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Micro-endemism pattern and *Wolbachia* infection of *Quedius obliqueseriatus* (Coleoptera, Staphylinidae), a montane rove beetle endemic of the North-Western Caucasus

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Abstract

We conducted an integrative taxonomic study of a forest floor dwelling montane rove beetle *Quedius obliqueseriatus* Eppelsheim, 1889. It is one of many endemic species of the North-Western Caucasus, a region considered a global biodiversity hotspot. Examination of the morphological characters in 93 specimens of *Q. obliqueseriatus* and phylogenetic assessment of the COI barcode for 28 of them, revealed that this species in fact consists of two distinct (p-distance of 4.0%) allopatric lineages, western and eastern. They subtly differ in the structure of the aedeagus, which was not noticed in the previous revision of this species. Nuclear DNA markers (H3, ITS1, ITS2, Wg and 28S) sampled in both lineages, did not show any divergence. Variation of the non-genitalic morphological characters, such as body size or coloration, is continuous across both lineages. Discovery of microendemic lineages within an endemic rove beetle species highlights how little is understood about the patterns and drivers of endemism in arthropods of the North-Western Caucasus. We refrained from the description of a new species due to shortage of data from the area where newly discovered western and eastern lineages meet. As *Q. obliqueseriatus* was found to be largely infected with *Wolbachia*, we gave a review of this infection among insects and other arthropods and its impact on speciation. Finally, we described our method of removal of the *Wolbachia* COI amplicon by endonuclease restriction enzyme in order to get the desired beetle amplicon from infected specimens.

Key Words

integrative taxonomy, molecular markers, morphology, endemism, COI

Introduction

The North-Western Caucasus is an area of the globe with a very rich temperate flora and fauna characterized by the high rate of endemism. In the west it is limited by the coast of the Azov and Black seas, in the north by the Manych Depression and a line between the mouth of the Don River and Lake Manych, in the south by the state border with Abkhazia and in the east by the valley of the river Urup and the watershed of the Urup and Bolshaya Laba rivers (Fig. 1A). Overall, it is very diverse terrain that covers about 87,000 km^2 (Zamotajlov et al. 2010). Its landscapes and elevations range from the lowland wetlands of the Kuban River to the mountain peaks like Mount Tsakhvoa that reaches 3346 meters above sea level. The North-Western Caucasus stretches across four administrative regions of Russia: the Krasnodar and Stavropol Territories, and the Republics of Adygea and Karachay–Cherkessia. This area is placed among the global biodiversity hotspots (Krever et al. 2001; Mittermeier et al. 2005; Egorov et al. 2020) and it is very attractive for naturalists and scientists experiencing and exploring biodiversity. Nevertheless, knowledge about the North-West Caucasian biodiversity remains incomplete, especially as far as insects and other arthropods are concerned.

Detailed entomological studies of the North-Western Caucasus are of broad scientific interest because they shed light on the speciation processes leading to significant diversification and originality of the regional entomofauna. According to Zamotajlov et al. (2010), out of 1939 beetle (Coleoptera) species recorded in the North-Western Caucasus, around 600 are endemic to this region. Many of them have very narrow distributions that occupy a mountain range or a system of close by ranges, with a sister species across a valley. In particular, the mainly predatory rove beetles (Staphylinidae) that inhabit soil, leaf litter or other ground-based microhabitats at various elevations of the North-West Caucasian mountains have such endemics, many of which are still unknown or poorly studied (Solodovnikov 1998, 2001).

One such example is a presumably monophyletic group of narrowly distributed, apterous rove beetle species that includes *Quedius humosus* Solodovnikov, 2005 confined to the low elevation forests around the Black Sea coast in the south-east, *Quedius lgockii* Roubal, 1911, confined to a few close alpine localities, and *Quedius obliqueseriatus* Eppelsheim, 1889 confined to a larger area of the mountain forests of low and middle elevations (Solodovnikov 2004, 2005; Salnitska and Solodovnikov 2019). All three species clearly differ from each other morphologically, especially by the structure of the aedeagus (Salnitska and Solodovnikov 2019). Among them, *Q. obliqueseriatus* has the widest distribution (Fig. 1A) where it occurs in forest leaf litter from 200–600 m in the foothills (Fig. 1B) to 1600–1800 m at the timber line. The habitus of *Q. obliqueseriatus* (Fig. 2) shows significant morphological variation. Beetles vary in body size, and in the coloration of their pronotum, elytra and to some extent abdomen (from pale, reddish, to dark, blackish). The aedeagus also shows a seemingly continuous variation in the pattern of sensory peg setae on the paramere (Fig. 3), and the shape of the apex of the median lobe (Fig. 4).

This study was prompted by the distribution range of *Q. obliqueseriatus*, which is significantly larger than in other similarly apterous species of this group, and the above mentioned morphological variation. It aims to test the integrity of *Q. obliqueseriatus* as a species using DNA and morphological data, as well as to explore potential geographic pattern within the morphological variation. Therefore, we sampled as much material as possible from the entire distribution area of this species and sequenced several commonly used mitochondrial and nuclear mark-

