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A fine line between species and ecotype: a case study of *Anoplistes halodendri* and *A. kozlovi* (Coleoptera: Cerambycidae) occurring sympatrically in Mongolia

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http://zoobank.org/0511AF60-C468-4DBA-870C-A745C560FE07

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Abstract

This paper discusses ecological adaptation based on a case study of *Anoplistes halodendri* and *Anoplistes kozlovi* (Coleoptera: Cerambycidae) that occur in the arid zone of Mongolia. By applying an integrative taxonomy approach, we revealed one of the first documented cases of sympatrically occurring ecotypes in Polyphaga and the second case of ecotypes in the family Cerambycidae. The results of the analysis of the ecological data, molecular analysis of mitochondrial (COI) and nuclear (ArgK and CAD) genes, as well as a detailed morphological examination, which consisted of a study on the male genitalia including the endophallic structures, supported the hypothesis that these two entities, which are commonly considered separate species, represent only ecologically adapted forms that are associated with rocky hills and sandy/gravelly plains, respectively. Therefore, a synonym is restored: *Anoplistes halodendri minutus* Hammarström, 1892 = *Asias kozlovi* Semenov and Znojko, 1934, **syn. res.** The differences in the elytral pattern and shade appear to be adapted to the different substrates in these distinct habitats. A probable scenario assumes that these two forms arose in parapatry, independently in multiple populations, under parallel speciation during the intensification of the aridification across the region in the period during which the Gobi Desert was formed (~ 24 to 2.6 Ma) and they may evolve into separate species in the future. The phylogenetic relationships of some taxa representing the most closely related genera of the tribe Trachyderini were analysed and the questioned species status of *Anoplistes jacobsoni* was confirmed. Low interspecific differences in the endophallic structures in the genus *Anoplistes* and between some species of the genus *Amarysius* indicate that they are evolutionarily relatively young groups. The practical differences between ecotype and subspecies are also discussed.

Key words

Central Asia, Gobi Desert, integrative taxonomy, non-allopatric speciation, parallel speciation, phenotypic variation.

1. Introduction

It is commonly acknowledged that species represent a unique level of self-organising entities in nature. However, delineating species is controversial and the variety of the mechanisms of speciation make recognising species even more problematic. The process by which one species diverges into two distinct phylogenetic lineages is one of the main issues in biology. Although it is commonly accepted that speciation occurs across a continuum over time, the various points along the process need to be studied in order to understand the nature of this phenomenon, the role of the reproductive isolating barriers during speciation and the point at which the process is completed (Lowry 2012). It has been proven that dividing speciation into stages could be quite useful for understanding the entire process better (e.g. Turesson 1922; Clausen 1951; Wu 2001; Nosil et al. 2009).

Adapting to different environmental conditions *via* divergent natural selection processes can generate phenotypic and genetic differences in the ecologically important characters between populations of the same species, which eventually may lead to the formation of a new species if the gene flow is negligible or absent (Schluter 2000; Nosil 2012). The intermediate stages in ecological speciation involve ecotypes—groups of populations within one species that are distinguished because they have adapted to local environmental conditions *via* a composite of variations in many traits and allele frequencies across loci over space (Lowry 2012).

The genus *Anoplistes* Audinet-Serville, 1833 (Cerambycidae: Cerambycinae: Trachyderini) is a relatively small taxonomic unit that comprises sixteen previously described species and several subspecies of *Anoplistes halodendri* (Pallas, 1773) (Danilevsky and Smetana 2010; Sláma 2010; Lazarev 2014; Danilevsky 2020; Karpiński 2020). It is one of the most poorly studied genera of the Palaearctic longhorned beetles and, taxonomically, it remains an intricate group. The representatives of the genus are distributed between SE Europe (Bulgaria) to the Pacific coast of Asia with the center of its occurrence located in still mostly unexplored areas of SE Kazakhstan, Kyrgyzstan, NW China and Mongolia. The bionomy and ecology of most of the taxa is largely unknown, and therefore many issues remain enigmatic and require a thorough investigation. This especially applies to the *Anoplistes halodendri* species-group recognised by Karpiński (2020)—originally distinguished as the "long-antennae" group by Plavilstshikov (1940) and Kostin (1974), which is mainly distributed in Russia, Kazakhstan, Mongolia, China and Kyrgyzstan. Besides *A. halodendri*, another four species: *Anoplistes churkini* Danilevsky, 2020, *Anoplistes degener* (Semenov, 1907), *Anoplistes jacobsoni* Baeckmann, 1904, and *Anoplistes kozlovi* (Semenov and Znojko, 1934) sensu Danilevsky and Smetana (2010) are herein regarded as belonging to this group. Within its range, *A. halodendri* was divided into six subspecies: *A. h. ephippium* (Steven and Dalman, 1817), *A. h. halodendri*, *A. h. heptapotamicus* (Semenov, 1926), *A. h. kasatkini* Lazarev, 2014, *A. h. minutus* Hammarström, 1892, and *A. h. pirus* (Arakawa, 1932) (Danilevsky and Smetana 2010; Lazarev 2014). According to the former authors, only one subspecies of *A. halodendri* (*A. h. minutus*) together with *A. kozlovi* is known to occur in Mongolia. Different opinions on the taxonomic position of *A. kozlovi* were previously published. While most authors have treated this taxon as a valid species (e.g. Danilevsky and Smetana 2010; Tavakilian and Chevillotte 2020) or a subspecies of *A. halodendri* (Özdikmen 2008), Namhaidorzh (1972) was the only author who questioned its specific distinctness and synonymised it with *A. halodendri* (along with its two other subspecies) by reducing it to a geographical form.

The interesting observation of these two taxa within one extensive site—nearly sympatrically but yet clearly sticking to the two distinct habitats—during our 2019 entomological expedition to southeastern Mongolia triggered us to thoroughly study this issue. It seemed prudent to investigate whether these two entities that possibly began to adapt to the different environmental conditions, despite the possible mating that had been observed in the contact zone, had not yet developed barriers in their gene flow (reproductive isolation), which consequently resulted in stable, diagnostic differences that are strong enough to consider them to be separate species. Here, we integrate the morphological, molecular, and ecological data that are used to understand the evolutionary relationship between the two taxa in question, which are distributed within the arid zone of Mongolia.

Secondary aims of this study were to verify the species status of the morphologically very similar *A. jacobsoni*, which has been considered a subspecies or even a synonym of *A. halodendri* (Kostin 1974; poor differential characters in Plavilstshikov (1940)), and to estimate phylogenetic relationships among the representatives of morphologically closely related genera of the tribe Trachyderini from the Palaearctic Region (*Amarysius*, *Purpuricenus*).

2. Material and methods

2.1. Material examined

This study is based on an examination of ~ 570 specimens of *Anoplistes halodendri minutus* and *A. kozlovi* sensu Danilevsky and Smetana (2010) from the territory of Mongolia (Figs 1, 2, 3) as well as additional ~ 110 specimens of *A. jacobsoni* from southern Kazakhstan and ~ 20 specimens of *A. halodendri halodendri* from eastern Kazakhstan. The specimens are housed in the following collections: CCH, the collection of Carolus Holzschuh, Villach, Austria; CLKR, the collection of Lech Kruszelnicki, Siemianowice Śląskie, Poland; CWTS, the collection of Wojciech Szczepański, Siemianowice Śląskie, Poland; HNHM, the Hungarian Natural History Museum, Budapest, Hungary; MAS, the Institute of Biology, Mongolian Academy of Sciences, Ulan Bator, Mongolia; MIZ, Museum and Institute of Zoology, Polish Academy of Sciences, Łomna, Poland; NMP, the National Museum Prague, Prague, Czech Republic; USMB, the Upper Silesian Museum, Bytom, Poland; ZIN, the Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia.

The individuals that were used for the detailed morphological and molecular analyses were collected by the authors during entomological expeditions to Kazakhstan (2017) and Mongolia (2019). Moreover, additional fresh material for the analyses was collected in SE Mongolia by Badamnyambuu Iderzorig (National University of Mongolia, Ulan Bator, Mongolia) earlier in 2019.

2.2. Morphological analyses

2.2.1. Measurements

Individuals of two nearly sympatric populations of *A. halodendri minutus* and *A. kozlovi* sensu Danilevsky and Smetana (2010) from the environs of the Choiriin Bogd Mountain were analysed. Thirty males and 20 females of both taxa were subjected to a detailed morphometric examination. Measurement data on the body length, ratio between the length and width of pronotum and between the length and width of the elytra are presented as boxplots and were tested using the non-parametric Mann-Whitney U test using PAST software (Hammer et al. 2001).

The beetles were examined using an Olympus SZH10 Stereo Microscope at 7–140 × magnification and a PROLAB MSZ Stereo Microscope at 7–90 × magnification.

2.2.2. Procedure for preparation of male terminalia

To examine the sclerotised parts of the male terminalia, the specimens were relaxed in distilled water for 12–24 h at room temperature. Then, the genitalia and last abdominal segment were separated from the other abdominal structures using pins and forceps (WTS, LKA) or a glass stick (DK) without removing the rest of the abdomen. The procedure for preparing the endophallus and the terminology for the endophallic structure were described in detail by Kasatkin (2006). In general, species of the genus *Anoplistes* have much softer membranes compared to the other Trachyderini and, therefore, they require more caution during the eversion and inflation of the endophallus. Moreover, minor damage to the membrane that frequently occurs during the work requires additional reparation.

2.2.3. Photography and preparation of the figures

The scanning electron microscope (SEM) images were taken using a Hitachi S-3400N SEM at the Museum and Institute of Zoology, Polish Academy of Sciences.

Photographs of the habitus were taken with a Canon EOS 50D digital camera equipped with a Canon 100 mm f/2.8 USM Macro lens and a Canon MP-E 65 mm f/2.8 1–5 × lens. Photographs of the endophallus were taken with a Canon EOS 5D Mark III digital camera equipped with a Canon MP-E 65 mm f/2.8 1–5× lens. The images that were produced were stacked, aligned, and combined using Helicon Focus (www.heliconsoft.com) and Zerene Stacker (www.zerenesystems.com) software. Photographs of the cerambycids in nature, their host plants and habitats were taken with Canon EOS 550D, EOS 600D and Panasonic Lumix DMC-ZS3 cameras. All plates were prepared using Adobe Photoshop CS5 and GIMP 2.10.14.

