

***Agrodiaetus shahkuhensis* sp. n. (Lepidoptera: Lycaenidae), a cryptic species from Iran discovered by using molecular and chromosomal markers**

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Abstract. We have found that a taxon usually identified as *A. kendeveni* Forster, 1956 is represented in the Elburz Mts., N. Iran by two reproductively isolated groups of butterflies that are morphologically very similar and fly in Shahkuh in the eastern part of the Elburz Mts., syntopically and synchronously. In Shahkuh these groups have different chromosome numbers, $n=15$ and $n=16$. They also differ from one another by 10 fixed nucleotide substitutions in a 448 bp fragment of the mitochondrial *COI* gene and form discrete clusters in neighbour-joining and maximum parsimony trees. Analysis of morphological, molecular and chromosomal data, field observations and study of type and historical material in museums of Munich, Berlin, Paris and London showed that one of these groups is represented by known taxa *A. kendeveni kendeveni* Forster, 1956 (Central Elburz) and *A. kendeveni pseudoxerxes* Forster, 1956 (East Elburz). Another group is described here as *Agrodiaetus shahkuhensis* sp.n. The new species is genetically very distant from all other representatives of the *A. cyaneus* (Staudinger, 1899) lineage of the genus *Agrodiaetus* Hübner, 1822.

Key words: *Agrodiaetus*, butterfly, chromosome, *COI*, cryptic species, Iran, karyotype, Lepidoptera, Lycaenidae, molecular marker.

INTRODUCTION

From the point of view of evolutionary biology, the genus *Agrodiaetus* Hübner, 1822 is a very interesting group of butterflies. It displays one of the highest known rates of species diversification (Kandul et al., 2004; Coyne, Orr, 2004) and karyotype evolution (Lukhtanov et al., 2005; Kandul et al., 2007) among all animals. The genus was estimated to have originated only about three million years ago (Kandul et al., 2004) and radiated

rapidly with more than 100 species distributed in the Western Palaearctic (Lukhtanov et al., 2005). From the point of view of taxonomy, *Agrodiaetus* is a complicated group and includes numerous cryptic species. Walter Forster, the author of the most comprehensive revision of the genus (Forster, 1956, 1960-1961), divided *Agrodiaetus* into several polytypic species based on geographic distribution and classic morphological characters. In particular, Forster established a polytypic

species *A. carmon* (Herrich-Schäffer, 1851) that included subspecies *A. carmon carmon*, *A. carmon cyaneus* (Staudinger, 1899), *A. carmon ciscaucasicus* Forster, 1956, *A. carmon kendevari* Forster, 1956, *A. carmon pseudoxerxes* Forster, 1956, and *A. carmon altaiensis* Forster, 1956 (Forster, 1956). The karyotype studies of de Lesse (1957, 1960a, b, 1962, 1963a, b), which appeared concurrently with Forster's revision, revealed that *Agrodiaetus* species had extremely diverse karyotypes. Furthermore, some of the species in Forster's system appeared to be complexes of sibling species that could be identified by their sharply differing karyotypes (de Lesse, 1960b, 1962, 1963a, 1963b; Lukhtanov, 1989; Lukhtanov et al., 1997, 1998). Karyotyping may provide the only reliable diagnostic character for many *Agrodiaetus* species, and as such become a necessary requirement for describing new taxa (de Lesse, 1960; Lukhtanov, Dantchenko, 2002a, 2003; Lukhtanov et al., 2003). The karyological investigations showed that *A. carmon* sensu Forster, 1956 was extremely heterogeneous (de Lesse, 1963a; Lukhtanov, 1989). On the basis of karyotype studies, this taxon was split into several species that can be characterized by species-specific haploid chromosome numbers (n) ranging from $n=15-16$ in *A. ciscaucasicus* (Lukhtanov, 1989) to $n=80$ in *A. carmon* (de Lesse, 1963a).

DNA based phylogenetic analysis demonstrated that *A. carmon* sensu Forster was an artificial polyphyletic assemblage consisting of representatives from two distantly related lineages (Kandul et al., 2004, 2007; Lukhtanov et al., 2005; Wiemers, 2003; Wiemers, Fiedler, 2007). One of these lineages is represented by taxa that are close to *A. carmon*. Another lineage is represented by taxa that are close to *A. cyaneus*. According to Wiemers (2003) and our data (Kandul et al., 2007), the last lineage includes from 15 to 20 species that are closely

related genetically. Most of them have similar phenotypes and allopatric distribution ranges, and in fact their systematics and real taxonomic status (species or subspecies) remain poorly understood.

Our paper addresses a more detailed analysis of the taxonomy of the *A. cyaneus* lineage with a special consideration of a species that was traditionally determined in current taxonomic literature as *A. kendevari* (Eckweiler, Häuser, 1977; Häuser, Eckweiler, 1997). The distribution range of *A. kendevari* is restricted to the Elburz Mountains in northern Iran. It is represented by two known subspecies: *A. kendevari kendevari* (described from the Kendevar Pass in the Central Elburz), and *A. kendevari pseudoxerxes* (described from vicinity of Shahkuh village in the Eastern Elburz) (Fig. 1).

De Lesse (1962) analyzed six male specimens from Kendevar Pass and pointed out that they were heterogeneous in wing colouration. Five of them had a wing upperside colour close to that in *A. altivagans* Forster, 1956 (i.e. blue), whereas one individual had wing upperside colour close to that in *A. carmon* (i.e. violet). Five specimens with blue colouration were found to have $n=16$ and were treated by de Lesse as *A. altivagans pseudoxerxes*. The *A. carmon*-like specimen with violet colouration was identified by de Lesse as *A. kendevari*. The conclusions of de Lesse on the taxonomy and the nomenclature were not supported by karyotype investigation of the *A. carmon*-like individual (its karyotype remained unstudied) and by analysis of the type material (it was not analyzed). In spite of that, the de Lesse's conclusion was interpreted as an evidence for nonconspecificity and sympatry of the taxa *A. kendevari* and *A. pseudoxerxes* (Wiemers, 2003).