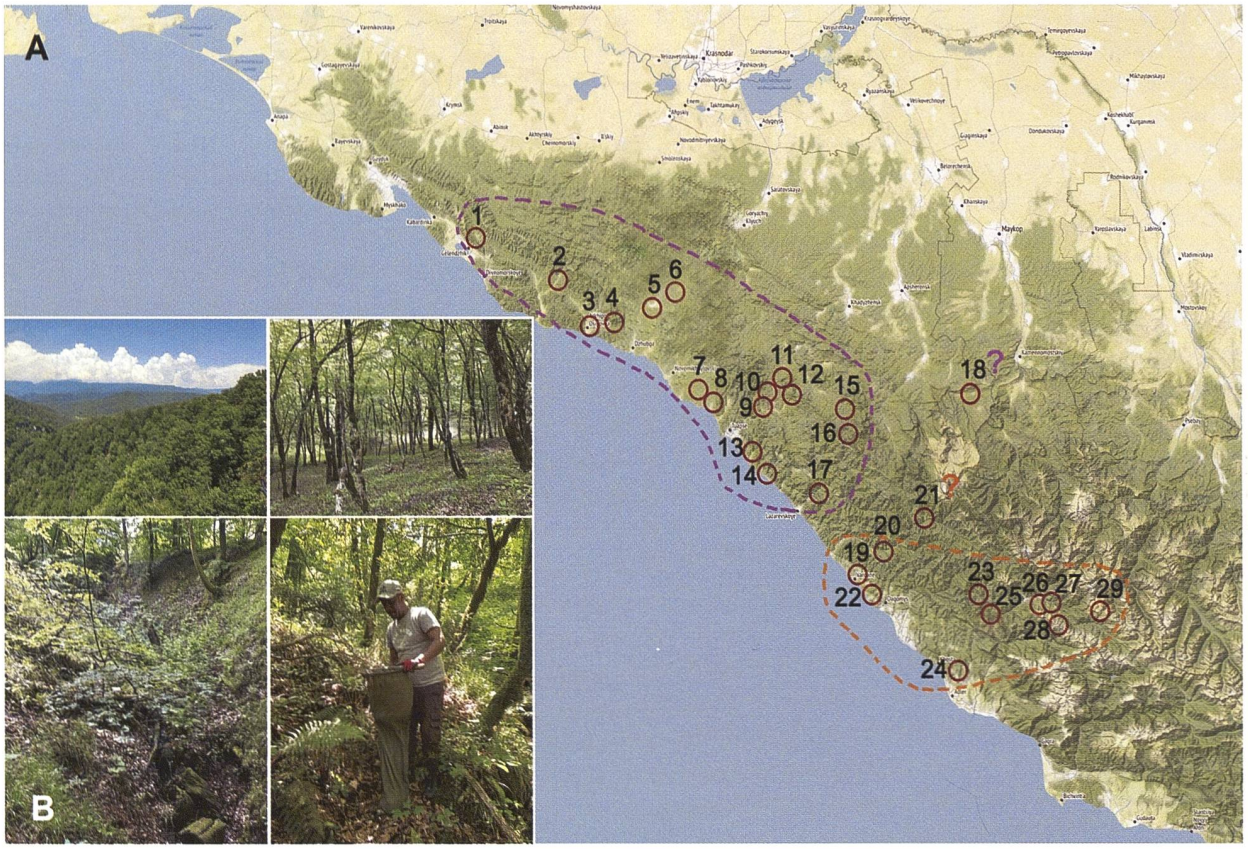

Figure 1. Distribution and sampling of *Quedius obliqueseriatus* in the North-Western Caucasus. **A.** North-Western Caucasus terrain and sampled localities. **B.** Example of sampled habitats of *Q. obliqueseriatus*. Numbers represent the names of localities as follows: 1. Aderbienka 2. Pshada 3. Archipo-Osipovka 4. Bzhyd 5. Defanovka 6. Moldavanovka 7. Olginka 8. Nebug 9. Kirpichnoe 10. Krivenkovskoe 11. Induk 12. Semashko 13. Shepsi 14. Druzhba 15. Terziyan 16. Apsheronsk 17. Tatyanovka 18. Temnolesskaya 19. Vardane 20. Solokh Aul 21. Babuk Aul 22. Gorniy Vozdukh 23. Medoveevka 24. Vardane Verino 25. Chvizhepse 26. Krasnaya Polyana 27. Esto-Sadok 28. Aibga 29. Atschischo. Dotted purple and orange lines or question marks indicate hypothesized distribution area of the western and eastern clades, respectively. Photos by M. Salnitska.

ers. In the course of the molecular work, we discovered that a significant proportion of specimens were infected with the microbial endosymbiont *Wolbachia*, whose COI sequence was amplified instead of the desired beetle COI fragment. Discovery of the *Wolbachia* infection became an interesting side-track, which necessitated an addition of a concise discussion about *Wolbachia* and design of an additional laboratory experiment to amplify beetle sequences from the infected specimens. Before proceeding to the presentation of the methods, results and conclusions of our study, it is necessary to introduce 1) our choice of molecular markers for species delimitation and 2) the phenomenon of *Wolbachia* infection in insects and its impact on speciation and species delimitation studies.

Choice of molecular markers for our study

Species delimitation using molecular markers is widely used in Coleoptera and Staphylinidae in particular, with an agreement that a combination of mitochondrial and nuclear gene fragments provide the most reliable results, especially if they show congruence with morphology (Song and Ahn 2014; Lee et al. 2020; Muñoz-Tobar and Caterino 2020; Tokareva et al. 2021; Yoo et al. 2022; Hansen and Jenkins Shaw 2023). A few studies on Staphylinidae used several molecular markers for species delimitation purposes. Song and Ahn (2014) assessed the accuracy of species delimitation and phylogenetic relationships of the *Aleochara fucicola* species complex using two mitochondrial (COI and COII) and three nuclear genes (CAD, EF1-α and Wg). Von Beeren et al. (2016a, 2016b) applied the mitochondrial COI and two nuclear genes (Wg and CAD) to survey species boundaries for the army ant symbionts. Muñoz-Tobar and Caterino (2020) used the mitochondrial COI and the nuclear Wg genes to examine the concordance of morphological characters and geography with hypothesized species boundaries in the genus *Panabachia*. Yoo et al. (2021) and Lee et al. (2020) sequenced the mitochondrial COI and the nuclear 28S genes to study species delimitation of *Phucobius* and some Korean *Oxyporus*, respectively. At the same time, a number of species delimitation studies in Staphylinidae were based on the COI fragments alone, or in combination with morphology. Using COI alone, Chatzimanolis and Caterino (2007) examined the phylogeographic structure of *Sepedophilus castaneus* (Tachyporinae), Caterino et al. (2015) explored relationships and gene flow among island and mainland populations of four species, and Serri et al. (2016) tested the intraspecific genetic variation in Steninae. Using the barcoding fragment of COI and morphology, Brunke et al. (2020a) explored species limits in the genus *Quedionuchus*, Lee et al. (2020) in the genus *Coprophilus*, Tokareva et al. (2021) in the genus *Oxyporus* and Hansen and Jenkins Shaw (2023) in the genus *Lobrathium*. Brunke et al. (2020b), Salnitska and Solodovnikov (2021) and Hansen et al. (2022) applied COI barcoding and morphological characters for delimiting species in the genus *Quedius*. Other molecular markers repeatedly used in species level studies in Coleoptera are the nuclear H3, ITS1 and ITS2 (Downie and Gullan 2004; Fossen et al. 2016; Svante et al. 2017). Based on the reviewed literature, we aimed to sequence mitochondrial COI and nuclear H3, ITS1, ITS2, Wg and 28S to explore integrity of *Q. obliqueseriatus* as a species.

Wolbachia and its impact on speciation and species delimitation studies in insects

Wolbachia are maternally inherited obligate intracellular alpha-proteobacteria and a member of the order Rickettsiales (Werren et al. 2008). *Wolbachia pipientis* and related species are known to infect arthropods and nematodes and cause diverse complex symptoms such as vitamin deficiency, cytoplasmic incompatibility and other reproduction abnormalities, as well as parthenogenesis in their hosts (Werren et al. 2008; Hosokawa et al. 2010; Zug and Hammerstein 2015; Nugapola et al. 2017; Schuler et al. 2018; Jiménez et al. 2019).