The distribution of the species was illustrated using Quantum GIS (QGIS) 3.6.0 'Noosa' and the raster layer was downloaded from OpenStreetMap (https://www.openstreetmap.org/). Additionally, Google's close-up satellite images were downloaded from Google Earth. Geographical coordinates of the localities are given in WGS 84 format.

2.3. Molecular analyses

2.3.1. Mitochondrial DNA

DNA barcoding, the analysis of a standardised segment of the mitochondrial cytochrome c oxidase subunit I (COI) gene, was performed on a representative selection of specimens of *A. halodendri minutus* (E Mongolia), *A. kozlovi* sensu Danilevsky and Smetana (2010) (SE Mongolia), *A. halodendri halodendri* (NE Kazakhstan), *A. jacobsoni* (S Kazakhstan), and *Anoplistes galusoi* (Kostin, 1974) (SE Kazakhstan) (Table S1). The individuals that were collected in the field were preserved in 96% ethanol, which was subsequently replaced at least five times (at increasing intervals) in order to avoid diluting the alcohol. In the case of *A. halodendri halodendri*, two dried specimens (collected in 2017 and killed with ethyl acetate) were utilised. The specimens were processed for DNA barcoding later in 2019 and in 2020. The right mid femur was cut open on both ends to expose the muscle tissue and then partly crushed with forceps and placed in a sealed well that con-

Table 1. Primer sequences used for PCR amplification.

Gene	Primer Name	Direction	Sequence	Reference
COI	LCO1490	F	GGTCAACAAATCATAAAGATATTGG	Hebert et al. 2003
COI	HCO2198	R	TAAACTTCAGGGTGACCAAAAAATCA	Hebert et al. 2003
CAD	CD338F	F	ATGAARTAYGGYAATCGTGGHCAYAA	Moulton and Wiegmann 2004
CAD	CD668R	R	ACGACTTCATAYTCNACYTCYTTCCA	Wild and Maddison 2008
CAD	CD688R	R	TGTATACCTAGAGGATCDACRTTYTCCATRTTRCA	Wild and Maddison 2008
AK	ForB2	F	GAYTCCGGWATYGGWATCTAYGCTCC	Danforth, Lin, Fang 2005
AK	RevB1	R	TCNGTRAGRCCCATWCGTCTC	Danforth, Lin, Fang 2005
AK	ForB4	F	GAYCCCATCATCGARGACTACC	Jordal 2007

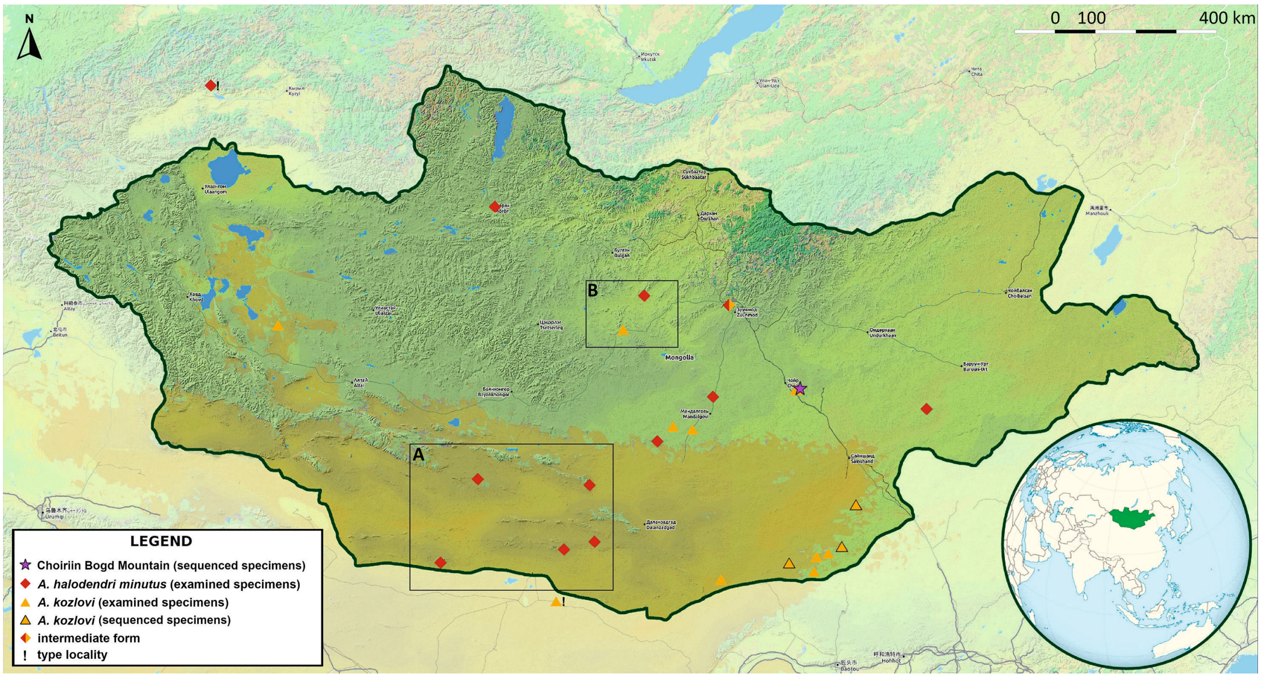

Figure 1. Distribution of the two ecotypes of *Anoplistes halodendri minutus* in Mongolia and the adjacent territory (OpenStreetMap contributors). Regions "A" and "B" are presented in detail in the following figures.

tained two drops of 95% ethanol on a standard 96-well microplate, which was used for the tissue submission.

All of the laboratory work for extracting, purifying, amplifying and sequencing the DNA was performed at the "Canadian Centre for DNA Barcode" (CCDB, http://www.ccdb.ca), University of Guelph, Ontario, Canada, following the standard protocol (Ivanova et al. 2006).

Twenty specimens were successfully sequenced for a 590–658 bp long DNA barcoding fragment. The sequences were submitted to GenBank under the accession numbers MW506917–MW506936 (Table S1).

The obtained sequences and additional relevant information such as the specimen images, primers, gel images and trace files were uploaded to the "Barcode of Life Database" (BOLD, http://www.boldsystems.org) in the public online dataset "Anoplistes Central Asia LK" (DS-LKCCAA; DOI: dx.doi.org/10.5883/DS-LKCCAA). All of the voucher specimens reported herein are part of the BOLD project "LECHK Cerambycidae Central Asia L. Karpinski" and were deposited in the LK's Cerambycid DNA-grade specimen bank at the Museum and Institute of Zoology, Polish Academy of Sciences (Warsaw, Poland).

2.3.2. Nuclear DNA

We used the DNeasy column extraction kit (Qiagen) to extract DNA from ethanol preserved samples. Whole leg tissue was taken from adult beetles, dried to remove ethanol, and ground with a pestle before an overnight lysis incubation at 55C. DNA was eluted into 150ul of Qiagen buffer AE.

Two nuclear protein coding regions (ArgK and CAD) which regularly inform species boundaries in beetles (Wild and Maddison 2008) were targeted. PCR amplification of these genes was carried out according to established beetle protocols using the primers in Table 1 and optimized if needed (Maddison 2012; McKenna et al. 2015). PCR success was confirmed using gel electrophoresis. PCR products were cleaned using EXO-SAP cleaning. Sequencing primers were CD338F and CD668R for CAD and ForB4 and RevB1for ArgK.

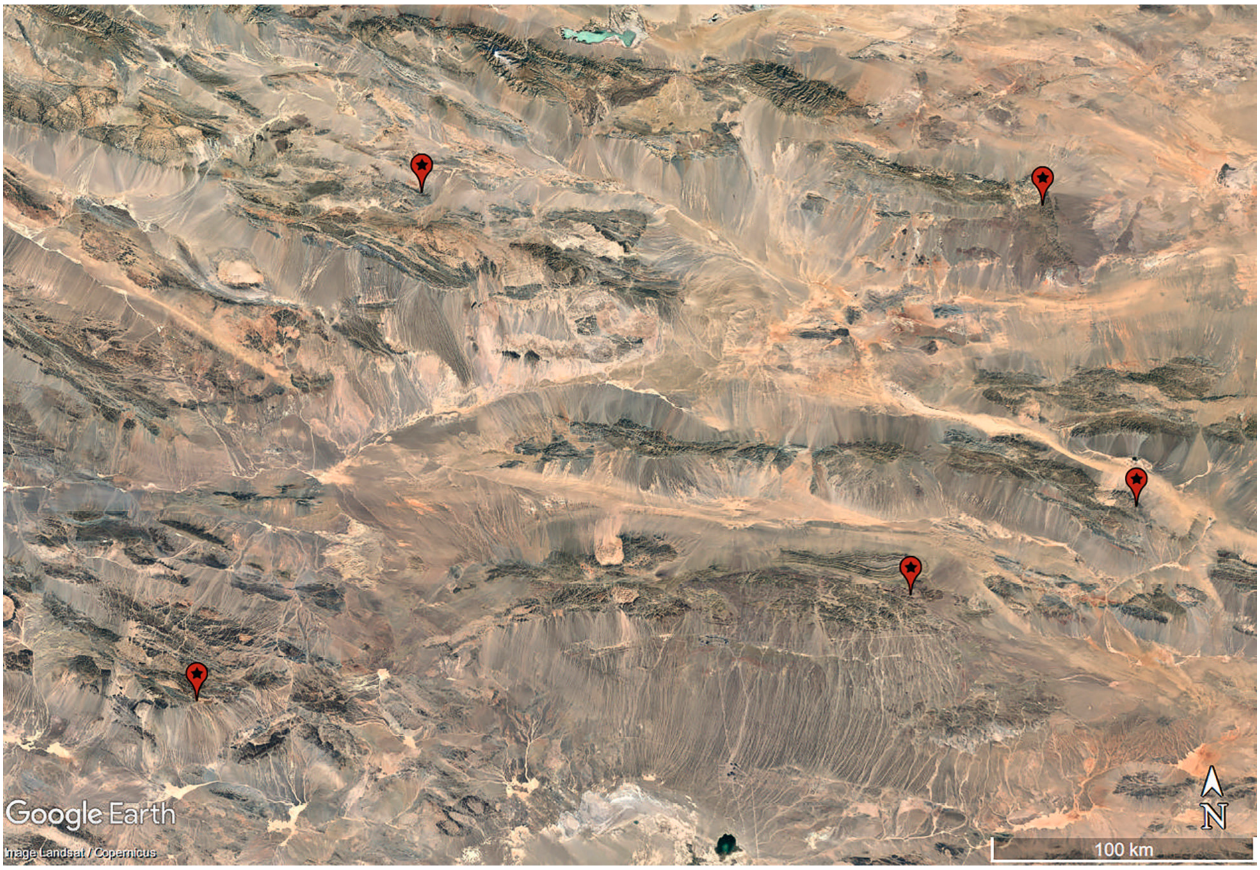

Figure 2. Google's close-up satellite image in a desert area in southern Mongolia (region "A" marked in Fig. 1) with the localities of the rock ecotype of *Anoplistes halodendri minutus* (Google Earth).