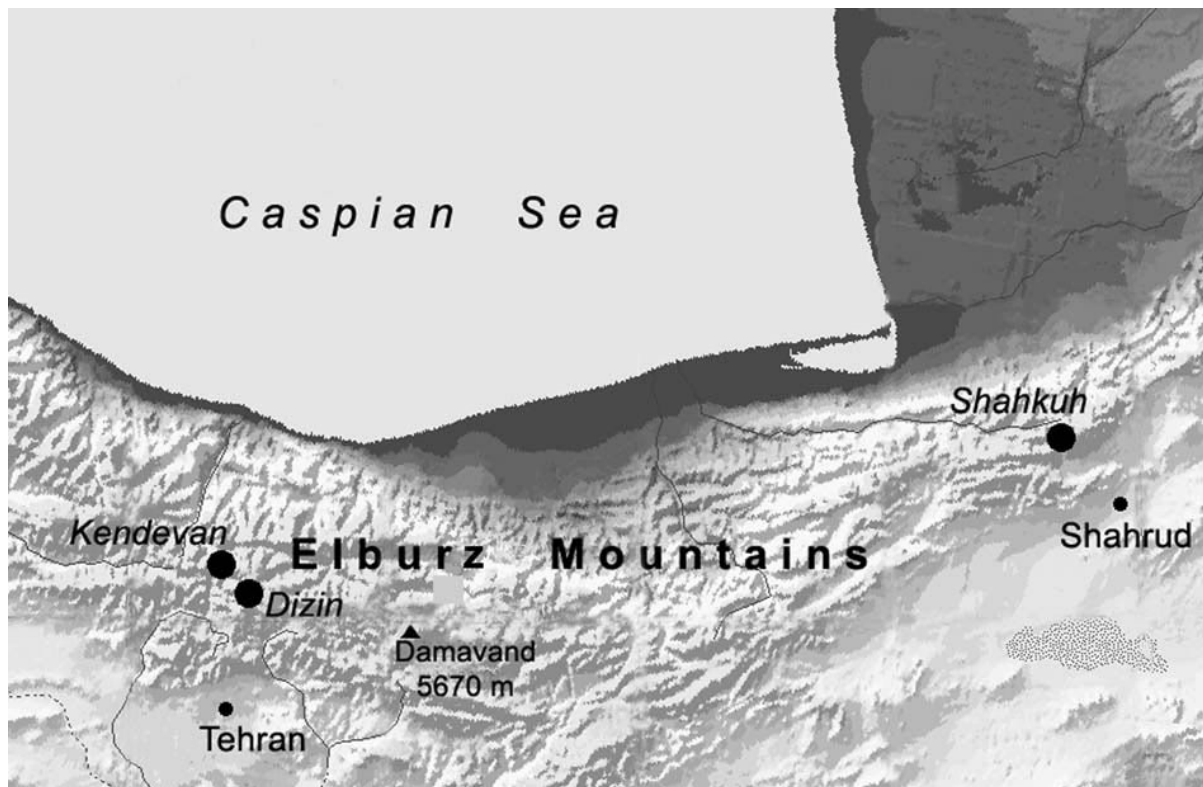


Fig. 1. Distribution of *A. shahkuhensis* sp. n. (Shahkuh), *A. kendeivani kendeivani* (Kendeivan, Dizin) and *A. kendeivani pseudoxerxes* (Shahkuh).

MATERIAL AND METHODS

Insects

Population samples of different taxa of the genus *Agrodiaetus* were collected in the period of 2000-2007 in Iran, Turkey, Armenia, Azerbaijan and Russia (N Caucasus, Dagestan). In particular, we visited the Kendeivan Pass in North Iran (the type locality of *A. kendeivani kendeivani*) five times, and we visited Shahkuh (the type locality of *A. kendeivani pseudoxerxes*) three times. In Kendeivan we collected material in two localities: 1) 5-10 km N of the pass (indicated as Kendeivan), and 15-30 km SE of the Kendeivan pass, close to the sky station Dizin (indicated as Dizin) (Fig. 1).

Fresh (not worn) adult males were used to

investigate the karyotypes. After capturing a butterfly in the field, it was placed in a glassine envelope for 1-2 hours to keep it alive until we processed it. Testes were removed from the abdomen and placed into 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 two sets of forceps: (i) a coarse or “flattened” set to hold the body and (ii) a much finer set to pinch off the wings. The wingless body was placed into a plastic, 2 ml vial with pure 100% ethanol. Each vial with ethanol has already been numbered. This ID number was also used to label a vial with the fixative and a glassine envelope in which the wings are preserved. Thus, each specimen was individually fixed.

After the fixation we had three components collected for each butterfly, each of which was identified by a common ID number: (a) a vial containing the butterfly testes (for karyotype analysis), (b) a vial containing the butterfly wingless body (for DNA analysis) and (c) a glassine envelope containing the wings. The set specimens of the donor butterflies (the butterfly wingless bodies in ethanol and wings in glassine envelopes) are kept in the DNA and Tissues Collection of Museum of Comparative Zoology (MCZH, Harvard University, Cambridge, MA, USA) (the material of 2000-2005) and in Zoological Institute of the Russian Academy of Science (St. Petersburg) (ZIN RAS) (the material of 2006-2007). All the testes are kept in the Department of Karyosystematics (ZIN RAS).

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 have used an original two-phase method of chromosome analysis (Lukhtanov, Dantchenko, 2002b; Lukhtanov et al., 2006). In the first phase, the stained testes were placed into a drop of 40% lactic acid on a slide, the gonad membranes were torn apart using fine needles and intact spermatocysts were removed and transferred into another drop of 40% lactic acid. During the metaphase I stage, each spermatocyst is a regular sphere and consists of 64 spermatocytes. Intact spermatocysts were studied and photographed, at first by using 40x and 60x objectives and then 100x objective. In the second phase, different stages of chromosome spreading were observed using a slight, gradually growing pressure on the coverslip. The second phase was

very useful for studying the bivalent structure, identifying bivalents and multivalents, and solving controversial cases of touching or overlapping bivalents. Scaling up the pressure on the coverslip, we were able to manipulate chromosomes by changing their position and orientation on the slide.