Since the first detection and description of *Wolbachia* in *Culex pipiens* (Hertig and Wolbach 1924; Hertig 1936), many other arthropod species were reported to harbor similar endosymbionts and to show equivalent mating incompatibilities. *Wolbachia* is now known as the most abundant and widespread intracellular bacterium on Earth (Roy et al. 2015; Boonsit and Wiwatanaratanabutr 2021). In a survey of 157 species of Neotropical arthropods from Panama, *Wolbachia* were detected in 26 of 154 insect species from all major orders (16.9%) (Werren et al. 1995). In a detailed screening of *Wolbachia* infection for 15 colonies of the very common soil-feeding termites *Cubitermes* spp. *affinis subarquatus* (Termitidae, Termitinae) in Central Africa, Roy et al. (2015) showed that 50% of the individuals were *Wolbachia* positive. *Wolbachia* infections were also common among four mosquito species in Sri Lanka (Nugapola et al. 2017), in moths from three different geographic regions of Thailand (Boonsit and Wiwatanaratanabutr 2021), or in an insular radiation of damselflies (Lorenzo-Carballa et al. 2019), to mention just a few diverse examples for insects.

Non-insect arthropods, such as crustaceans (Rousset et al. 1992; Bouchon et al. 1998), arachnids (Breeuwer and Jacobs 1996) and mites (Sourassou et al. 2014) were also found to harbor *Wolbachia*. While *Wolbachia* is a frequent reproductive parasite in arthropods, in filarial nematodes it is an obligate mutualist (Lo et al. 2007; Hilgenboecker et al. 2008; Wasala et al. 2019; Manoj et al 2021).

The transmission of *Wolbachia* from one infected organism to another is maternal, via the cytoplasm of the egg. It was observed that *Wolbachia* are evenly distributed within female germ lines, but concentrate in the future oocyte during oogenesis (Werren 1997; Stouthamer et al. 1999). Once the oocyte is built, *Wolbachia* again disperse throughout the egg. There is evidence that *Wolbachia* utilize their host's microtubule cytoskeleton to localize in particular parts of the cell (Ferree et al. 2005). There is further evidence that *Wolbachia* are capable of moving from outside

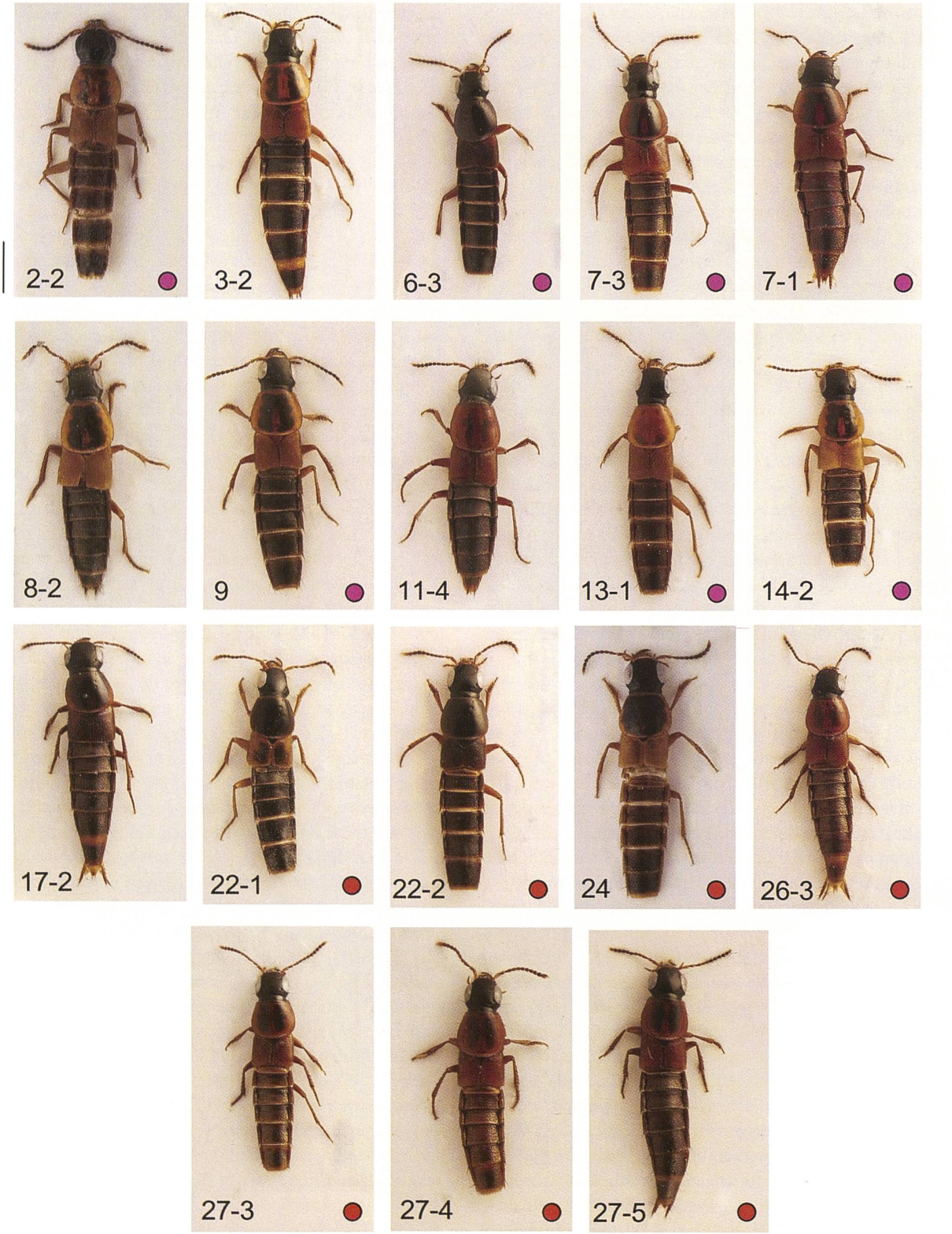

Figure 2. Habitus diversity of *Quedius obliqueseriatus*. Numbers correspond to the specimen code in the Suppl. material 1: table S2 that consists of the locality code (from 1 to 29, also used in Fig. 1) and sequential number of a specimen from a given locality. Purple dots indicate specimens from the western clade, orange dots indicate specimens from the eastern clade, as in Fig. 5. Scale bar: 1 mm.

the reproductive tissues into the female germ line. Frydman et al. (2006) have shown that *Wolbachia* can cross different tissues to reach the germ line when injected into *Drosophila melanogaster*. *Wolbachia* are not always exclusive to the reproductive tissues of their hosts. In some insect species they were also found in somatic tissues like muscles (Dobson et al. 1999) or nerves (Rigaud et al. 1991). Infection of the nervous tissue with this bacterium suggests a possible influence on the host's behavior. Therefore, interactions of *Wolbachia* with the host species can range from parasitic

to symbiotic. The success of *Wolbachia* is attributed to its efficient maternal transmission and to the impact on host reproduction that favors infected females, called the sperm-egg cytoplasmic incompatibility (Serbus et al. 2008).