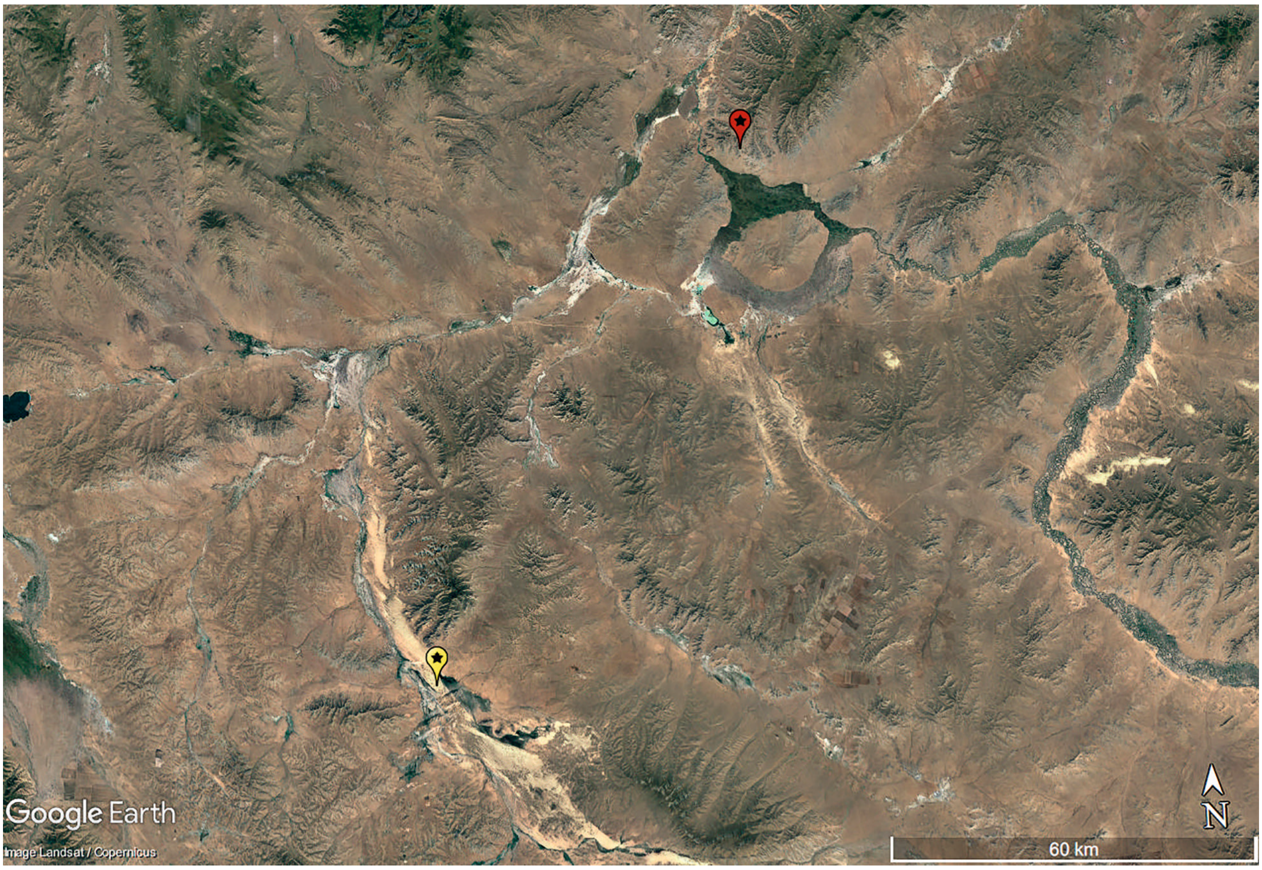

Figure 3. Google's close-up satellite image in the northern steppe area in central Mongolia (region "B" marked in Fig. 1) with the localities of the sand (yellow spot) and rock (red spot) ecotypes of *Anoplistes halodendri minutus* (Google Earth).

Sequencing was performed on an Applied Biosystems 3730xl DNA Analyzer. Resulting chromatograms were loaded into Sequencher software v. 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA) to manually edit, pair and export the resulting consensus gene sequences. Heterozygous sites in nuclear genes are coded with IUPAC ambiguity codes. This resulted in ~ 783bp arginine kinase (ArgK), and ~ 940bp carbamoyl-phosphate synthetase domain of rudimentary (CAD) for downstream use. These sequences have been submitted to GenBank under the accession numbers MW527463–MW527471 and MW527472–MW527489, respectively (Table S1).

There were several processing and quality checks performed on the gene data. Within Sequencher, chromatograms were assembled into contigs, and the primer regions were trimmed. Bases of low quality or that conflicted between forward and reverse reads were manually edited. After export from Sequencher, each gene was aligned using MAFFT v. 7 (Katoh and Standley 2013). Gene matrices were then constructed by importing the aligned sequence .fasta files into the Mesquite software package v. 3.5 (Maddison and Maddison 2019). Within the Mesquite editor, sequences were trimmed and translated to amino acids to check for problematic stop codons. Any stop codons were checked against the raw chromatogram to confirm the nucleotides. The resulting gene matrices were combined into one multi-matrix .nexus file in Mesquite that gives various export options for downstream analysis.

2.4. Analyses of phylogeny

2.4.1. Partitioning

For MrBayes and RAxML-NG, the three gene concatenated dataset of 2350 sites was analyzed in Partitionfinder v2.1.1 (Lanfear et al. 2016) using unlinked branch lengths, the greedy search algorithm, and AICc as the selection metric. With a potential statistical problem stemming from gamma + I models (Yang 2014), the model set in Partitionfinder was reduced to those not combining these two parameters (Kim et al. 2018). Models were also reduced to those available in the phylogenetic estimation programs used. The preferred scheme partitioned the data into three subsets: 1) first codon positions, 2) 2nd codon positions+AK_pos3, 3) CAD_pos3, 4) COI_pos3. The best models for MrBayes were: 1) GTR+I, 2) HKY+I, 3) GTR+G, 4) GTR+G. For RaxML-ng, the models were 1) GTR+I, 2) HKY+I, 3) GTR+G, 4) TN93+G. A 1723bp nuclear gene only dataset was also partitioned. This resulted in two partitions: 1) CAD_pos3 2) all others. The best-fitting models were GTR+G and GTR+I, respectively. The COI dataset was partitioned into each codon position, using GTR+I for positions one and two and GTR+G for position three.

2.4.2. Phylogeny estimation

The concatenated three gene dataset was analyzed using MrBayes v. 3.2.6 (Ronquist et al. 2012) using two runs with four MCMC chains each (one cold). The sequence was partitioned according to the best model determined by Partitionfinder. It was run for 10 million generations, with sampling every 1000 generations. Stationarity and convergence were evaluated by deviation of split frequencies < .01 and potential scale reduction factor values ~ 1.00. The tree files were summarized with the sumt command in MrBayes with a burnin of 25% to produce a consensus tree. The same method was used to estimate a concatenated two marker nuclear protein-coding tree and a COI tree each with runs of 5 million total generations sampling every 1000. All trees were viewed and manipulated for output in FigTree v. 1.4 (https://github.com/rambaut/figtree).

The three gene supermatrix was also run using maximum likelihood in RaxML-NG (Kozlov et al. 2019). The concatenated dataset ran using the optimal partitioning scheme and model breakdown from PartitionFinder (see section 2.4.1). 1000 bootstrap replicates and 10 parsimony starting trees were run.

In addition, the following sequences were obtained from GenBank as the outgroups: *Purpuricenus temminckii* (Guérin-Méneville, 1844) [COI: LC484442.1 and MN905245.1; CAD: MN886365.1], *Purpuricenus desfontainii* (Fabricius, 1793) [COI: KM449194.1], *Purpuricenus kaehleri* (Linnaeus, 1758) [COI: KM286081.1], *Purpuricenus sideriger* Fairmaire, 1888 [COI: KF737809.1], *Amarysius sanguinipennis* (Blessig, 1872) [COI: MN905184.1; CAD: MN886315.1], *Amarysius altajensis* (Laxmann, 1770) [COI: KY683593.1 and MN905183.1; CAD: MN886314.1], *Aromia moschata* (Linnaeus, 1758) [COI: MH020478.1], and *Aromia bungii* Faldermann 1835 [COI: MN905189.1; CAD: MN886320.1], and respectively used in four separate phylogenetic analyses: (1) Bayesian and (2) maximum likelihood analysis of combined COI + CAD + ArgK data, (3) Bayesian analysis of CAD + ArgK data and (4) Bayesian analysis of COI data.

Analyses of the intra- and interspecific mitochondrial genetic distances were conducted in MEGA v. 7.0 software (Kumar et al. 2016) using the Maximum Composite Likelihood model (Tamura et al. 2004) (Tables 2 and S2).