DNA Extraction and Sequencing

A mitochondrial gene, cytochrome oxidase subunit I (*COI*) was used to analyze the clustering the specimens. DNA extraction, purification and sequencing were conducted in the "Taxon" Laboratory in the Zoological Institute of Russian Academy of Sciences. First five abdominal segments were used for DNA extraction. The segments were homogenized in CTAB buffer and digested with proteinase K (10 mg/ml) for three hours at 60°C. DNA was purified through successive ethanol precipitations and stored in dd H₂O at -20°C. Mitochondrial DNA fragments were amplified using DNA thermal cycler ABI Gene Amp PCR System 2700. Primers for the amplification were designed using PreimerPremier v 5.0 software:

S1 (5'-TAATACCTCATTCTTTGACCCAGC-3')
 A1 (5'-ATAAAACATAATGGAAATGAGCAACTAC-3')
 A2 (5'-CTATCCGGATAATCTGAATAACGTCG-3')

The primers S1 and A1 were used to amplify a 448 bp fragment. The primers S1 and A2 were used to amplify 643 bp fragment. PCR reactions (50 µl) contained 1 µl of each primer, 1 mM dNTPs, 10x PCR Buffer (0.01 Tris Cl, 0.05M KCl, 0.1% Triton X-100: pH 9.0) 1 unit Taq DNA Polymerase (Helicon) and 5 mM MgCl₂ and were conducted using the following profile: initial 4 minutes denaturation at 94°C and 30 cycles of 30 seconds denaturation at 94°C, 1 minute annealing at 55°C, 1 minute extension at 72°C and 5 minutes final elongation at 72°C. PCR products

were analyzed on 1.5% gel, and purified using QiaQuick PCR purification kit (QiaGen). Sequencing of double-stranded product was carried out using BigDye v.3.1 sequencing kits on ABI 3100 automated sequencer (Applied Biosystems/Hitachi).

Sequence alignments and phylogeny inference

The sequences were edited and aligned using CHROMAS, Finch TV v.1.2.1 and BioEdit 7.0.3 (Clustal W algorithm). The alignment was unambiguous, as all the sequences were of equal length and included no insertions/deletions. Primer sequences were cropped. Missing data or ambiguities were designated by "N". Since *Agrodiaetus stempfferi* (Brandt, 1938) was earlier inferred as an outgroup to the *Agrodiaetus cyaneus* complex (see Kandul et al., 2007), we used one specimen of this species (GenBank sample AY954000) to root the phylogeny.

Additional sequences of the *Agrodiaetus cyaneus* species group were found in GenBank (Wiemers, 2003; Kandul et al., 2007; Lukhtanov et al., 2005) and were included into analysis in cases where such sequences completely overlapped with our fragment.

Neighbour-joining (NJ) analysis was performed using Kimura's two-parameter model of base substitution as implemented in MEGA4 (Tamura et al., 2007). MEGA4 was also used to perform bootstrap analysis (1000 replicates).

Maximum parsimony (MP) analysis was performed using a heuristic search as implemented in MEGA4 (Tamura et al., 2007). 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 nonparametric bootstrap values (Felsen-

stein, 1985) to estimate branch support on the recovered tree. The bootstrap consensus tree inferred from 1000 replicates using MEGA4 software.

Tests of the homogeneity of substitution patterns between sequences and estimation of net base composition bias disparity between sequences were performed using MEGA4 software.

Museum work

The Zoologische Staatssammlung (Munich, Germany) (ZSSM) was visited in order to study the holotypes and paratypes of *A. carmon kendevari* and *A. carmon pseudoxerxes*. Additional paratypes of these taxa were studied in the Museum für Naturkunde der Humboldt-Universität (Berlin, Germany) (MNHU) and the Natural History Museum (London, UK) (BMNH). The Muséum National d'Histoire Naturelle (Paris, France) (MNHN) was visited in order to reinvestigate the morphology of the individuals of "*A. altivagans pseudoxerxes*" and "*A. kendevari*" that were studied and karyotyped by de Lesse (1962; see Introduction).

RESULTS

Analysis of type and historical materials

Holotypes and paratypes of the taxa *A. kendevari* and *A. pseudoxerxes* preserved in Munich, Berlin and London were studied and photographed. The specimens that were karyotyped by de Lesse (1962) and identified by him as *A. altivagans pseudoxerxes* were found in Paris, studied and photographed. Comparison of de Lesse's samples with the types and with recent material from Iran showed that these samples were phenotypically much closer to *A. kendevari kendevari* than to *A. kendevari pseudoxerxes*. This result was very expected

Table 1. Upperside male wing colouration, chromosome numbers and mitochondrial *COI* haplotypes of the specimens studied. «-» – data are not available; «blue/violet» – colour intermediate between blue and violet (e.g. Fig. 2g); *sh* and *k* – two groups of mitochondrial haplotypes found in the *A. kendevari* complex. These haplotype groups differ from one another by 10 fixed nucleotide substitutions in 448 bp fragment of mitochondrial *COI* gene and form discrete clusters in both NJ and MP phylogenetic reconstructions (Figs 4, 5); *ca* (circa) – approximately determined chromosome number; MI – first metaphase of meiosis; MII – second metaphase of meiosis.

Taxon, locality	Code number of the specimen	Wing colour	Haploid chromosome number (MI)	Haploid chromosome number (MII)	Diploid chromosome number	COI gene
<i>A. shahkuhensis</i> , Shahkuh (group I)	VL284_Nz7	violet	-	n=16	-	sh
	VL286_ AY953998	blue	n=16	-	-	sh
	VL300_Nz5	blue/ violet	-	n=16	2n=32	sh
	J516	blue	n=16	-	-	sh
<i>A. kendevari</i> <i>pseudoxerxes</i> , Shahkuh (group II)	AD306_Nz1	violet	n=15	n=15	-	k
	AD307_ AY953991	violet	n=15	n=15	-	-
	AD308_Nz4	blue	-	n=15	-	k
	VL285	blue	n=15	-	2n=30	-
	VL291_NZ6_	violet	n=15	-	-	k
	VL298_Nz2	violet	-	-	2n=30	k
	VL301_Nz10	violet	n=15	-	-	k
	J512	violet	-	-	2n=30	k
	J513	violet	-	-	-	k
	J514	violet	n=15	-	-	k
	J515	violet	-	-	-	k
	J532	violet	-	-	2n=30	k
	J534	violet	-	-	2n=30	k
	J535	violet	n=15	-	-	-
	J536	violet	n=15	n=15	-	-
	J537	violet	-	-	-	k
	VL288_Nz3	violet	n=15	n=15	2n=30	k
J533	violet	n=15	n=15	-	k	
<i>A. kendevari</i> <i>kendevari</i> , Kendevan (group III)	VL305	blue	-	-	2n=30	-
	VL312_Nz11	violet	n=15	-	-	k
	VL313	blue	n=15	-	-	-
	VL314_Nz9	violet	n=15	-	-	k
<i>A. kendevari</i> <i>kendevari</i> , Dizin (group IV)	VL433_Nz8	violet	-	n=16	-	k
	VL442_ AY954005	blue	n=16	-	-	k
	VL443	violet	-	-	2n=ca32	-
	J118	blue	-	n=16	-	-

taking into account the fact that de Lesse collected his material in Kendevan Pass, i.e. in the type-locality of the taxon *A. kendevari*.