Manipulation of host reproduction by *Wolbachia* plays an important role in a variety of evolutionary processes of the host species (Werren 1998; Bordenstein 2003). The presence of diverse symbionts is thought to increase speciation rates through the spread of symbiont strains that encode cytoplasmic incompatibility in their hosts, and which may produce barriers to gene flow (Bordenstein et al. 2001; Telschow et al. 2005). In fact, the first suggestion that cytoplasmic elements have the potential to influence host speciation, given postzygotic isolation, was published long ago (Laven 1959). *Wolbachia* infections can, for example, be responsible for reproductive isolation between sister species or for lethality of hybrid males. In particular, *Wolbachia* can induce a mating incompatibility in their hosts that terminates or at least reduces offspring production between infected males and uninfected females. In particular, *Wolbachia* induced cytoplasmic incompatibility where male and female gametes were unable to form viable offspring due to differences in parental *Wolbachia* infection status. When *Wolbachia* infected males mated with uninfected females (unidirectional infection), few or no offspring were produced, while all other crosses were fertile (Hoffmann and Turelli 1997). As a result, once infection levels in a population surpass a threshold, *Wolbachia* is predicted to sweep through the host population (Kriesner et al. 2013; Schuler et al. 2016). The reproductive advantage of *Wolbachia* infected individuals can result in rapid spread of the endosymbiont (Schuler et al. 2013). Moreover, the co-maternally inherited mitochondrial DNA can hitchhike with the spreading *Wolbachia*, replacing mitochondrial haplotypes associated with uninfected individuals (Schuler et al. 2016). Thus, *Wolbachia* infected populations typically exhibit lower mitochondrial diversity than uninfected populations (Jiggins 2003; Hurst and Jiggins 2005). Reproductive isolation can be also favored for example, when both partners are infected with different strains of *Wolbachia* causing bidirectional cytoplasmic incompatibility, with both mating directions being infertile (Bordenstein et al. 2001; Kodandaramaiah et al. 2013). All these have generated interest in the possible role of *Wolbachia* in promoting speciation or maintaining species boundaries (Werren 1998; Bordenstein 2003; Brucker and Bordenstein 2012; Schuler et al. 2016).

A number of studies explored species boundaries for insects infected with *Wolbachia*. Gebiola et al. (2012) conducted an integrative taxonomic study for delimiting wasp species within the *Pnigalio soemius* complex. They confirmed a trend towards host specificity within the presumed polyphagous *P. soemius* and suggested that *Wolbachia* infection could have played a major role in the reproductive isolation and genetic diversification of at least two species. Ritter et al. (2013) tested for cryptic speciation for two butterfly species, *Phengaris teleius* and *Phengaris nausithous*, based on a comprehensive sample across their Palaearctic ranges using COI gene sequences, nuclear microsatellites and tests for *Wolbachia*. In both species, a deep mitochondrial split occurring 0.65–1.97 million years ago was observed that did not correspond with microsatellite data but was concordant with *Wolbachia* infection. Ritter et al. (2013) rejected the hypothesis of cryptic speciation within *P. teleius* and *P. nausithous* in favor of the explanation that the major splits in the mtDNA phylogeny in both species were caused by *Wolbachia* infections. Furthermore, they concluded that geographic isolation during Pleistocene glaciations contributed to differentiation of mitochondrial and nuclear genomes. Kodandaramaiah et al. (2013) showed that the satyrine butterfly *Coenonympha tullia*, a species uniform in nuclear genes and morphology, having a deep split between two mitochondrial clades, each infected by two different *Wolbachia* strains, respectively. Plewa et al. (2018) used different sets of data (morphology, genetics and ecology) to verify the taxonomic status of *Monochamus sartor sartor* and *M. s. urussovii* (Coleoptera: Cerambycidae) across their entire range. Their morphological and molecular data showed that both subspecies have distinct but very weakly diverged mitochondrial haplogroups. Moreover, each subspecies is infected by different strains of the intracellular bacterium *Wolbachia*, which could be one of the factors causing their genetic isolation, regardless of geographic isolation.

Material and methods

Specimen acquisition

Material for this study was examined from the collections of the Zoological Institute of the Russian Academy of Science in St. Petersburg (**ZIN**) and the University of Tyumen (**UTMN**). A special field trip to sample *Q. obliqueseriatus* in the mountain foothills along the Black Sea coast in Russia was organized in 2022 (Fig. 1B). All geographic localities for the material in this study are shown in Fig. 1A, which was produced using QGIS 2.18.22 based on coordinates given on the labels or found by us when toponyms on the labels were recorded only verbally. All examined material is listed in the Suppl. material 1: table S2.

Morphological character examination and documentation

For the study of morphological characters, beetles were examined under the dissecting microscope (Zeiss Stemi 305) and photographed with a Canon EOS 5D Mark III DSLR (Canon Inc.) digital camera with a macro lens Canon MP-E 65mm F2.8 1–5x (Canon Inc.) mounted on a Cognisys Stackshot 3X macro rail connected with a controller and a macro flash Macro Twin Lite MT-24EX Flash (Canon Inc.). Aedeagi were dissected under the dissecting microscope, and the paramere was detached from median lobe. The median lobe and underside of the paramere with peg setae were photographed from soft preparations of these structures in glycerin using the