3. Results

3.1. Analysis of field data

Two nearly sympatric *Anoplistes* populations were found in one extensive site (LK1) in the environs of the Choiriin Bogd Mountain [Чойрын Богд Уул] (46.24, 108.77), approx. 30 km SEE of Choir [Чойр] ("purple star" in Fig. 1; Fig. 4) on 17 and 19 July and 1 August 2019. The first population, which was found in a small canyon and on rocky slopes of the surrounding mountains (plot 128 in Fig. 4; Fig. 5A, B), consisted nearly exclusively of individuals with an almost completely blackened elytra with two reddish spots in the basal part and a single stripe along

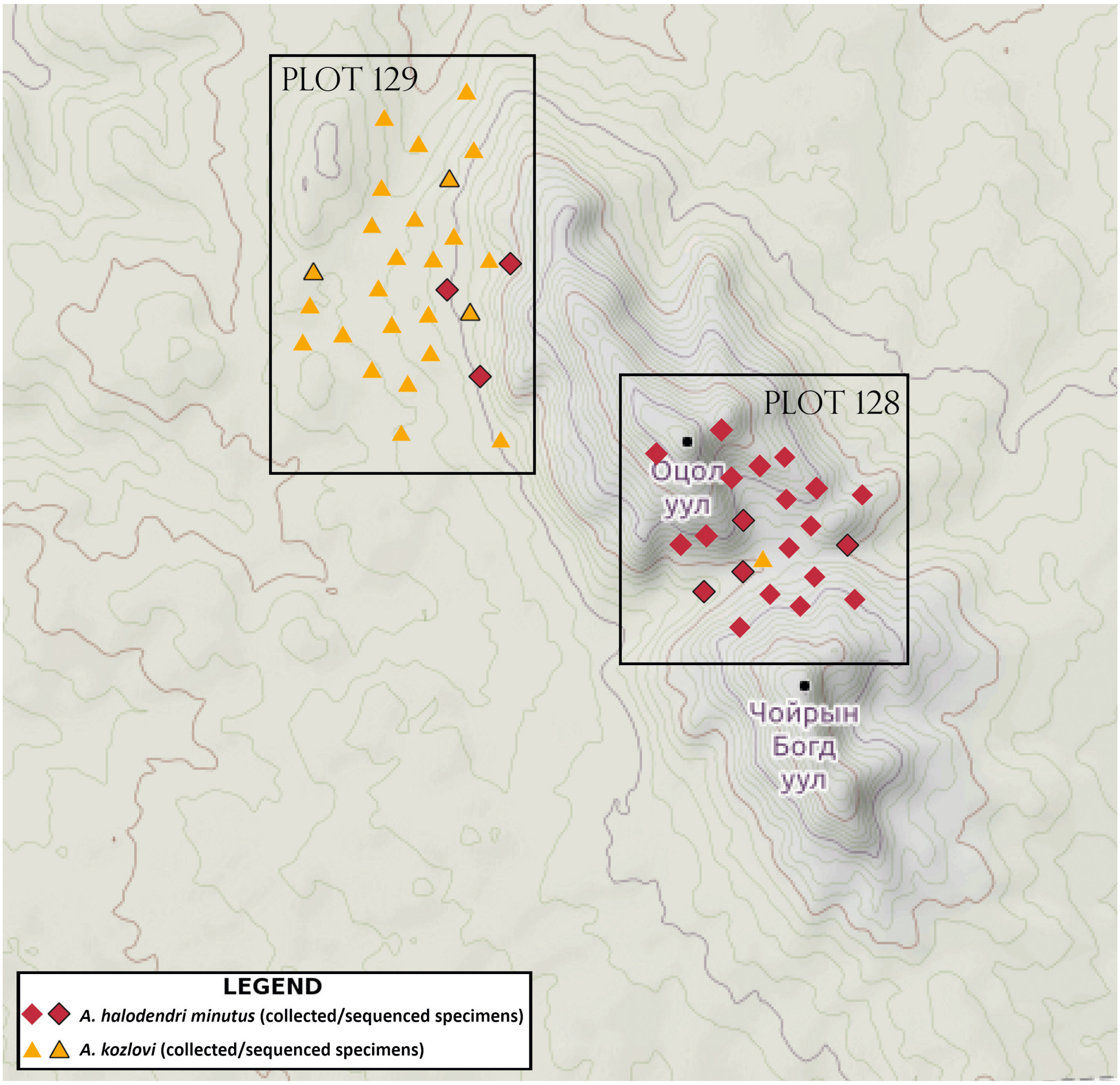

Figure 4. Detailed view of the main study site in the Choiriin Bogd Mountain (plots no. 128 and 129) in eastern Mongolia, where both ecotypes of *Anoplistes halodendri minutus* occur sympatrically (OpenStreetMap contributors).

each epipleuron (Fig. 5C, D), which corresponded to the habitus of *A. halodendri minutus*—a widely distributed subspecies in Mongolia. However, after leaving the rocky area and continuing our sampling towards the flat terrain with a gravelly-sand surface with no rocks (plot 129 in Fig. 4; Fig. 5E), we began to find almost exclusively individuals with a reduced main black spot and predominance of pale orange on the elytra (Fig. 5F, G), which seemed to correspond to *A. kozlovi*—another taxon of the genus that is widespread in southern Mongolia and northern China (Inner Mongolia). It is worth noting that while only a few (< 5%) individuals with a reduced black spot were found in the rocky part (plot 128) of the site (and only at lower elevations), in the second part of the site (plot 129), the other forms were slightly more numerous (~ 10%). An incidence of copulation between two different forms was observed in several pairs, mainly in the transitional zone (Fig. 5H). The detailed structure of the site with the approximate proportions of the individuals of both forms is presented in Fig. 4.

All of the individuals were found sitting on *Caragana* Fabr. (Fabaceae) shrubs. Although the pea-shrubs that grow in both plots of the above-described site appeared somewhat different in their structure, it was determined by local botanists that the pea-shrub individuals within the entire locality belong to the same species—*Caragana leucophloea* Pojark (Fig. 6A). Additionally, a second species—*Caragana bungei* Ledeb. (Fig. 6B)—occurs in the rocky part of the site. However, the adults of *Anoplistes* in the stony habitat were commonly sitting on both *Caragana* species. The development of the larvae of *A. halodendri minutus* in the discussed plant species was confirmed by the literature data (Karpiński, Szczepański, Boldgiv et al. 2018). Therefore, the two *Anoplistes* forms that were observed seem to be ecologically associated with the same hosts and some of the differences in the

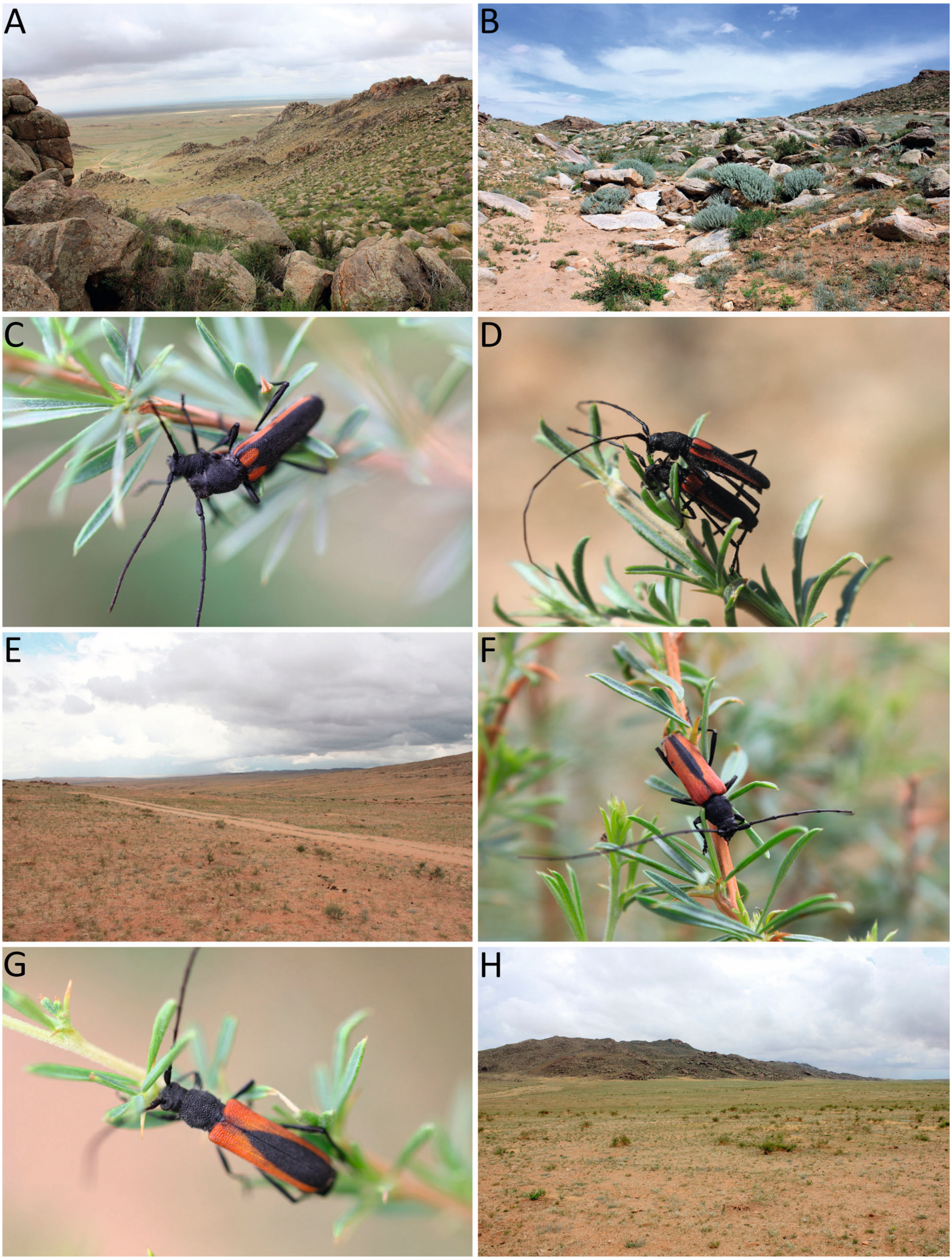

Figure 5. Field photographs of imagines in nature and the habitats of *Anoplistes halodendri minutus*. **A**, **B**: view on rocky habitat. **C**, **D**: the rock ecotype of *A. h. minutus*. **E**: view on sandy/gravelly habitat. **F**, **G**: the sand ecotype of *A. h. minutus* from the Choiriin Bogd Mountain. **H**: view on transition zone from sandy to rock habitat.