Male wing colouration

Analysis of butterflies from Kendevan Pass (Kendevan and Dizin) and Shahkuh supported the de Lesse's observation that males are heterogeneous in male wing colour (Table 1, Fig. 2). The males with blue and violet tint of the colour upperside were almost equally frequent in Kendevan. In Shahkuh the violet colouration was predominant. It should be also noted that the difference between "blue" and "violet" colours was not strong. In fact one can speak here rather about two variations of the blue than about two different colours.

Karyotypes

Depending on karyotypes and localities, four groups of individuals can be separated (Table 1 and see below). The groups I and II were found in Shahkuh flying together, at the same place and at the same time. The groups III and IV represent two allopatric chromosome races that were found near the Kendevan pass, north and south-east of the pass correspondingly.

Group I (will be described below as *A. shahkuhensis*) (Fig. 3, a-b)

The haploid chromosome number $n=16$ was found in meiotic metaphase I (MI) and meiotic metaphase II (MII) cells of four studied individuals. The number $2n=16+16=32$ was found in diploid chromosome sets that could be observed in sister MII cells. In MI cells, all bivalents formed a gradient size row. The karyotype contained no exceptionally large or small bivalents.

Group II. (*A. kendevari pseudoxerxes*) (Fig. 3, c-f)

The haploid chromosome number $n=15$ was found in MI and MII cells of eleven stud-

ied individuals. In six specimens the diploid chromosome number $2n=30$ was observed in male asynaptic meiosis. This number was also supported by counts of diploid chromosome sets that could be observed in sister MII cells ($2n=15+15=30$). In MI cells, all bivalents formed a gradient size row. The karyotype contained no exceptionally large or small bivalents.

Group III. (*A. kendevari kendevari*, chromosome race Kendevan) (Fig. 3, g-h)

The haploid chromosome number $n=15$ was found in MI cells of three studied individuals. In the specimen VL305, the number $2n=30$ was found in a diploid chromosome sets that could be observed in male asynaptic meiosis. In MI cells, all bivalents formed a gradient size row. The karyotype contained no exceptionally large or small bivalents.

Group IV. (*A. kendevari kendevari*, chromosome race Dizin) (Fig. 3, i)

The haploid chromosome number $n=16$ was found in MI and MII cells of two studied individuals. In the specimen VL443, we could approximately count $2n=ca32$ in male asynaptic meiosis. The last count was done with approximation due to the overlapping of some chromosomes. In MI cells, all bivalents formed a gradient size row. The karyotype contained no exceptionally large or small bivalents.

Phylogenetic analysis of molecular data

We have analyzed two *COI* data sets. The first data set included 48 shorter sequences (448 bp). 22 of them were obtained in the present study, and 26 sequences were obtained earlier (Kandul et al., 2004, 2007; Lukhtanov et al., 2005). In fact, 29 of these 48 samples represented fragments of longer (832 bp) sequences analyzed in the second data set (see below). The final first data set alignment included 448 sites, 45 sites were variable, and 28 sites were parsimony-informative. The av-

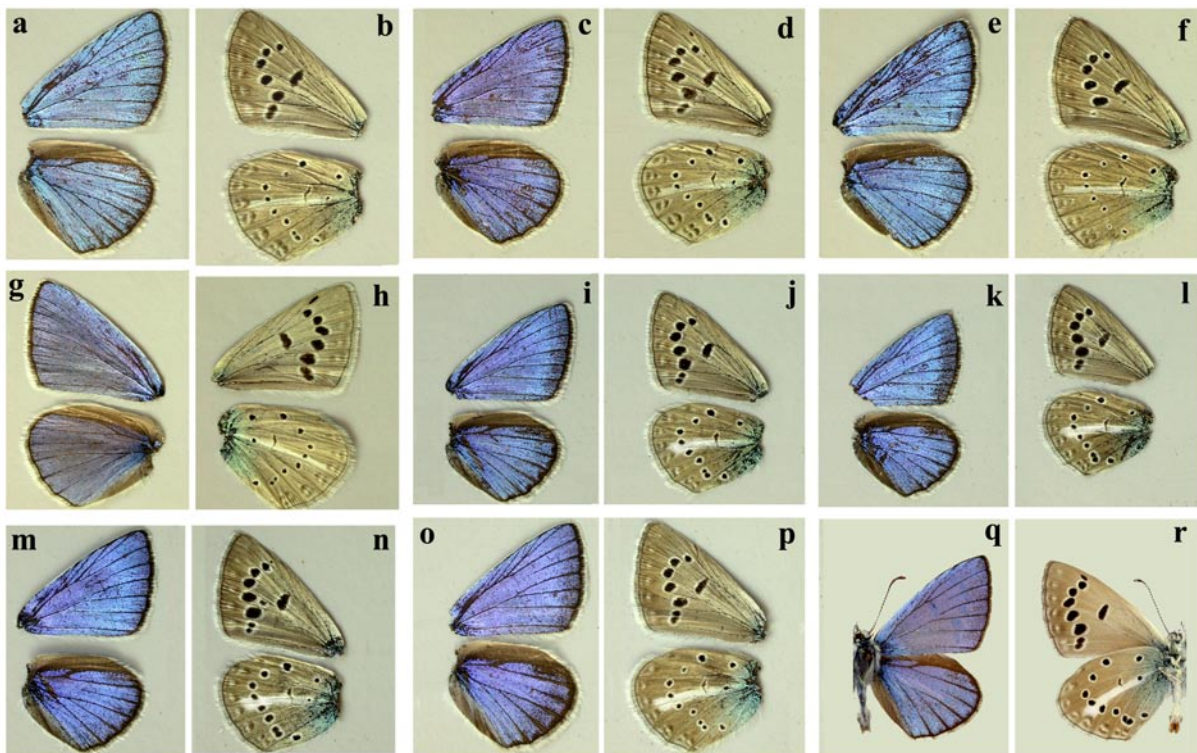


Fig. 2. *Agrodiaetus* from Shahkuh, N. Iran. Upperside (left) and underside (right) of the male wings. **a-f** - *A. shahkuhensis* sp. n.; **a, b** - holotype VL286; **c, d** - paratype VL284; **e, f** - paratype J516; **g, h** - paratype VL300. **i-r** - *A. kendevari pseudoxerxes*; **i, j** - AD307; **k, l** - AD308; **m, n** - J512; **o, p** - VL298; **q-r** - paratype of *Agrodiaetus carmon pseudoxerxes* Forster, 1956, "Shahkuh, N. Persia, Funke [leg.], 1898", BMNH. See also Table 1 for the explanations.