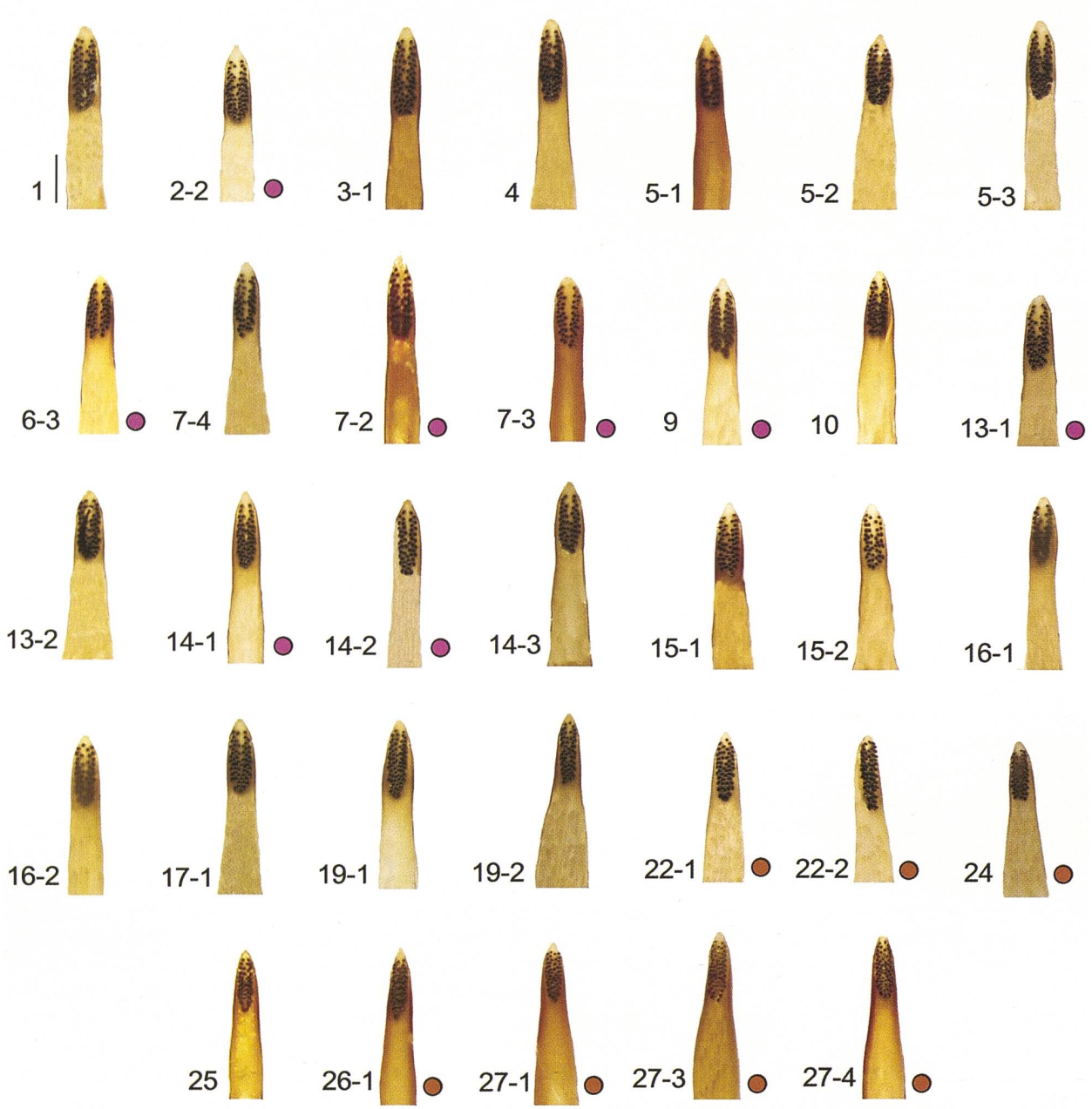

Figure 3. Variability of the sensory peg setae arrangement on the underside of the parameral apex in *Quedius obliqueseriatus*. Numbers correspond to the specimen code in the Suppl. material 1: table S2 that consists of the locality code (from 1 to 29, also used in Fig. 1) and sequential number of a specimen from a given locality. Purple dots indicate specimens from the western clade, orange dots indicate specimens from the eastern clade, as in Fig. 5. Scale bar: 0.25 mm.

same photo system described above. All dissected aedeagi were preserved in the same vials separated with cotton tissue under their respective specimens.

Molecular work

Molecular work was performed mainly in the Laboratory of Insect Systematics and Phylogenetics of the Institute of Environmental and Agricultural Biology (X-BIO), University of Tyumen (Tyumen, Russia). Some of the molecular work was performed in the Antimicrobial Resistance Laboratory of the same institute.

DNA extraction

The DNA extraction is carried out using Qiagen's DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). In the first step, 180 µl of buffer ATL was added to the vacuum dried specimens in the Eppendorf tube and immediately 20 µl of proteinase K solution was added, after a short vortex it was incubated at 55 °C for 24 hours. In the second step, 200 µl of buffer AL was added and immediately a short vortex was required followed by incubation for 10 minutes at 70 °C. After the addition of 200 µl of 96% ethanol, the total volume was transferred to a labelled mini spin column and centrifuged at 8000 rpm for 1 minute.

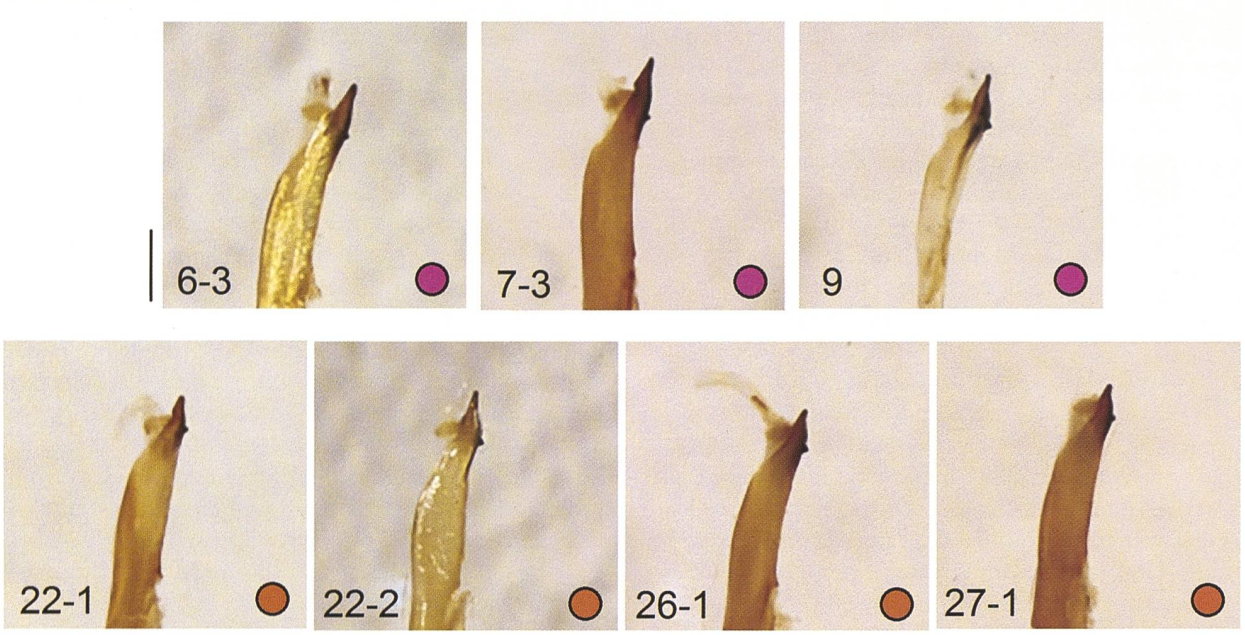

Figure 4. Variability of the apical portion of the aedeagus median lobe in *Quedius obliqueseriatus*, in lateral view. Numbers correspond to the specimen code in the Suppl. material 1: table S2 that consists of the locality code (from 1 to 29, also used in Fig. 1) and sequential number of a specimen from a given locality. Purple dots indicate specimens from the western clade, orange dots indicate specimens from the eastern clade, as in Fig. 5. Scale bar: 0.25 mm.

Next, the mini spin column was moved to new collection tube and the flow through was discarded. 500 µl of buffer AW1 was added followed by centrifugation at 8000 rpm for 1 minute and discarding the flow through. Another 500 µl of buffer AW2 was added, followed by centrifugation at 14000 rpm for 3 minutes and discarding the flow through. Finally, 60 µl of elution buffer AE was added, followed by centrifugation at 8000 rpm for 1 minute. The flow through containing the DNA extract was transferred to a new Eppendorf tube and stored at -20 °C.