C. leucophloea structure most likely result from growing on a different type of subsoil—rocky (Fig. 6C) and sandy/gravelly (Fig. 6D). *Anoplistes jacobsoni*, on the other hand, is most likely monophagous on *Halimodendron halodendron* (Pall.) Voss. (Fabaceae) (Karpiński, Szczepański, Plewa et al. 2018).

Figure 6. Field photographs of imagines in nature, host plants and habitats of *Anoplistes halodendri minutus*. **A**: *Caragana leucophloea*. **B**: *Caragana bungee*. **C**: *C. leucophloea* on rocky ground. **D**: *C. leucophloea* on sandy/gravelly ground. **E**, **F**: the sand ecotype of *A. h. minutus* from southern Mongolia. **G**, **H**: the desert habitats in southern Mongolia; (photos E–H by Badamnyambuu Iderzorig).

In order to confirm the relationship between the specific form and the type of environment, the habitus and habitat of the specimens from other regions of Mongolia (Figs 1, 2, 3) were analysed. First, we examined specimens of two populations (BI1 and BI2; Fig. 6E, F) that were recently collected in desert habitats (Fig. 6G, H) in

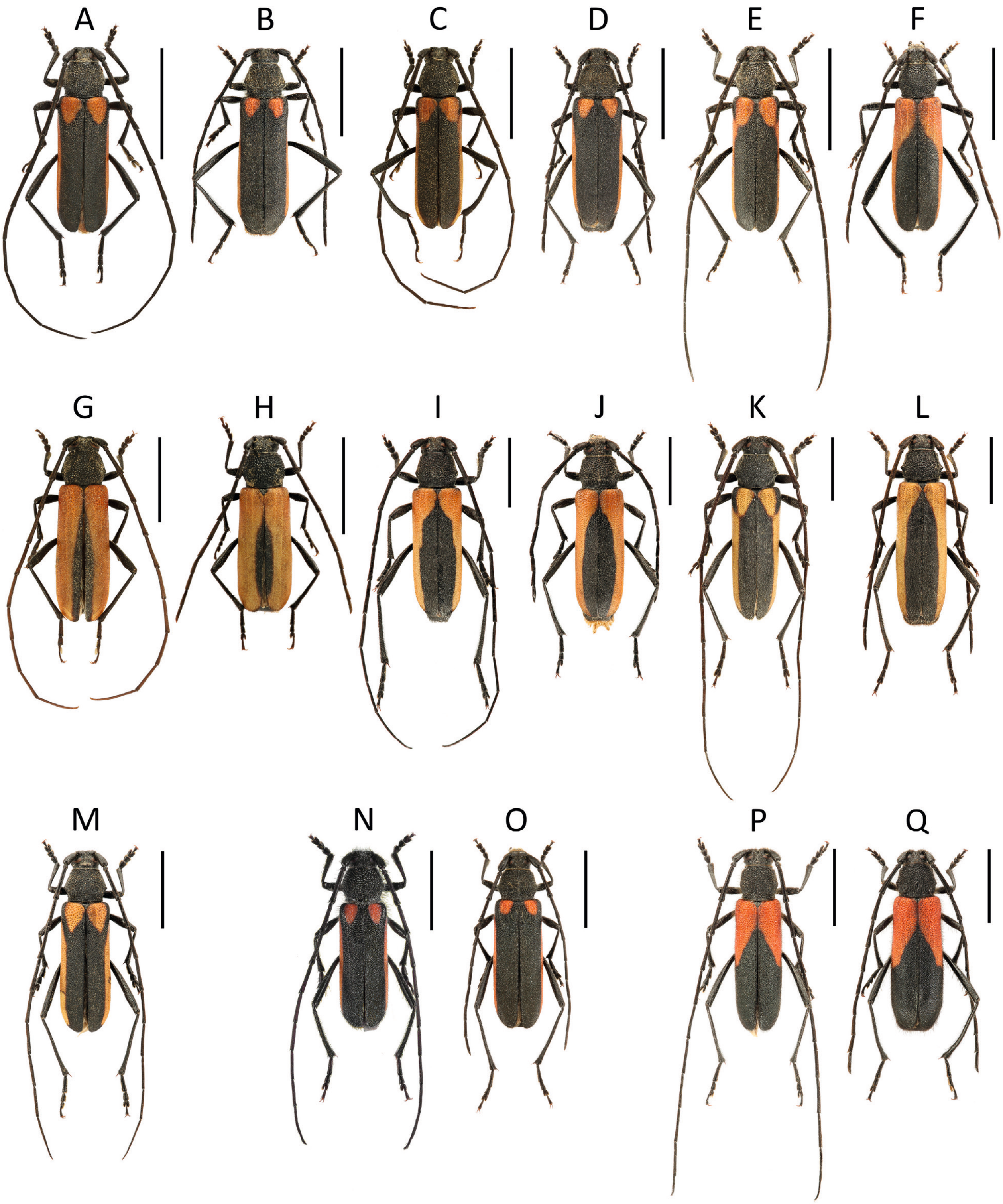

Figure 7. Habitus of some taxa of the *Anoplistes halodendri* species-group. **A**–**M**: *Anoplistes halodendri minutus*. **A**–**F**: rock ecotype; **G**–**L**: sand ecotype—*Anoplistes kozlovi* sensu Danilevsky and Smetana (2010). **M**: intermediate form. **N**, **O**: *Anoplistes halodendri halodendri*. **P**, **Q**: *Anoplistes jacobsoni*. **A**, **C**, **E**, **G**, **I**, **K**, **M**, **N**, **P**: males. **B**, **D**, **F**, **H**, **J**, **L**, **O**, **Q**: females. Scale bar: 5 mm.

the southern part of the country (Fig. 1: "framed triangles") whose DNA sequences were also used for a molecular analysis. All of the specimens (N = 20) were almost uniform in their elytral pattern and bright colouration and corresponded to the habitus of *A. kozlovi* sensu Danilevsky and Smetana (2010) from the previously discussed locality. Subsequently, we studied additional museum and private specimens of numerous other populations in order to determine, where possible, the exact habitat in which the beetles were collected—either using precise geographical coordinates (when provided) or the detailed descriptions of the sites provided by Zoltán Kaszab in his expedition reports (Kaszab 1964, 1965, 1966, 1967, 1968, 1969). In some cases, there was only one type of habitat in a region, thus the precise localisation of the plot was not necessary. The obtained data confirmed a

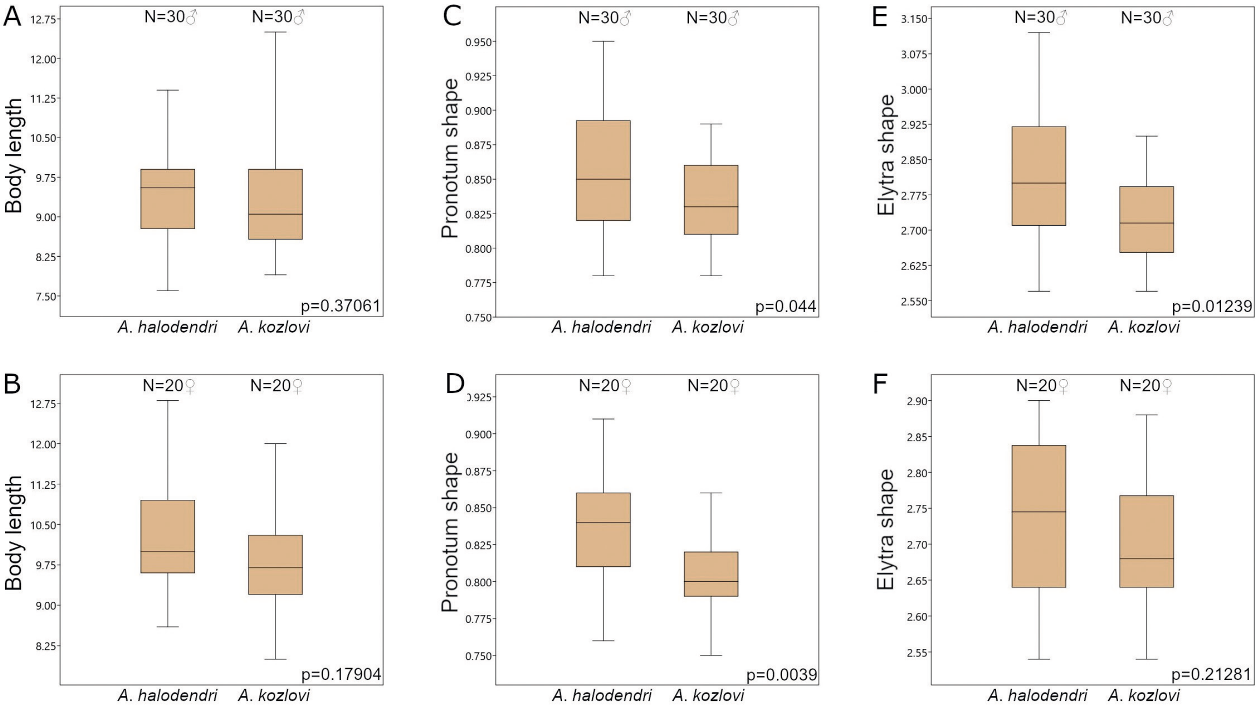

Figure 8. Measurements of the body lengths and pronotum and elytra shapes (ratio of its total length to its maximum width) in the males and females of *Anoplistes halodendri minutus* and *Anoplistes kozlovi* sensu Danilevsky and Smetana (2010).