erage nucleotide frequencies were 0.319 (A), 0.392 (T), 0.13 (C), and 0.159 (G). The test of the homogeneity of substitution patterns between sequences did not reject the null hypothesis that the 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 second data set included 29 longer sequences (832 bp). Three of them were obtained in the present study, and 26 sequences were obtained earlier (Kandul et al., 2004, 2007; Lukhtanov et al., 2005). The final second data

set alignment included 832 sites, 102 sites were variable, and 53 sites were parsimony-informative. The average nucleotide frequencies were 0.319 (A), 0.374 (T), 0.159 (C), and 0.148 (G). The test of the homogeneity of substitution patterns between sequences did not reject the null hypothesis that the 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 first data set alignment completely overlapped with the second one. The sequences from both data sets were similar

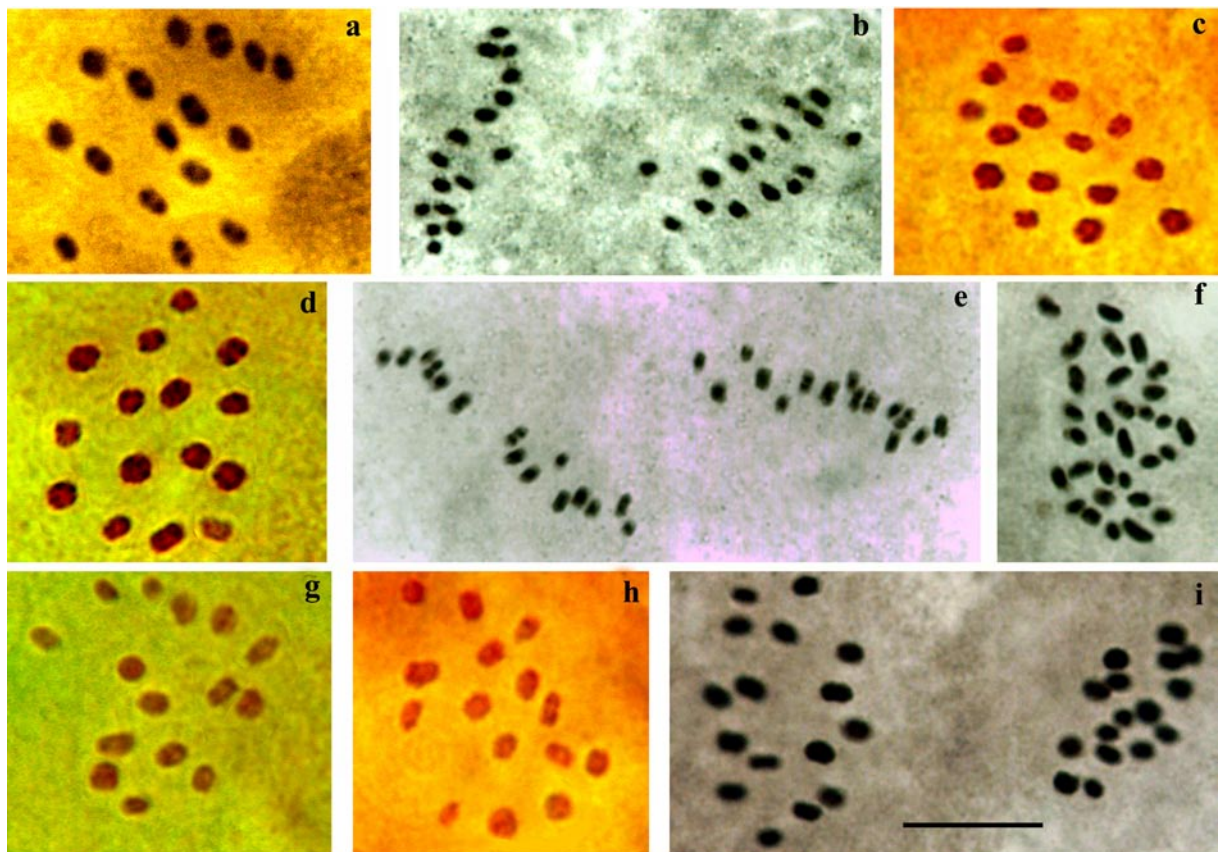


Fig. 3. a-b - *A. shahkuhensis* sp. n.; a - holotype VL286, MI, n=16; b - paratype VL300, two sister MII plates, each demonstrates n=16. c-f - *A. kendevari pseudoxerxes*; c - AD306, MI, n=15; d - VL285, MI, n=15; e - AD308, two sister MII plates, each demonstrates n=15; f - VL298, asynaptic meiosis, 2n=30. g-h - *A. kendevari kendevari*, Kendevan, VL313, MI, n=15. i - *A. kendevari kendevari*, Dizin, VL442, MI, n = 16. Bar = 10µm.

in the base composition, and both data sets demonstrated the homogeneity of substitution pattern. Therefore we combined the 19 shorter and 29 longer sequences and analyzed them together using options “Pairwise Deletion” for missing data (in NJ) and “Use All Sites” (in MP) as implemented in MEGA4 (Figs 4, 5).

Both analyses demonstrated that individuals from Shahkuh form two discrete, statistically supported discrete clusters. These clusters differ from one another by 10 fixed nucleotide substitutions in 448 bp fragment of mitochondrial *COI* gene, and uncorrected “p” distance between them is 2.23%. In fact these

clusters are more distant from one another than the great majority of other species pairs of the *A. cyaneus* lineage.

