

## Molecular genetic confirmation of the subspecies status of blues *Agriades orbitulus pheretimus* (Staudinger, 1892) and *A. orbitulus jugnei* Churkin, 2004 (Lepidoptera: Lycaenidae: Polyommatainae)

## Молекулярно-генетическое подтверждение подвидового статуса голубянок *Agriades orbitulus pheretimus* (Staudinger, 1892) и *A. orbitulus jugnei* Churkin, 2004 (Lepidoptera: Lycaenidae: Polyommatainae)

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KEY WORDS: fauna, COI, ITS2, entomology, South Siberia, Mongolia.

КЛЮЧЕВЫЕ СЛОВА: фауна, COI, ITS2, энтомология, Южная Сибирь, Монголия.

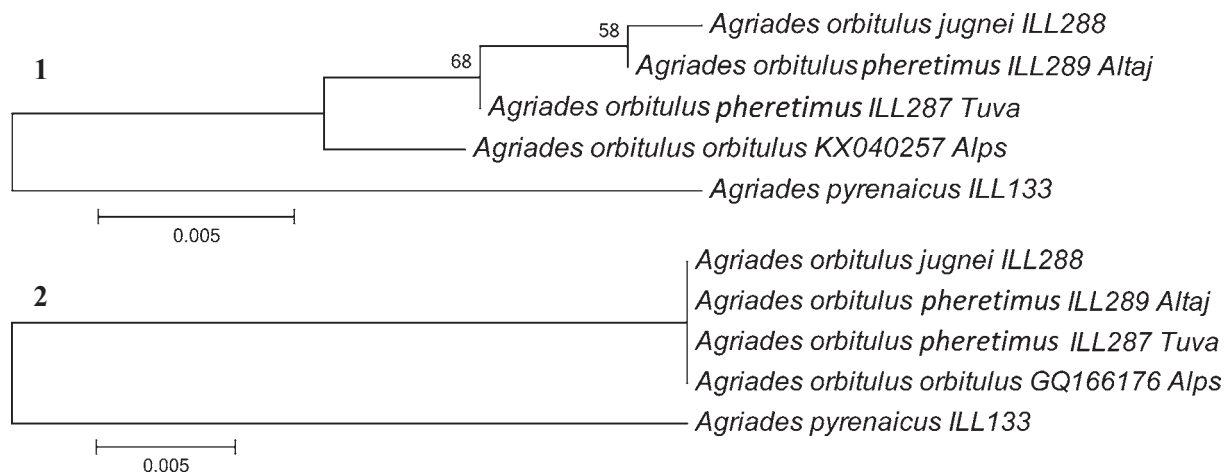
**ABSTRACT.** When analyzing gene sequences COI and ITS2, we revealed minor differences (in COI) between the following taxa: *Agriades orbitulus orbitulus* (de Prunner, 1798), *A. orbitulus pheretimus* (Staudinger, 1892) and *A. orbitulus jugnei* Churkin, 2004, which confirms the subspecies status of these blues.

**РЕЗЮМЕ.** При анализе последовательностей генов COI и ITS2 были выявлены незначительные отличия по COI между таксонами: *Agriades orbitulus orbitulus* (de Prunner, 1798), *A. orbitulus pheretimus* (Staudinger, 1892) и *A. orbitulus jugnei* Churkin, 2004, что подтверждает их подвидовой статус.

### Introduction

The Alpine Argus — *Agriades orbitulus* (de Prunner, 1798) — was described from Piemonte (Northern Italy). This is a rather widespread blue (Lepidoptera, Lycaenidae, Polyommatainae). Currently, it is indicated in Alps, Norway, Sweden, in several points of the Urals, in the mountains of South Siberia, the Lower Amur region, northern Sikhote-Alin, northeast China and Mongolia [Henriksen, Kreutzer, 1982; Gorbunov, 2001; Korshunov, 2002]. The subspecies structure is quite

simple. The described later *A. atys* (Hübner, 1805) and *A. pheretes* (Hübner, 1805), both as *Papilio*, are synonyms of the nominative subspecies. From the Asian part of the habitat, three taxa of the subspecies level were described much later: *Lycaena pheretes pheretimus* Staudinger, 1892 (type locality: Kentei [Buryatia and Transbaicalian Regions, Malkhansky Mts.]), *Lycaena pheretes sajana* Heyne, [1895] (type locality – Südliches Sibirien) and *Albulina orbitulus jugnei* Churkin, 2004 (type locality “ S. Mongolia, Gobi-Altai aimak, Tzakhir Khalgyn Nuruu, Detyin Davaa Pass). Some authors consider the above listed Asian taxa to be synonymous [Tshikolovets et al., 2009]. A point of view about the synonymy of *phetimus* and *sajana* [Gorbunov, 2001] is also common. There is an opinion that *phetimus* and *sajana* are bona subspecies [Zhdanko, 2000; Korshunov, 2002]. Chinese colleagues expressed the idea about the species status of *Albulina sajana* (Heyne, [1895]) [Wang, Fan, 2002]. The difficulty of analyzing the taxonomic situation is that the typical habitat of *Lycaena pheretes sajana* is completely unclear. In our opinion, the populations from Altai and Sayans are well distinguished from the Transbaicalian ones, see also images [Tuzov et al., 2000: plate 75]. But we can not clearly attribute the Altai and Sayan specimens with the taxon *sajana* up to specifying their typical