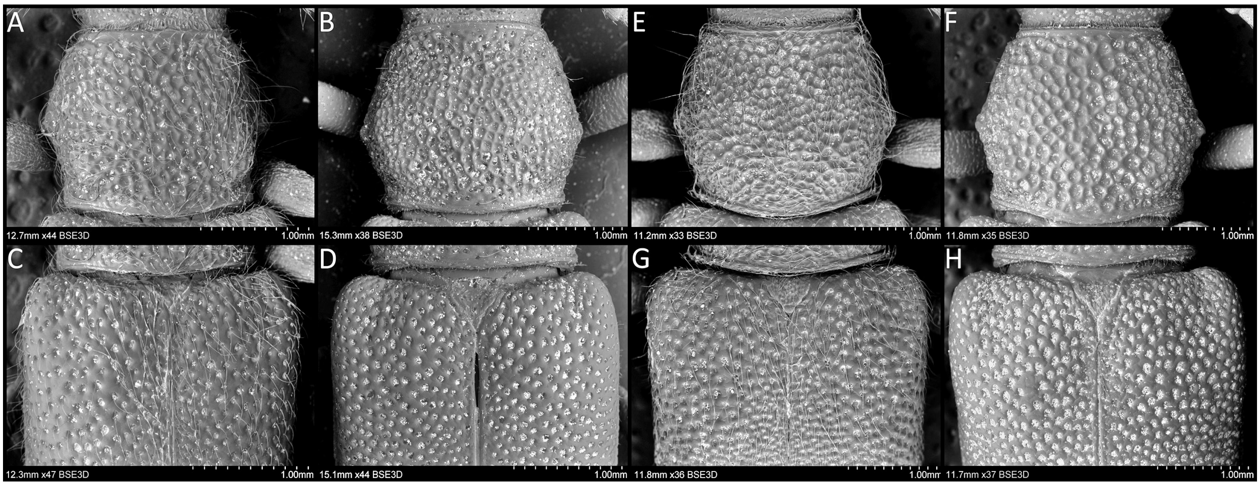

Figure 9. SEM images of the pronotum and elytra of *Anoplistes halodendri minutus* presenting the differences in pubescence density. **A**–**D**: rock ecotype. **E**–**H**: sand ecotype.

connection between the given form and one of the two habitat types.

An interpretation of the ecological field data suggests the ecotypic, rather than a specific differentiation.

3.2. Analysis of morphological data

Over 500 specimens of *Anoplistes halodendri minutus* (Fig. 7A–F) and *A. kozlovi* sensu Danilevsky and Smetana (2010) (Fig. 7G–L) from the territory of Mongolia were studied including more than 200 that were collected in the main site (Choiriin Bogd Mountain).

Results of the detailed morphometric measurements of 100 individuals of both forms revealed no statistically significant differences in the body length. Total body length in the males ranged from 7.6 to 11.4 mm ("minutus", N = 30) vs 7.9 to 12.5 mm ("kozlovi", N = 30); in the females, it ranged from 8.6 to 12.8 mm ("minutus", N = 20) vs 8.0 to 12.0 mm ("kozlovi", N = 20); 25–75% quartiles practically overlapped between the examined specimens of the two forms (Fig. 8A, B). It is worth noting that the individuals that represented the southern populations of *A. kozlovi* sensu Danilevsky and Smetana (2010) (BI1 and BI2; Fig. 1: "framed triangles") were relatively bigger than those from the Choiriin Bogd Mountain population (LK1). In a sample of 13 specimens, most were larger than 10 mm (mean for males: 11.25 mm, N = 8; for females: 11.2 mm, N = 5). Regarding the maximum value of the ratio of pronotal width and length, it

Figure 10. Variability of the elytral pattern in the specimens of *Anoplistes halodendri minutus*. **A**: rock ecotype. **B**: sand ecotype.

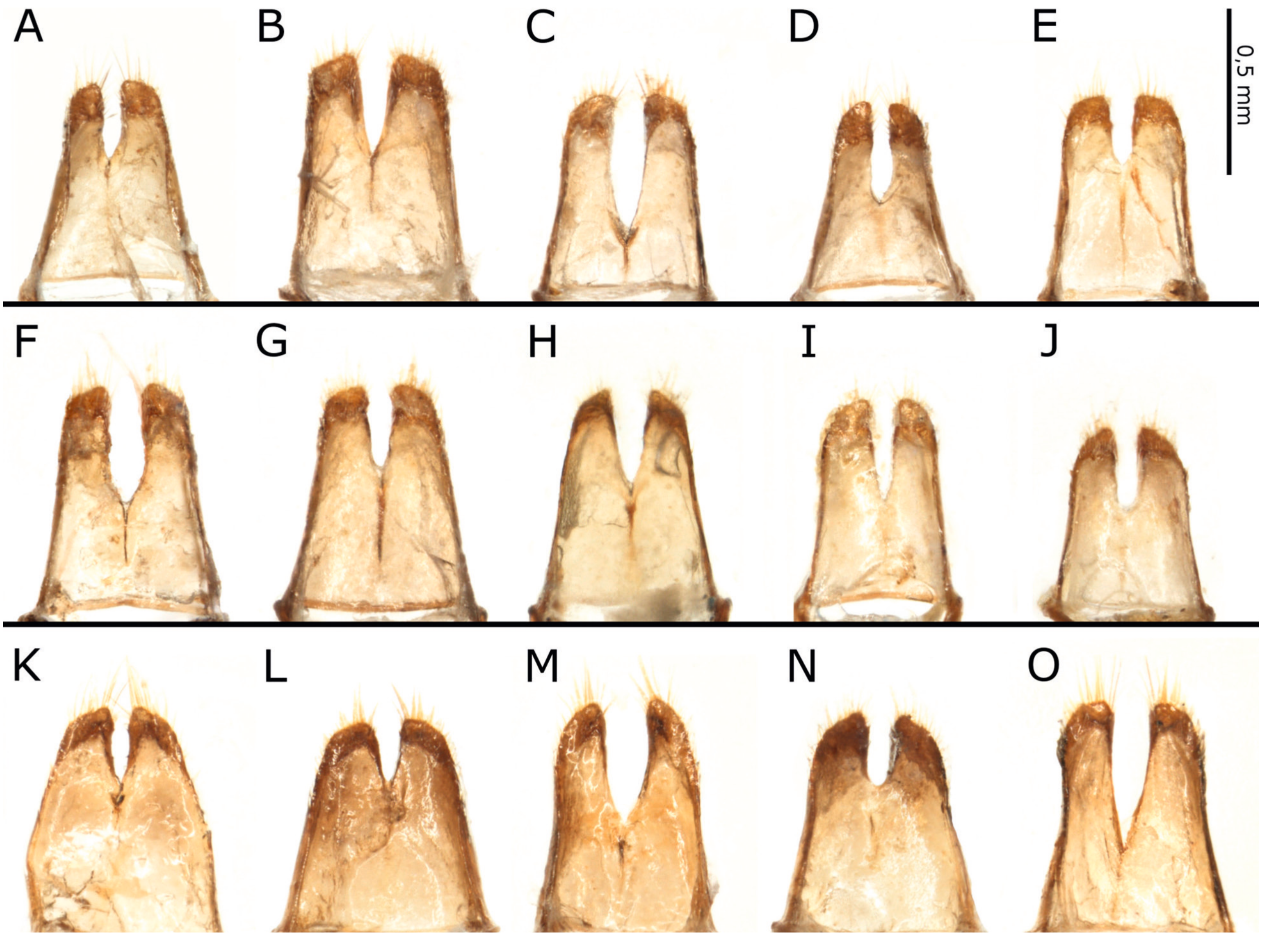

Figure 11. Variability of the lateral lobes of the tegmen in the *Anoplistes halodendri* species-group. **A**–**J**: *Anoplistes halodendri minutus*. **A**–**E**: rock ecotype. **F**–**J**: sand ecotype—*Anoplistes kozlovi* sensu Danilevsky and Smetana (2010). **K**–**O**: *Anoplistes jacobsoni*.

was significantly smaller in the "kozlovi" form for both the males and females (Fig. 8C, D). However, this trait cannot be diagnostic as the values for most of the individuals of both subpopulations were in the same ranges. In the case of the elytral shape, although there were some differences in the maximum values for the males, the values for the females had a nearly total overlap (Fig. 8E, F). Similarly, when the ratios of the individual antennomeres to antennomere 1 were considered, no statistically significant differences were found (Fig. S1).

Comparative analysis of the pubescence density and length using SEM technology indicated that within both forms, densely pubescent and almost completely hairless individuals (considering the elytra and pronotum) occurred (Fig. 9). This applied to the newly hatched individuals, which were collected in the same place and at the same time and did not appear to be connected to "wiping off" of the beetles, but rather to a natural individual variability. A closer examination of the SEM images also enabled it to be confirmed with certainty that there were no differences in the sculpture and punctation of the pronotum and elytra.

Clearly, the best trait for distinguishing both ecotypes is the shape and the range of the elytral spot, and, to a lesser extent, the colouration of their lighter part. The full range of the variability of these traits for both forms is presented (Fig. 10). In both ecotypes, there were patterns with or without bright spots in the basal part of the elytra, however, generally, the main black elytral spot was clearly narrower and spread differently in the "kozlovi" compared to the "minutus" (Fig. 10A vs B). The colouration of the lighter part of the elytra was almost always pale orange in the "kozlovi" and usually reddish in the "minutus".

An examination of the lateral lobes of the tegmen revealed no differences between the discussed ecotypes (Fig. 11). Generally, these structures had a relatively high variability and numerous specimens within both environmental forms had an almost identical structure (e.g. Fig. 11A vs G). Additionally, the parameters of the seemingly very close species of this group—*A. jacobsoni* (Fig. 11K–O) were also analysed. Although most of the individuals appeared to have distinctly different lateral lobes, after studying a sufficiently large series, it was found that some of them had structures that were very close to those of *A. halodendri* (Fig. 11O vs C, respectively). This highlights the need for future studies to examine the genitalia of many specimens before drawing conclusions about the separateness of two entities. Notwithstanding, there was a clear stronger dissimilarity of *A. jacobsoni*.