DISCUSSION

Two cryptic species of the *A. kendevari* complex in Shahkuh

We have found that a taxon usually identified as *A. kendevari* is represented in Shahkuh, N. Iran by two groups of individuals. The representatives of these groups have different chromosome numbers, n=15 and n=16. They also have fixed differences at ten nucleotide

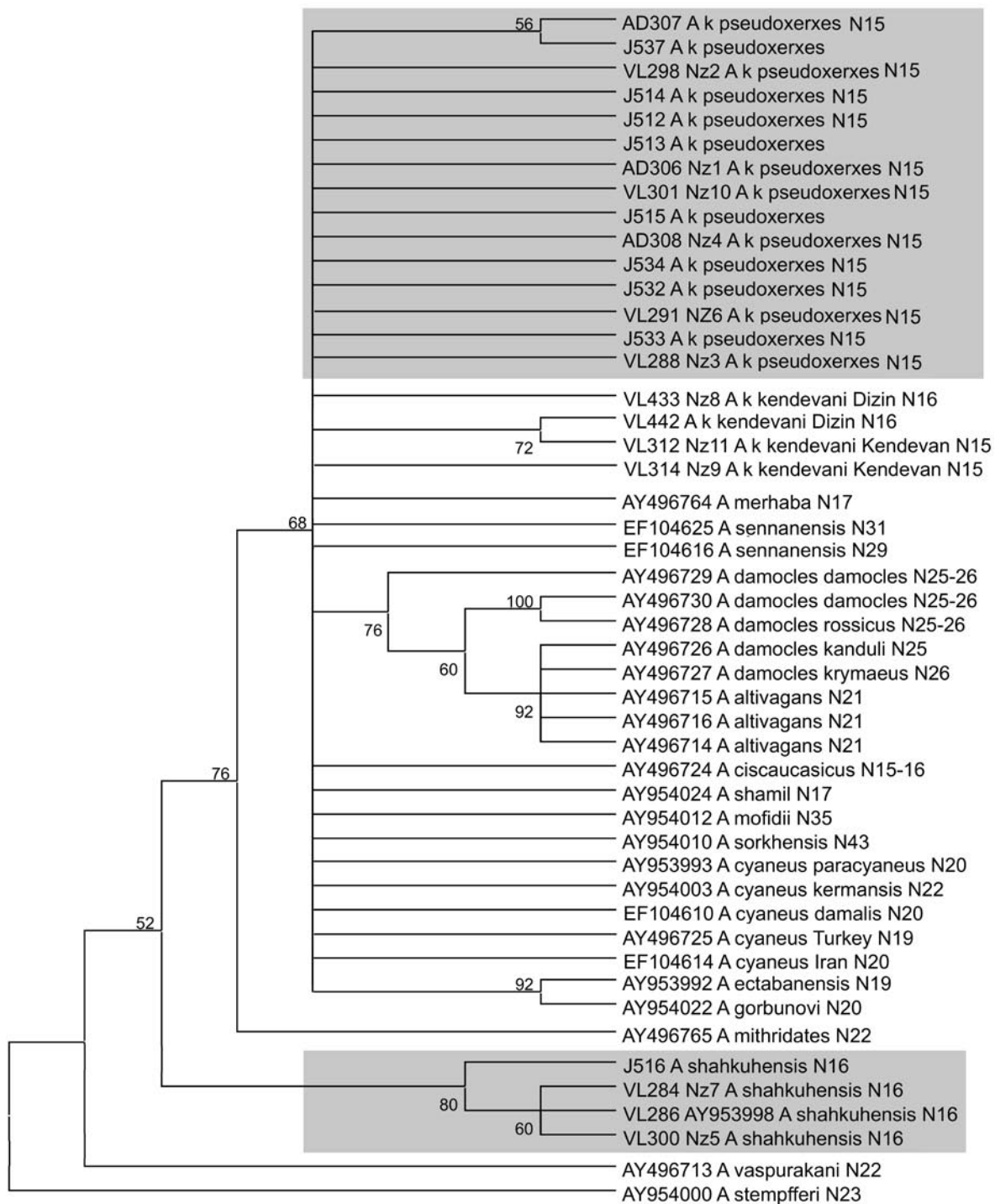


Fig. 4. Bootstrap consensus NJ tree of *Agrodiaetus cyaneus* complex inferred from 48 sequences of COI.

The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The bootstrap support is shown at the nodes of recovered branches. Haploid chromosome numbers are shown to the right of the names of specimens. The shaded blocks highlight the genotypic and karyotypic clusters (*A. kendevari pseudoxerxes* and *A. shahkuhensis*) found in sympatry in Shahkuh, Iran.

