. nov

HOLOTYPE. Male. Forewing length 16.0 mm. Field code 2003VL474, GenBank accession no. AY954006. North Iran, Gilan, vicinity of Masuleh, 37.185°N, 48.906°E, 2200 m alt., 3 August 2002, A. Dantchenko & V. Lukhtanov leg. In Zoological Institute of the Russian Academy of Science (St. Petersburg).

PARATYPES. Six males. Forewing length 15.0–16.5 mm in six specimens. Field codes 2002Mas3, 2002Mas4, 2002VL476, 2002VL477, 2014A07 and 2014A08 (see GenBank accession codes in the Table 1). One female. Forewing length 15.5. Field code A06 (GenBank accession no. KR265485). All specimens have same data as holotype.

Males

Upperside: ground colour brown with slightly darker veins. Discoidal, submarginal and antemarginal marking absent on both fore- and hindwings. Forewings with a well-developed sex brand and scale-tuft. Fringe brown as ground colour.

Underside: ground colour brown. Greenish blue basal suffusion almost invisible. Basal black spots present only on hindwings. Discoidal black spots present on fore- and hindwings. Postdiscal black marking large and circled with white on both fore- and hindwings. Submarginal marking well pronounced and sharp on hindwing, antemarginal marking strongly reduced. On forewings submarginal marking well developed, more pronounced to anal end of row. White streak on hindwings underside clearly visible, usually enlarged distally. Fringe brown with greyish tint.

Females

Several females were found in the same biotope, several copulating pairs were observed but only a single specimen with known COI haplotype was included in the type series.

Upperside: ground colour as in males, but with small discoidal stroke and with clear visible marginal spots on hindwings that are slightly darker than ground colour. Fringe brown.

Underside: ground colour and general design as in males but submarginal markings well pronounced. Greenish blue basal suffusion near invisible. White streak on hindwings underside sharp and enlarged distally. Fringe brown, inner part lighter in colour.

Diagnosis

Phenotypically very close to *P. rjabovianus rjabovianus* Koçak, 1980, but ground colour is darker. Antemarginal row of spots on upperside of hindwings are sharper than in *P. rjabovianus*. There is a fixed karyotype difference between these two subspecies: $n = 49$ in *P. rjabovianus rjabovianus* and $n = 43$ in *P. rjabovianus masul*.

Bionomy

Polyommatus rjabovianus masul ssp. nov. inhabits ravines with steppe tragacanth vegetation at about 2000 m altitude. Flight period lasts from end of July to middle of August. Egg-laying was observed on undetermined plant species of the genus *Onobrychis* (Fabaceae).