The shape of the median lobe (Fig. S2) appears to be a taxonomically uninformative character owing to its variability between taxa, being similar to the genus *Ropalopus* Mulsant, 1839 (Callidiini) of the same subfamily

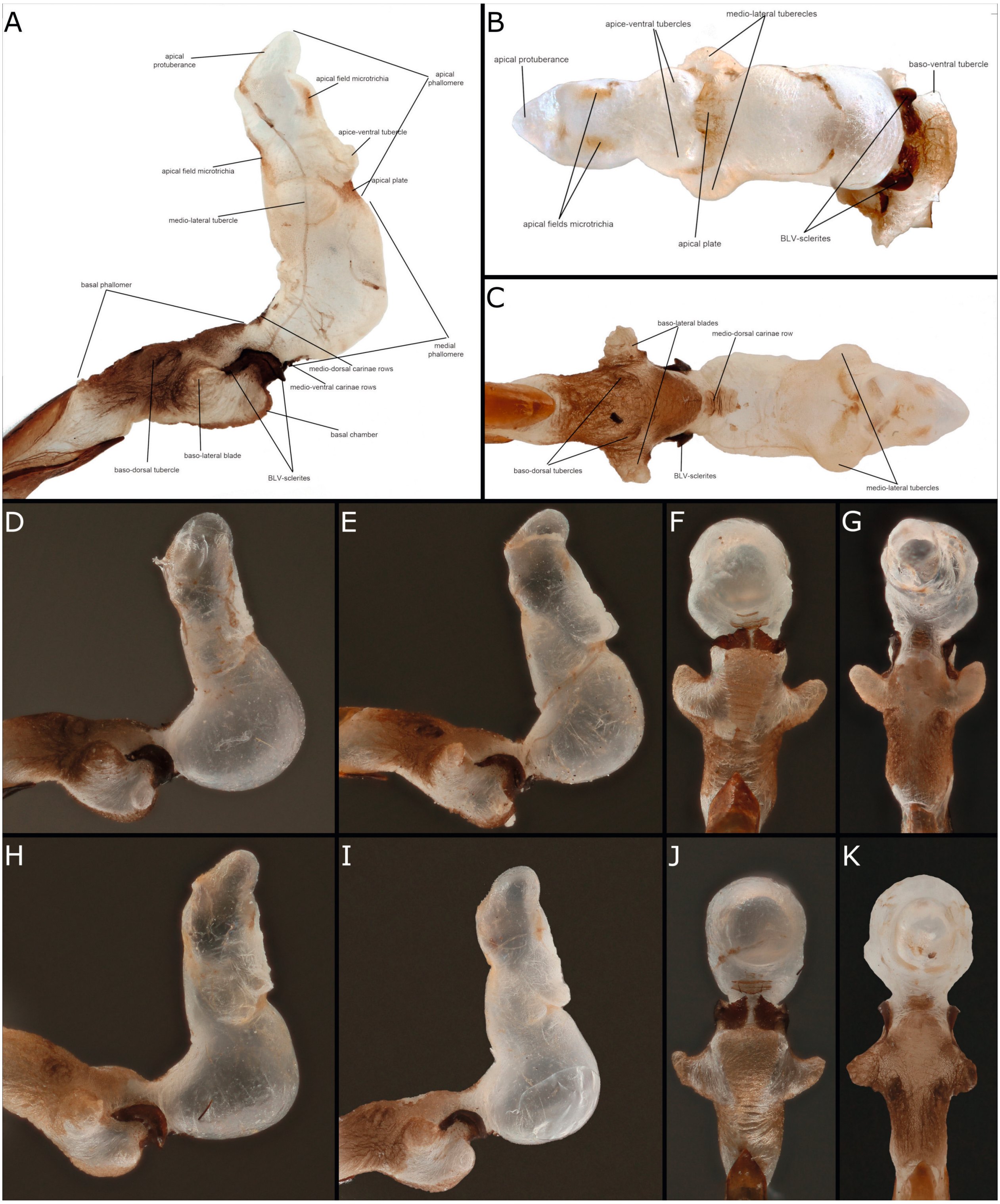

Figure 12. Variability of the endophallic structures in the *Anoplistes halodendri* species-group. **A**–**C**: *Anoplistes jacobsoni*, with the terminology used in this paper. **D**–**K**: *Anoplistes halodendri minutus*. **D**–**G**: rock ecotype, two different specimens. **H**–**K**: sand ecotype—*Anoplistes kozlovi* sensu Danilevsky and Smetana (2010), two different specimens. **A**, **D**, **E**, **H**, **I**: lateral view. **B**, **F**, **J**: ventral view. **C**, **G**, **K**: dorsal view.

(Karpiński et al. 2020). More importantly, an examination of the endophallic structures did not reveal any stable differences between *A. halodendri minutus* and *A. kozlovi* sensu Danilevsky and Smetana (2010). Some differences between these two forms (e.g. in the length of the baso-ventral tubercles, in the shape of the apical tubercles and the chambers) were weak and not stable and they result from individual variability (Fig. 12). Regarding *A. jacobsoni*, the most distinct difference was the shape of the BLV-sclerites. While in *A. halodendri* the lateral arc of the BLV-sclerites was crescent- (or C-) shaped and moderately short, in *A. jacobsoni* this structure was longer and comma-shaped.

Table 2. Summary of the intraspecific and mean interspecific genetic distances that were estimated using maximum composite likelihood model implemented in MEGA7. See Supplementary Table (S2) for the genetic distances between all of the specimens that were analysed. 'n/a' indicates a species in which only a single specimen was sequenced and therefore the intraspecific distance could not be calculated.

Intraspecific distances [%]	**Species**	**Mean interspecific distances [%]**				
			1	2	3	4
n/a	*Anoplistes galusoi*	**1**				
0.18	*Anoplistes jacobsoni*	**2**	4.67			
0.0–0.89	*Anoplistes kozlovi*	**3**	4.54	4.82		
0.0–0.18	*Anoplistes halodendri minutus*	**4**	4.80	5.29	0.76	
0.00	*Anoplistes halodendri halodendri*	**5**	4.78	5.26	0.73	0.03

A detailed examination of the morphology of the specimens supported the ecological data in concluding that the two discussed entities seem to represent only ecologically adapted forms and therefore should not be treated as separate species.

3.3. Analysis of molecular data

3.3.1. Mitochondrial genetic distances

The COI genetic distances that were calculated based on the sequences obtained from 15 individuals: seven *A. halodendri minutus* (both plots of the locality LK1) and eight *A. kozlovi* sensu Danilevsky and Smetana (2010) (four localities: LK1, BI1, BI2, including one larva collected in a strongly desertified habitat—LK2_larva) are presented in Tables 2 and S2.

The intraspecific distances only varied from 0.0% to 0.18% in *A. halodendri minutus* and from 0.0% to 0.89% in *A. kozlovi* sensu Danilevsky and Smetana (2010). Regarding the latter, when only the individuals from the Choiriin Bogd Mountain (LK1) population (N = 3) were compared, the distance was 0.0%. The individuals of the southern populations (BI1, BI2 and LK2_larva; Fig. 1: "framed triangles") (N = 5) were much more genetically diverse (0.0% to 0.89%), though this can be attributed to a single distinctive individual that was much closer to the individuals of *A. halodendri minutus*, but still exhibited the phenotypic characters that are typical for *A. kozlovi* sensu Danilevsky and Smetana (2010). The individuals that represent the southern populations differed from the Choiriin Bogd Mountain population by between 0.18% and 0.71% (mean, 0.28%). Finally, the overall genetic distance between the two putative ecotypes was very low and only ranged from 0.18% to 1.07% (mean, 0.76%).

Afterwards, we barcoded two additional specimens of *A. halodendri halodendri* from northeastern Kazakhstan (very close to the type locality of the nominative subspecies). Interestingly, their COI sequences proved to be almost identical (0.0% for six and 0.18% for one specimen) with those of the rock ecotype of *A. halodendri minutus* from the Choiriin Bogd Mountain population, which exists nearly two thousand kilometers to the east and on the opposite side of the Altai Mountains. Although the barcoding data indicate no divergence and the habitus of the Kazakh specimens (Fig. 7N, O) corresponds to the rock ecotype of *A. halodendri minutus* (Fig. 7A–F), further study must be conducted before a final decision is made on the possible synonymisation of these two subspecies (in prep.).

We also added two individuals of the morphologically close *A. jacobsoni* (S Kazakhstan) for additional comparison with this seemingly closely related taxon from outside Mongolia. The genetic distance between this species and *A. halodendri minutus* and *A. kozlovi* sensu Danilevsky and Smetana (2010) was much higher and ranged from 5.17% to 5.55% and from 4.58% to 5.36%, respectively. Similar values were obtained for *A. galusoi* (SE Kazakhstan), in both cases indicating a specific divergence and thereby determining approximate values for species separation in the genus *Anoplistes*.

3.3.2. Phylogenetic analysis

The dataset used for the phylogenetic reconstruction contained 627 bp of COI, 783 bp of ArgK and 940 bp of CAD, for a total of 2350 bp of nucleotide sequence. We considered Maximum likelihood bootstrap values (BS) 90–100% as very strong, 75–89% as strong, 50–74% as moderate and < 50% weak support, Bayesian posterior probability from MrBayes (PP) 100% as very strong, 90–99% as strong, 75–89% as moderate, and 50–74% as weak support.

Both the Bayesian (Fig. 13) and Maximum Likelihood (Fig. 14) inference trees for all three genes showed congruent topologies. The only difference is a soft polytomy in the Bayesian analysis regarding the *Anoplistes halodendri* clade that is poorly resolved in all analyses. Interestingly, the genera *Amarysius* and *Purpuricenus* were revealed as paraphyletic, however, representation of these genera (particularly for *Purpuricenus*) might not be comprehensive enough to draw far-reaching conclusions (see more in the Discussion). The genus *Anoplistes* was recovered as monophyletic with very strong (PP) or strong (BS—87%, thus almost very strong) support. *Amarysius altajensis*, which of the entire tribe is morphologically closest to the genus *Anoplistes* (yet still differs in a number of characters), was found sister to the clade that includes all *Anoplistes* species.