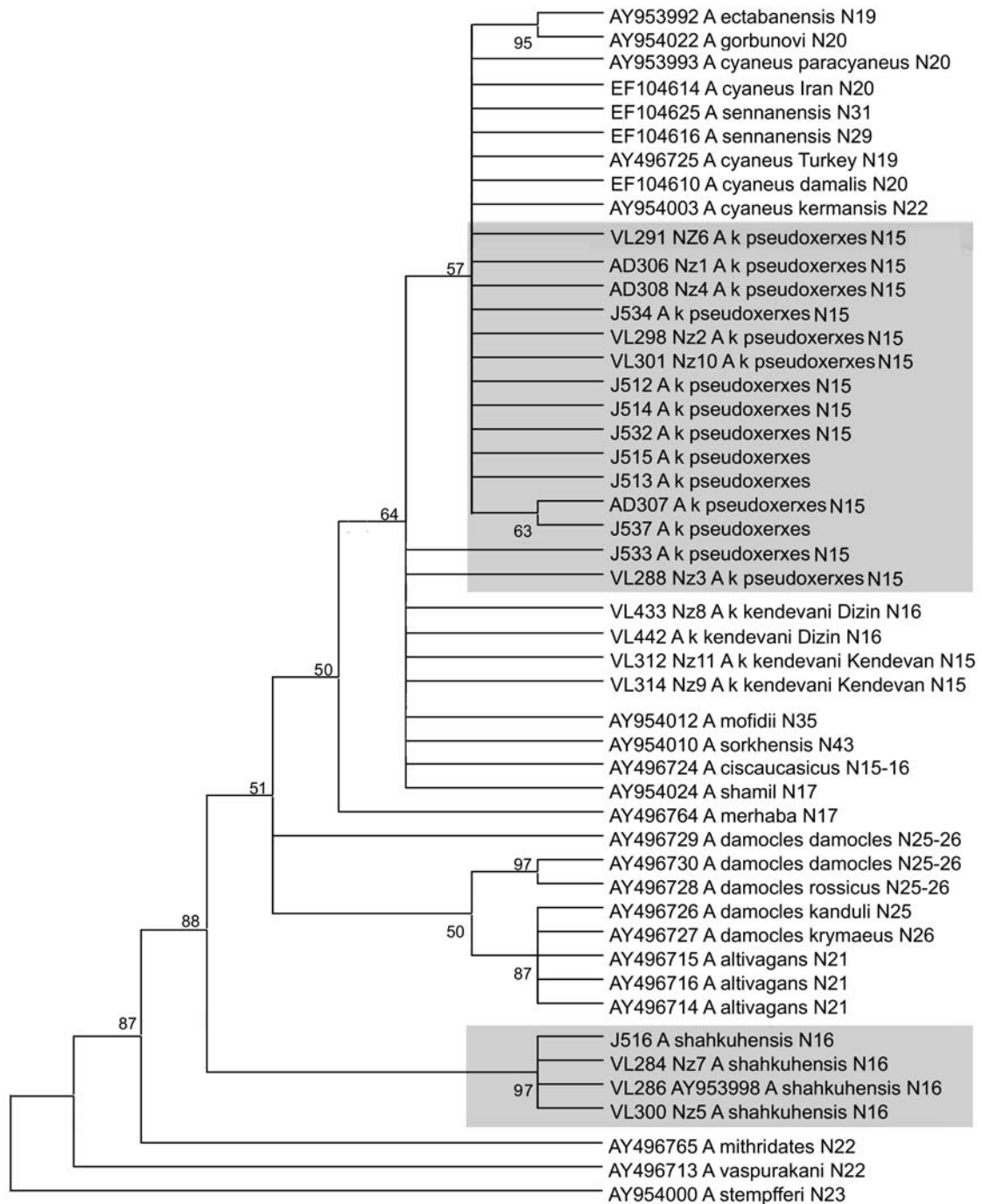


Fig. 5. Strict consensus MP tree of *Agrodiaetus cyaneus* complex inferred from 48 sequences of *COI*.

The strict consensus tree inferred from 57 most parsimonious trees is shown: Total length is 162, consistency index is (0.568966), the retention index is (0.825784), and the composite index is 0.570912 (0.469843) for all sites and parsimony-informative sites (in parentheses). The bootstrap values >50% are shown at the nodes of recovered branches. Haploid chromosome numbers are shown to the right of the names of specimens. The shaded blocks highlight the genotypic and karyotypic clusters (*A. kendevari pseudoxerxes* and *A. shahkuhensis*) found in sympatry in Shahkuh, Iran.

positions in 448 bp fragment of mitochondrial *COI* gene and form discrete clusters in both NJ and MP trees. At the same time the representatives of these groups are similar in the male wing upperside and underside (Table 1, Fig. 2) and practically identical in genitalia. There are two possible explanations for the existence of these two discrete groups in sympatry. First, these chromosomal and genotypic clusters may represent variants of intra-population variability. Second, they may represent two different biological (i.e. reproductively isolated) species.

These two hypotheses can be tested by using chromosomal and mitochondrial characters analyzed in our study. The chromosome sets (karyotypes) and the mitochondrial gene *COI* belong to two different, physically not linked parts of the genome: to nuclear (karyotypes) and mitochondrial (*COI*) markers. In 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. Additionally, in case of intra-population variability we should expect that heterozygotes for nuclear markers will be found.

In 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. Additionally, in case of two cryptic species, no or very limited number of heterozygotes for nuclear markers are expected to be found.

Analysis of our data show that the situa-

tion we found in Shahkuh corresponds exactly to the pattern expected for two cryptic species, not for intra-population variability. First, the karyotype $n=16$ is always accompanied by the mitochondrial haplotype sh, and the karyotype $n=15$ is always accompanied by the mitochondrial haplotype k. Thus, there is strong linkage disequilibrium of these physically unlinked markers. Second, no evidence for chromosomal heterozygotes was found. In case of hybridization between $n=16$ and $n=15$ forms, we should expect that (1) multi- or/and univalents will be observed in the MI stage of meiosis, (2) in the sister MII cells different chromosome number, $n=16$ and $n=15$, will be found, and (3) an intermediate chromosome number $n=31$ will be found in diploid cells. Such karyotypes were not observed in individuals studied from Shahkuh indicating absence of hybridization between $n=16$ and $n=15$ races.

Thus, both kinds of the data obtained – the conjugacy of chromosomal marker $n=16$ with mitochondrial marker sh and the conjugacy of chromosomal marker $n=15$ with mitochondrial marker k, as well as the absence of chromosomal heterozygotes – show that there is no gene flow between two clusters found in Shahkuh in sympatry. These clusters represent two different species, not a case of intra-population variability.

Status and taxonomic position of the $n=15$ chromosome race from Shahkuh

Two sympatric species found in Shahkuh are morphologically very similar, but not completely identical. The specimens with $n=16$ and *COI* haplotype sh are on average larger, usually blue and, most importantly, have a white stripe on the hindwing underside that is not contrasting, and has nearly parallel borders (*A. shahkuhensis*, Fig. 2, b, d, f, h). The specimens from Shahkuh with $n=15$ and *COI* haplotype k are on average smaller, usually violet and have white stripe on the hindwing underside

that is very contrasting and gradually becoming broader distally. The last characters correspond exactly to morphology of holotype and paratypes of *Agrodiaetus carmon pseudoxerxes* (Fig. 2, r). Taking into account the phenotype similarity and the fact that our material was collected exactly in the type-locality, we believe that the race n=15 (COI haplotype k) represents the taxon that was originally described under the name *Agrodiaetus carmon pseudoxerxes* (Forster, 1956).

Genetically, chromosomally and morphologically, the last taxon is close to *A. kendelevani* described from the Kendevan pass and can be treated as a subspecies – *A. kendelevani pseudoxerxes*.

Status and taxonomic position of the n=16 chromosome race from Shahkuh

This taxon represents a species that is independent from the above mentioned, sympatric *A. kendelevani pseudopsaxes*. It can be also easily distinguished by karyotype (Kandul et al., 2007; Figs 4, 5) and in some cases by morphology (see Wiemers, 2003) from *A. altivagans* Forster, 1956 (n=21), *A. brandti* Forster, 1956 (n=19), *A. cyaneus* (n=17-22, different numbers are found in different populations), *A. damocles* (Herrich-Schäffer, 1844) (n=25-26), *A. ectabanensis* de Lesse, 1964 (n=19), *A. mithridates* (Staudinger, 1878) (n=22), *A. mofidii* de Lesse, 1963 (n=35), *A. sennanensis* de Lesse, 1959 (n=29-31), *A. sertavulensis* Koçak, 1979 (n=20, Wiemers, 2003); *A. sorkhensis* (Eckweiler, 2003) (n=43), and *A. vaspurakani* (Lukhtanov, Dantchenko, 2003) (n=22).