PCR

The targeted regions were amplified using the primers listed in Suppl. material 1: table S1. The primers (HCO 3198 and LCO 1490) were used to amplify a 658 bp fragment of the cytochrome oxidase (COI) mitochondrial gene. Typical polymerase chain reaction (PCR) was conducted with a PCR master mix containing a total volume of 20 µl in each PCR tube. This premix contains 4 µl of Red Buffer (Evrogen), 2 µl of primers (1 µl each), 0.4 µl of dNTPs, 0.5 µl of Taq Polymerase, 2 µl of DNA extract and 11.1 µl of sterile distilled water. PCR was performed under the following cycling conditions: initial denaturation for 3 minutes at 94 °C; 35 cycles of: 30 seconds at 94 °C, annealing temperature of 51 °C for 30 seconds and 72 °C for 30 seconds; followed by a final extension temperature of 72 °C for 5 minutes (Hebert et al. 2003; Schomann and Solodovnikov 2017).

Amplification of a 464 bp fragment of Wg was performed using a nested reaction. The first reaction used the external primers (Wg550F–WgAbRZ) and consisted of an initial denaturation for 3 min at 94 °C, 35 cycles of: 30 s of denaturation at 94 °C, 30 s of annealing at 53 °C and 1.5 min of extension at 72 °C, followed by a 5 min final extension at 72 °C. The product of this is then used as a template for a reaction using the internal primers (Wg578F–WgAbR) and consisting of the same temperature profile for the external primers (Wild and Maddison 2008; Schomann and Solodovnikov 2017).

The amplification profile of a 802 bp fragment of 28S consisted of an initial denaturation for 2 min at 94 °C, 35 cycles of: 45 s at 94 °C, 30 s at 53–58 °C and 1 min at 72 °C, followed by a 2 min final extension at 72 °C (Yoo et al. 2021).

The amplification profile of a 308 bp fragment of H3 consisted of an initial denaturation for 5 min at 95 °C, 35 cycles of: 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C, followed by a 8 min final extension at 72 °C (Fossen et al. 2016).

The amplification profiles of 477 bp and 602 bp fragments of both ITS1 and ITS2, respectively, were the same: an initial denaturation for 2 min at 94 °C, 35 cycles of: 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C, followed by a 7 min final extension at 72 °C (Downie and Gullan 2004; Ashfaq et al. 2010).

In all cases the final PCR products were separated in 1% agarose gel by electrophoresis and the gel was docked under UV-illuminator apparatus to visualize the DNA bands.

DNA cleaning, sequencing and alignment

Post PCR products were cleaned using the Cleanup S Cap kit, according to manufacturer's instructions

(https://evrogen.ru/kit-user-manuals/BC041.pdf). Sequencing was performed commercially by Evrogen (https://evrogen.ru). All sequences were generated in both directions (with forward and reverse primers) and confirmed with sense and antisense strands. Sequences were cleaned and aligned manually using MEGA version 6.0 (Tamura et al. 2013) and BIOEDIT 7.0 (Hall 1999). All genes are in protein-coding regions, so manual alignment of sequences was straightforward.

Detection of *Wolbachia* infection

Sequences from all 23 samples were blasted using GenBank nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For 23 specimens, COI fragments were identified as belonging to *Wolbachia* rather than *Q. obliqueseriatus*. Of these, we further tested 13 of them for *Wolbachia* infection by amplifying the gene *Wolbachia* surface protein (wsp), commonly used as markers to detect the presence of the bacteria (Conte et al. 2019; Shaikevicha et al. 2019; Kirik et al. 2020). We used *Wolbachia*-specific primer pairs, wsp81F and wsp691R (Shaikevicha et al. 2019), amplifying a ~ 550 bp fragment of the wsp gene. The amplification profile for wsp consisted of an initial denaturation for 5 min at 94 °C, 35 cycles of: 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, followed by a 10 min final extension at 72 °C (Shaikevicha et al. 2019).

Removal of *Wolbachia* COI amplicon

We compared *Wolbachia* and *Q. obliqueseriatus* COI sequences using NEBcutter V2.0 (http://nc2.neb.com/NEBcutter2/) and found a SspI restriction site that belongs to *Wolbachia* and is absent in *Q. obliqueseriatus* sequences. An endonuclease restriction enzyme (SibEnzyme, http://sibenzyme.com/pcr-fragments-restriction/) was used to differentiate the *Q. obliqueseriatus* DNA from *Wolbachia* COI amplicon by cutting the *Wolbachia* mitochondrial COI DNA segment at the AAT↑ATT / TTA↓TAA site. Endonuclease restriction enzyme was applied into the cleaned PCR DNA product of *Q. obliqueseriatus* with *Wolbachia* infection. The standard protocol was as follows: total reaction volume of 20 µl, which contained restriction buffer (X10) – 2 µl, restriction endonuclease – 1 µl, PCR DNA fragment – 10 µl and purified water – 7 µl. The reaction mixture was incubated at optimum temperature of 37 °C for 2 hours. 20 µl of the reaction mixture was applied to 1% agarose gel for control electrophoresis and to separate the DNA bands. One clear band approximately 600 bp and two clear bands approximately 300 bp were obtained. We cut out the 600 bp length band from the gel and cleaned it. DNA cleaning was conducted on the excised gel using the Evrogen cleaning kit (https://evrogen.ru/kit-user-manuals/BC041.pdf). Sequencing was performed commercially by Evrogen (https://evrogen.ru).

Molecular phylogenetic analysis for species delimitation

First, we performed alignment of sequences individually for all sequenced markers in MEGA version 6.0 (Tamura et al. 2013); including an outgroup *Quedius maurorufus* COI sequence. No variation was found for any of them except COI. For COI, the substitution model GTR + G was selected based on the Akaike information criterion (AIC), using JMODELTEST (Guindon and Gascuel 2003) run on CIPRES (https://www.phylo.org/index.php/; Miller et al. 2010). The Bayesian analysis was performed using MrBayes 3.2.7a (Ronquist et al. 2012). The analysis was run twice using 4 simultaneous chains for 10 000 000 generations with tree sampling every 1000 generations and discarding 25% of each run as burn-in. Convergence was judged by stabilization of the standard deviation of the split frequencies around 0.01. The overlay plot was checked for even distribution in both runs. The values of estimated sample size (ESS) and potential scale reduction factor (PSRF) were checked to reach a value of more than 100 for almost per all parameters, and 1.0, respectively.

The estimates of evolutionary divergence (p-distance) between sequences were computed in MEGA version 6 (Tamura et al. 2013) and differences in the composition bias among sequences (Tamura and Kumar 2002) were accounted for. The haplotype network was produced in PopART, using integer NJ network parameter (Leigh and Bryant 2015).