Figs 1–2. *Agriades orbitulus* ssp.: ML-cladogram for DNA sequences: 1 — COI; 2 — ITS2.

Рис. 1–2. *Agriades orbitulus* ssp.: ML-клатограмма для ДНК сиквенсов: 1 — COI; 2 — ITS2.

habitat, so currently we accept the point of view that *Lycaena pheretes pheretimus* Staudinger, 1892 = ? *Lycaena pheretes sajana* Heyne, [1895]. Currently the genus *Albulina* is included in the genus *Agriades* [Talavera et al., 2013].

## Material and methods

For molecular genetic studies, we used fresh material collected by a butterfly net in Russia (the Tuva Republic and the Altai Republic) and Mongolia (Gobi-Altai aimak): *Agriades orbitulus pheretimus*: ♂, Russia, Tuva, Kozhagar Mts., Kara-Sug vall., 1750 m, 5-Jun-2017, R. Yakovlev (voucher ILL\_287); ♂, Russia, Altaj, Aktash, 7-Aug-2016, R. Yakovlev (voucher ILL\_289); *A. orbitulus jugnei*: ♂, Mongolia, Gobi-Altai aimak, near Dotijin-Davaa pass, 1950 m, 5-Jul-2017, R. Yakovlev (voucher ILL\_288).

We amplified DNA 5' section of the mitochondrial gene Cytochrome Oxidase subunit I (COI) and the nuclear noncoding sequence internal transcribed spacer 2 (ITS2) on the Mastercycler gradient (Eppendorf). The following cycling protocols were used: an initial 4 min denaturation at 95°C and 40 cycles of 30 s denaturation at 95°C, 30 s annealing at 53°C and 60 s extension at 72°C. We used the following PCR primer pairs: forward, 5'-GTC AAC AAT CAT AAA ATA TTG G-3' with reverse, 5'-TTG CTC CAG CTA ATA CAG GTA A-3' were used to amplify COI. ITS2 was amplified with forward, 5'-GGG CCG GCT GTA TAA AAT CAT A-3' and reverse, 5'-AAA AAT TGA GGC AGA CGC GAT A-3' [Stradomsky, 2016].

The amplified fragments were separated using an automated sequencing machine (Applied Biosystems 3500). The analysis of primary nucleotide sequences was made with the help of the application BioEdit Sequence Alignment Editor, version 7.0.5.3 [Hall, 1999]. COI and ITS2 nucleotide sequences were treated quantitatively using MEGA6 [Tamura et al., 2013] methods Maximum Likelihood (ML) and were represented as ML-cladograms.

*Agriades pyrenaicus* voucher ILL\_133 (accession NoNo GenBank KC759734 (COI), KC759736 (ITS2)) was used as an outgroup to root the tree. *Agriades orbitulus orbitulus* (accession NoNo GenBank KX040257 (COI), GQ166176 (ITS2)) was used for comparison.

## Results and discussion

The obtained results of molecular-genetic study are presented in the form of ML-cladograms of DNA sections for mitochondrial COI gene (Fig. 1) and nuclear sequence ITS2 (Fig. 2). The presented data suggest that mitochondrial DNA sequences in the examined specimens of *A. orbitulus* are very close to each other and differ only by 0.2–0.4%, and from *A. orbitulus orbitulus* they differ by 0.7–1.1%, which is characteristic only of the subspecies level.

All nuclear DNA sequences of ITS2 were generally identical to each other, which again speaks in favor of the species *A. orbitulus* unity.

Thus, molecular genetic characters demonstrate minor differences in the mitochondrial COI gene sequences of populations from the mountains of South Siberia (ssp. *phetimus* =? *sajana*) and the Mongolian Altai highlands (ssp. *jugnei*) from the nominative subspecies, which indicates (in conjunction with the already known external differences) the subspecies status of these forms.

**Acknowledgments.** We express our gratitude to the colleagues for their assistance in the field studies on the territory of Mongolia (A. Fomichev, V. Rudoj, and A. Cherepanov). The authors are grateful to Anna Ustjuzhanina (Tomsk, Russia) for language improvements. The results were obtained within the framework of the state task No. 6.2884.2017/4.6 Ministry of Education and Science of Russian Federation.

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