Theoretically, the new species from Shahkuh can be considered as a subspecies of *A. ciscaucasicus* Forster, 1956 (n=15-16, N. Caucasus), or *A. shamil* Dantchenko, 2000 (n=17, Dagestan), or *A. merhaba* (De Prins, van der Poorten, Borie, van Oorschot, Riemis, Coenen) (n=17, E. Turkey) (see Figs 4, 5), or

A. wagneri Forster, 1956 (n=18, Turkey), or *A. maraschi* Forster, 1956 (n=16, Turkey) (see: Wiemers, 2003). All these taxa are allopatric in distribution, morphologically similar and have the same or similar chromosome number. However, genetically *A. ciscaucasicus*, *A. shamil*, *A. merhaba* (this study, Figs 4, 5) as well as *A. maraschi* and *A. wagneri* (Wiemers, 2003) are much closer to *A. kendelevani* than to the new species from Shahkuh. So, from the point of view of a “lumper” who would like to lump together a maximal number of allopatric populations of the *A. cyaneus* species group, it would be more logical to unite *A. ciscaucasicus*, *A. shamil*, *A. merhaba*, *A. maraschi*, and *A. wagneri* with *A. kendelevani*, but not with the species (n=16) found in Shahkuh.

Thus, the new taxon from Shahkuh seems to remain a separate species even if the broadest polytypic species concept and “lumpers” approach to taxonomy will be applied.

Status and taxonomic position of the chromosome races from Kendevan and Dizin

Our data do not support the de Lesse’s hypothesis (de Lesse, 1962; Wiemers, 2003) about two sympatric species of the *A. kendelevani* complex in the Kendevan pass. We found that the difference between “blue” and “violet” males was not strong, and, most importantly, the butterflies with different tinges were identical in COI sequences. The wing colour is not connected with a particular karyotype. The blue as well as violet males were found in both n=15 and n=16 individuals. It seems that the differences in tinge are subject of intra-population variability but not species-specific characters.

We also found no reason to use the name *A. pseudoxerxes* for the population from Kendevan as it was suggested by de Lesse (1962). It seems much more logical to use the name *A. pseudoxerxes* for the population of *A. kende-*

evani from Shahkuh. The name *A. kendevari* *kendevari* should be preserved for the taxon from Kendevar pass. At the same time our data demonstrate that the subspecies *A. kendevari* is represented, most probably, by two chromosomal races: the race n=15 (north of the Kendevar pass) and the race n=16 (south-east of the Kendevar pass). However, the last conclusion is based on limited material and should be checked in future.

Species description

Agrodiaetus shahkuhensis sp. n. (Fig. 1 – map, Fig. 2, a-h – wings, Fig. 3, a-b – karyotype, Figs 4-5 – phylogeny)

Holotype. ♂ (MCZH), Iran, Prov. Golestan, Elburs Mts., Shahkuh (about 45 km NW of Shahrud), 2000-2200m, 30.VII.2002, V. Lukhtanov and A. Dantchenko leg. Specimen field code VL286. GenBank code for mitochondrial cytochrome oxidase subunit I (*COI*) gene (partial cds), *tRNA-Leu* gene (complete sequence), and cytochrome oxidase subunit II (*COII*) gene (partial cds) is AY953998. The type-locality described in details by Naumann (2000).

Paratypes. 3 ♂♂, VL284, the same locality, date and collectors as the holotype. VL300, the same locality and collectors as the holotype, 31.VII.2002. J516, the same locality as the holotype, 21.VII.2005, V. Lukhtanov leg. All the paratypes are in MCZH.

Derivatio nominis. The new taxon is named after the village Shahkuh where it was found.

Description. *Male upperside.* Forewing length 17 mm, ground colour blue (in specimens VL286 and J516) or blue with violet tint (in VL300), or violet (in VL284) with very narrow black marginal line; forewing costal area bordered by white pubescence; veins indicated with fine black over their entire length;

costal and anal areas of hindwings black; basal half of fringes of both wings dark grey, distal half of fringes of both wings white.

Male underside. Forewing ground colour light grey with brownish tint; discal spot black, longitudinal, bordered with white; postdiscal spots relatively large, bordered with white; submarginal and marginal lunules only faintly indicated; basal half of fringes grey; distal half of fringes white. Hindwings ground colour light grey with brownish tint; white stripe narrow, not contrasting, with nearly parallel borders; bluish suffusion of basal area is developed, but not strong; covering basal area; discal spot inconspicuous; postdiscal spots very small, bordered with white; submarginal and marginal lunules better indicated than in forewing; basal half of fringes grey; distal half of fringes white.

Female. The female remains unknown.

Male genitalia similar to those in *A. kendevari* and other species of the *A. cyaneus* group. No species-specific characters in genitalia of *A. shahkuhensis* are found.

Molecular differentiation. *A. shahkuhensis* differs from all other taxa of *Agrodiaetus* by fixed differences in mitochondrial gene *COI* (Figs 3, 4) and nuclear sequence *ITS2* (Lukhtanov, Shapoval, 2008).

Morphological differentiation. In Shahkuh males of *A. shahkuhensis* are on average larger than males of *A. kendevari pseudoxerxes*, usually blue and, most importantly, have a white stripe on the hindwing underside that is not contrasting, and has nearly parallel borders (Fig. 2, b, d, f, h). Males of *A. kendevari pseudoxerxes* have the white stripe on the hindwing underside that is more contrasting and gradually becoming broader distally (Fig. 2 j, l, n, p, r). Thus, the shape of the white stripe on the hindwing underside is a useful character to distinguish between local sympatric populations of *A. shahkuhensis* and *A.*

kendevani pseudoxerxes found in Shahkuh. However, this character may be more variable in other populations of *A. kendevani*. In particular, some individuals of *A. kendevani kendevani* from Kendevan and Dizin have the white stripe with nearly parallel borders, similar to *A. shahkuhensis*.

Karyotype. It should be noted that the chromosome number $n=16$ is not species-specific for *A. shahkuhensis* and was also found in other taxa of the *A. cyaneus* species group. The chromosome number may be also variable, e.g. $n=15$ and $n=16$ have been found in *A. kendevani kendevani* (this study). However, the karyotype can be used as a specific genetic marker to demonstrate absence of gene flow between *A. shahkuhensis* and *A. kendevani* (see above).

Ecology. Xeromorph mountain steppe at 2000–2200 m altitude. Butterflies fly together with *A. kendevani pseudoxerxes*, but on average at lower altitude. *A. kendevani* was collected in Shahkuh, Kendevan and Dizin at different altitudes from 2000 to 3200 m.

Distribution. Only known from the type locality.

Flight period. From July to August.

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