Results

As shown in detail in the Suppl. material 1: table S2, our sample consisted of 93 specimens (55 males and 38 females) of the traditionally (e.g., Solodovnikov 2004; Salnitska and Solodovnikov 2019) morphologically defined *Q. obliqueseriatus*, with samples collected from 29 localities across nearly its entire distribution (Fig. 1A). Of these, we were able to extract DNA from 70 specimens, which represented nearly all localities. From these extracts, we successfully amplified beetle COI from 22 specimens, Wg from 31 specimens, 28S from 11 specimens, H3 from 9 specimens, ITS1 from 5 specimens and ITS2 from 5 specimens. For 23 specimens, we were not able to amplify beetle COI because primers always picked up a COI fragment that blasted as *Wolbachia*. Of the 23 *Wolbachia* infected *Q. obliqueseriatus* specimens, 6 specimens were successfully freed from the *Wolbachia* amplicon using the endonuclease restriction enzyme as described above in the Material and Methods section. As a result, we obtained 28 COI sequences for *Q. obliqueseriatus* for our study.

Sequence alignments conducted for each gene individually revealed that only COI showed variation within *Q. obliqueseriatus* (fasta files of the aligned sequences for each marker are included in the Suppl. material 1 and named respectively as H3, ITS1, ITS2, Wg, and 28S).

Therefore, we conducted phylogenetic analysis with the COI gene fragment only.

Phylogenetic analysis of the COI data revealed with strong statistical support that *Q. obliqueseriatus* consists of two clades, here designated as "western" and "eastern" (Fig. 5A). Both clades are genetically divergent from each other (Fig. 5B) with p-distance of 4.0% (Suppl. material 1: table S3). Populations of the western clade are mainly distributed from around Pshada and Moldavanovka villages in the west to about Mt. Semashkho, and Shepsi and Druzhba villages in the east. Populations of the eastern clade are distributed from around Vardane and Solokh-Aul villages in the west to Vardane Verino and Krasnaya Polyana villages in the east (Fig. 5C). It should be noted that nuclear markers that we sampled from specimens across both "western"and "eastern" clades, did not show any variation.

Examination of the external morphology of the sequenced *Q. obliqueseriatus* specimens revealed no characters associated with either eastern or western clades. Smaller and larger specimens, darker and paler, with or without spots on pronotum and elytra, with variously shaped spots, occur among specimens in both clades (Fig. 2). Examination of the paramere revealed that most of the specimens from the western clade have sensory peg setae arranged in two irregular longitudinal groups with a distinct setae free area in between, at least apically (see examples marked with purple dot in Fig. 3). At the same time, all specimens from the eastern clade (see examples marked with orange dot in Fig. 3) have these rows indistinct, the distribution of sensory peg setae strongly diffuse, at most with an unclear setae free area between them.

The sensory peg setae arrangement revealed that additional specimens without COI data could be assigned to either of eastern and western molecular clades. For example (Fig. 3) specimen 1, all specimens in the same row from 3-1 to 5-3, the specimens 7-4, 10, 13-2, and all from 15-1 to 16-2 share a pattern characteristic of the western clade. At the same time, specimens 19-2, 22-1 and 25 (Fig. 3) share a pattern characteristic of the eastern clade. However, there are specimens (e.g., 17-1 or 19-1) that are difficult to assign to either of the clades based on morphology.

Examination of the median lobe in lateral view revealed that sequenced specimens from the western clade usually have the subapical tooth located at a slightly longer distance from the apex of median lobe (Fig. 4, upper row of examples marked with purple dot), while in the sequenced specimens from the eastern clade this tooth is usually located slightly closer to the apex of median lobe (Fig. 4, lower row of examples marked by orange dot). However, this morphological character is more subtle than the difference in sensory peg setae fields and does not show a distinct gap between both molecular clades. For example,

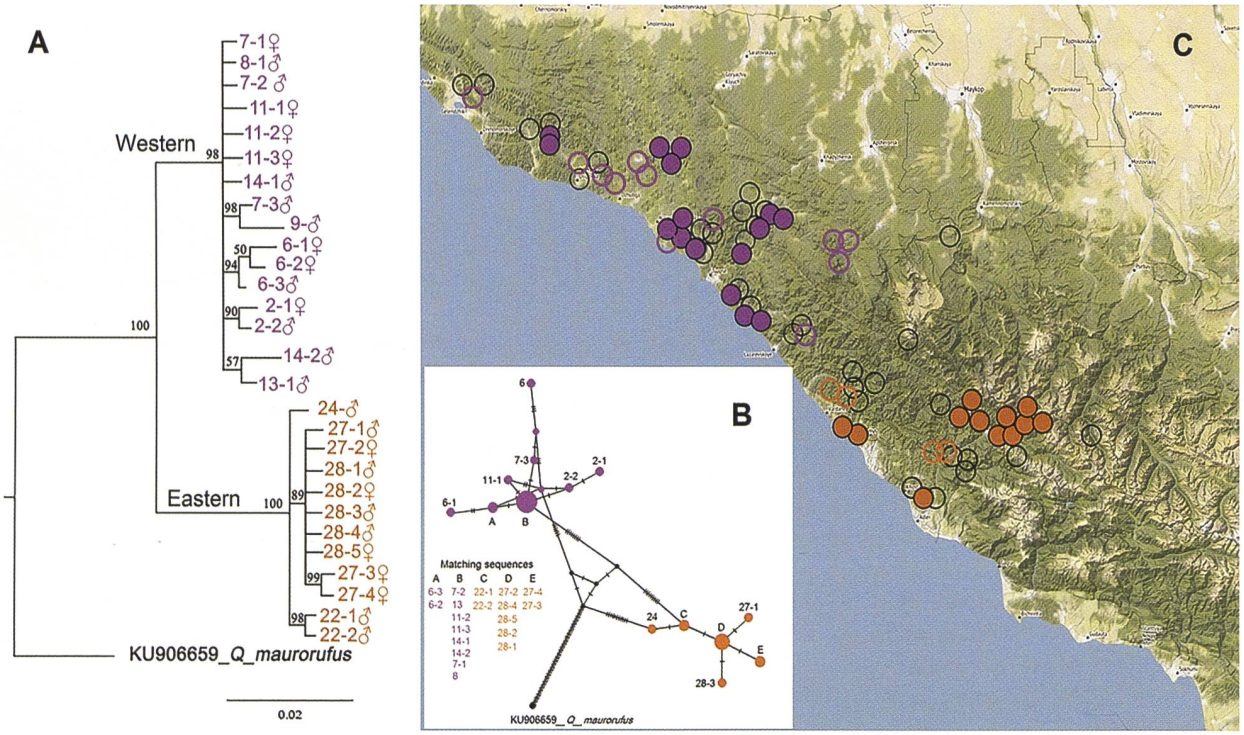

Figure 5. Micro-endemic forms of *Quedius obliqueseriatus* in the North-Western Caucasus, their phylogeny, genetic diversity, and geographic distribution. **A.** COI barcode-based Bayesian phylogenetic tree. **B.** Haplotype network. **C.** Geographic distribution. Purple color indicates specimens from western clade, orange color indicates specimens from eastern clade. Filled circles in **C** (the map) indicate sequenced specimens; open colored circles in **C** (the map) indicate non-sequenced males assigned to either western or eastern clade by the shape of the paramere only; open non-colored (black) circles in **C** (the map) indicate non-sequenced material without available males, thus unassigned to any clade. Numbers at terminals (branches) of the tree in A correspond to the specimen code in the Suppl. material 1: table S2 that consists of the locality code (from 1 to 29, also used in Fig. 1) and sequential number of a specimen when there is more than one from a given locality. Numbers at nodes in the tree in **A** indicate posterior probability in percentage.

the specimens 9 and 22-2 in Fig. 4 have the same distance between the subapical tooth and the apex of median lobe, even though they belong to different molecular clades.

Specimens infected by *Wolbachia* are widespread across the entire range of *Q. obliqueseriatus*. They belong to both western and eastern molecular clades (Suppl. material 1: fig. S1) and include males and females (Suppl. material 1: table S2). There is not any appreciable morphological trait associated with the *Wolbachia*-infected specimens. Generally, 24% of the specimens are infected with *Wolbachia*; of the infected specimens, 13 are males and 10 females.

Discussion

Our molecular and morphological examination revealed that *Quedius obliqueseriatus* consists of two lineages, here called western and eastern, that are clearly separable by COI barcoding and subtly separable by the characters of the aedeagus, mainly by the arrangement of sensory peg setae of the paramere. Because of the limited DNA-grade material or males for morphological examination, we still do not know exactly how both lineages are distributed. Based on the available data, these clades are allopatric and replace each other from west to east. However, it is not possible to confident in that due to the lack of the DNA-grade material from the area located between sequenced specimens from both clades (i.e., from such localities as Tatyanovka, Temnolesskaya, Vardane, Solokh-Aul and Babuk-Aul) (Figs 1A, 5C). There may be a zone of transition from one form to another, or a zone where they co-occur. Denser specimen sampling and more DNA-grade material is desired, especially from the geographic area where both forms meet.

The molecular divergence between both western and eastern lineages of *Q. obliqueseriatus* in COI with p-distance of 4.0% is strong enough to consider them as separate species by analogy with some other cases in beetles and other insects (e.g., Salnitska and Solodovnikov 2021; Yoo et al. 2022). However, there is no easy or uniform numerical threshold of molecular distance to determine species boundaries, and these instead vary case by case (Lukhtanov 2019). Examples in Staphylinidae vary too and depend on various considerations. Salnitska and Solodovnikov (2021) considered 4.4–7.7% distance among COI barcodes as inter-specific among species of the *Q. umbrinus* complex given the subtle morphological difference among them and distributional considerations. Yoo et al. (2022), also exploring morphology and nuclear marker along with the COI barcode data, considered interspecific divergence in COI barcode among the *Cafius* species ranging from 4.90% to 14.59%. In Tokareva et al. (2021) COI barcode-based distance among species of *Oxyporus* was lower than in either of the mentioned *Q. umbrinus* or *Cafius* complexes, but clearly supported by a hiatus in the endophallus structure.

Due to lack of divergence in the sampled nuclear molecular markers of *Q. obliqueseriatus*, the rather subtle nature of morphological difference between both COI-based lineages, and lack of any molecular data from the zone of potential contact of both forms, we consider the description of this potential new species premature, pending more material to be examined. Moreover, since the precise geographic origin of the type material for *Q. obliqueseriatus* collected by Hans Leder in the second half of the XIX-th century in "Circassia" (a broad area of Western Caucasus) is not clear (Eppelsheim 1889), we can only assume that the lectotype designated and illustrated in Solodovnikov (2004) belongs to the western clade because it has sensory peg setae clearly divided into two irregular rows. Therefore, the eastern clade represents a potential new species.

Discovery of two forms of an apterous and relatively narrowly distributed montane species that are clearly molecularly distant and somewhat subtly morphologically different, stresses a high degree of micro-endemism of the North-Western Caucasus that is not easy to detect by morphological investigations alone. Exploring and mapping distributions of such species in detail across various taxonomic and ecological groups of beetles and other arthropods may recover common patterns that in turn may reveal the origins of such endemism.

In case of the presumably monophyletic *Q. obliqueseriatus*-group, *Q. humosus* is restricted to the forest litter at low elevations of the south-eastern part of the North-Western Caucasus. Another species, *Q. lgockii*, is confined to high elevations above the timber line of the core montane area of the North-Western Caucasus. Finally, the here discovered western and eastern clades of *Q. obliqueseriatus* are both restricted to the forest belt stretching through a wide range of elevations and replacing each other from west to east, respectively. Interestingly, their ranges roughly coincide with two geo-botanical provinces in the western Transcaucasia recognized by Shiffers (1953). The western province is drier and of a Mediterranean aspect, the eastern is more humid and subtropical. These areas are thus used by various entomologists to define local regions in the faunistic works on the North-Western Caucasus (Ohrimenko 1992; Zamotailov 1992; Solodovnikov 1998). A thorough test for the monophyly of *Q. obliqueseriatus*-group along with the detailed molecular investigation of all endemic species of this complex may shed light on the ecological dimension of their diversification.

Finally, a *Wolbachia*-infection could have acted as a trigger for speciation in *Q. obliqueseriatus*, a process discussed for other *Wolbachia*-infected insects (Shoemaker et al. 1999; Sun et al. 2011; Leronzo-Carballa et al. 2019). It may be an interesting research program to further investigate strains of *Wolbachia* within both clades of *Q. obliqueseriatus*, to screen other narrowly distributed endemics for *Wolbachia* infections and compare their infection rates to widespread species. Our study does not have enough data to speculate on the potential role of *Wolbachia* in the micro-endemism pattern displayed by *Q. obliqueseriatus*. However, the discovered high rate of infection is noteworthy. On the practical side, we here demonstrated how to remove the *Wolbachia* amplicon that hinders the

barcoding of the desired gene fragment in an infected insect via the application of endonuclease restriction enzyme and subsequent excision following gel electrophoresis.

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Supplementary material 1

Suuplementary files in zip archive

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Data type: zip

Explanation note: **table S1.** Primers used in this study. **table S2.** All examined *Quedius obliqueseriatus* specimens. **table S3.** Genetic divergence of COI (*Quedius obliqueseriatus*) using uncorrected p-distance. **figure S1.** Distribution of the sequenced Wolbachia-free (green) and infected (yellow) individuals among the sequenced specimens of *Quedius obliqueseriatus* in the North-Western Caucasus. Dotted purple and orange lines indicate rough distribution area of the western and eastern clades, respectively. **QO- DNA** sequences for all markers used (in fasta files).

Link: https://doi.org/10.3897/alpento.7.111214.suppl1