Regarding the *Anoplistes* clade, the early-branching position of *A. jacobsoni* was emphasised. Inside the *A.*

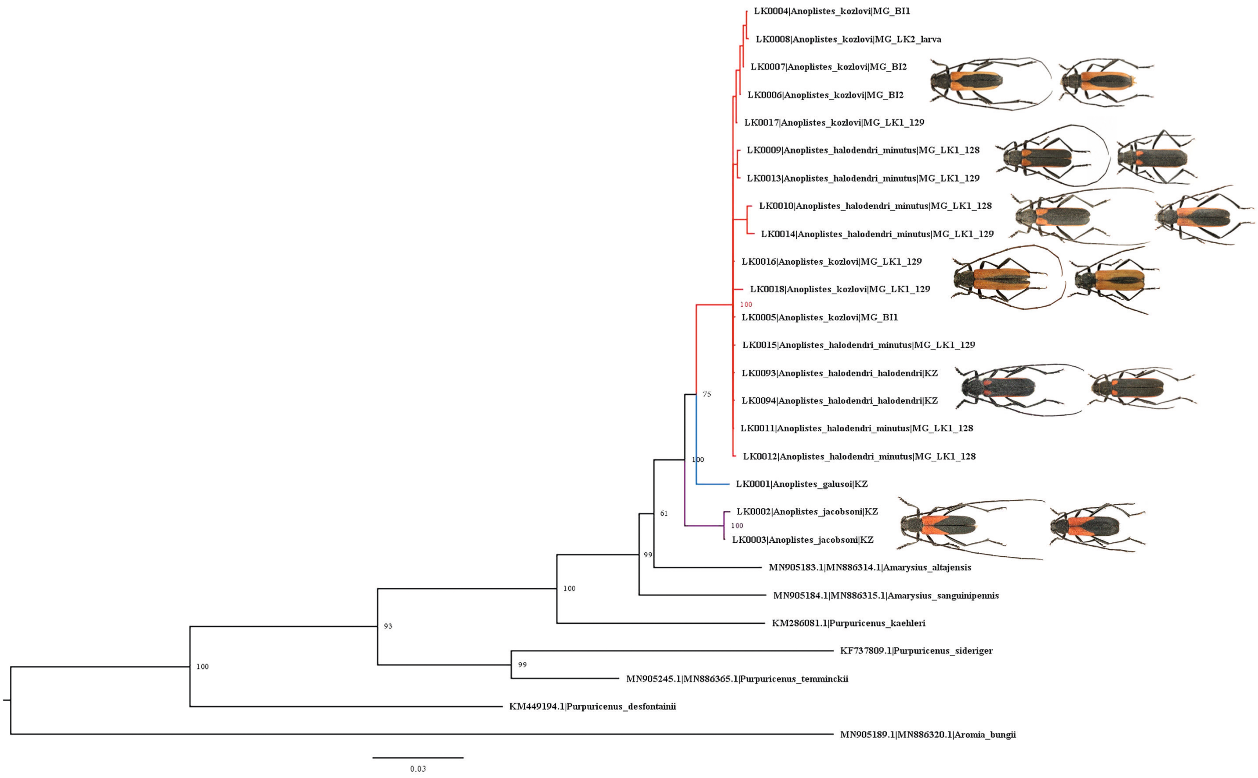

Figure 13. Phylogenetic tree obtained by the Bayesian analysis of the combined data matrix (AK, CAD and COI). Bayesian posterior probabilities are indicated adjacent to main nodes. *Anoplistes* clades are marked by colors: purple—*A. galusoi*, blue—*A. jacobsoni*, red—*A. halodendri*.

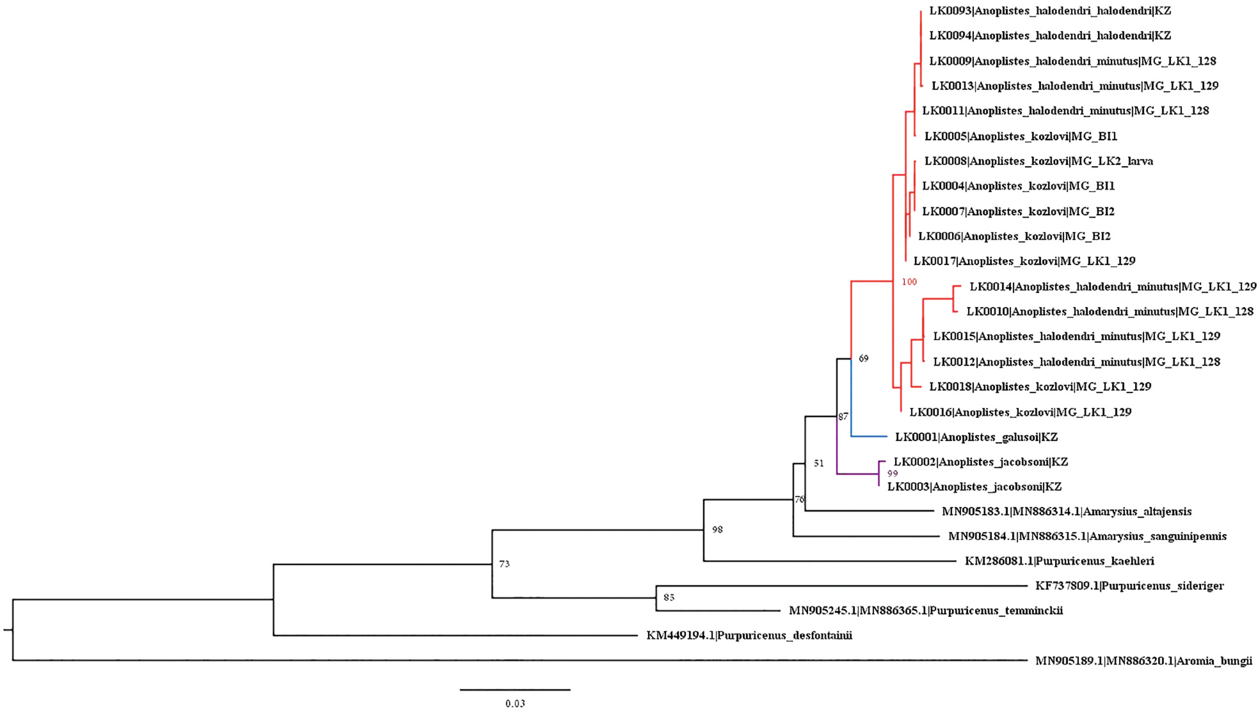

Figure 14. Phylogenetic tree obtained by the maximum likelihood analysis of the combined data matrix (AK, CAD and COI). Maximum likelihood bootstrap scores are indicated adjacent to main nodes. *Anoplistes* clades are marked by colors: purple—*A. galusoi*, blue—*A. jacobsoni*, red—*A. halodendri*.

halodendri section of the trees node support was low and the two putative ecotypes were mixed.

Additional trees were run on the genetic datatypes as evolution can proceed at different pace. In the BI nuclear-only tree (Fig. S3), *A. galusoi* showed more disjunction, but the *A. halodendri* clade had no supported structuring. These genes normally distinguish species, and do that for *A. galusoi*, *A. jacobsoni* and the clade including putative ecotypes of *A. halodendri*. By analyzing the Bayesian COI tree (Fig. S4), *Anoplistes halodendri* was interestingly divided into two distinct clades that correspond to two putative ecotypes, except for a single individual from the desert region (LK0005). Additionally, the *Anoplistes* clade, although still revealed as monophyletic, received only moderate support (PP 87%) and showed a strong (PP 99%, thus practically very strong) association with *Amarysius*.

The results of the molecular analysis, which were fairly consistent with the ecological and morphological data, revealed generally very low genetic divergence between the two Mongolian entities in question, a situation where classification under a single taxon is standard. Therefore, we propose the synonymy *Anoplistes halodendri minutus* Hammarström, 1892 = *Asias kozlovi* Semenov and Znojko, 1934, as previously proposed by Namhaidorzh (1972).

4. Discussion

4.1. Ecotype formation event

It is common among the sedentary species that occur in isolated populations to exhibit a strong intraspecific genetic differentiation, which is driven either by geographical isolation or by a local ecological adaptation to specific environmental conditions. While the first factor has frequently been studied, the genetic effects from a local ecological adaptation are much less recognised (Schluter 2009; Lechner et al. 2015). Ecotypes are intermediate stages in ecological speciation. They are groups of populations within one species that are distinguishable due to their adaptation to local environmental conditions (Lowry 2012). They increase the distribution area of a species by adjusting to diverse habitats. Such an adaptation, which depends on various variables (e.g. the type of response, trait, population, environmental conditions), can take many shapes and can occur at different speeds (Kristensen et al. 2018). Adjusting to different conditions via divergent natural selection can generate phenotypic and genetic differences in the ecologically important characters between local populations of the same species. If the gene flow is sufficiently reduced, these eventually may lead to the formation of new species (Schluter 2000; Nosil 2012). In that case, however, before the entire process is completed, some characteristic stages occur that can be identified.

Kruckeberg (1986) proposed a model for the evolution of a plant endemic in which he identified several such steps. It seems that a slightly modified model could also be used for animal ecotypes:

0 Some form of preadaptation in order to develop a tolerance to a specific environmental variable exists in the normal population of a species. This provides the conditions that permit the evolution of an ecotype that is better adapted to the new conditions;
1 Disruptive selection causes the separation of the species into different gene pools. This is the formation of an ecotype;
2 Further genetic divergence in the phenotypic and functional traits occurs within the ecotype;
3 Isolation between the two ecological forms becomes genetically fixed, such that the two gene pools are unable to exchange genes;
4 Further divergences of the incipient species occur, which is "put in motion by the initial genetic discontinuity".

However, according to Pandeya and Lieth (1993), the origin of ecotypes remains a difficult issue in population ecology as genetic variability, which is the basis for the formation of an ecotype, will constantly undergo natural selection before it is established. A mutation and recombination of genes must be the residual effect of the action, interaction and reaction between the environment and an organism and cannot be spontaneous. In other words, it is the environment that is a dominating factor in determining population differences.

4.2. Ecotype versus subspecies

It can be problematic to recognise and classify intraspecific forms and even more so to further distinguish possible ecotypes and subspecies, especially since both categories can occur within one species, as, for instance, is the case with the reindeer *Rangifer tarandus* (Linnaeus, 1758) (Gravlund et al. 1998).

A subspecies rank is commonly recommended to be used to recognise geographic distinctiveness. This term usually refers to one of two or more populations of a species that live in different regions of the species' range and that differ from one another by a relatively weak morphological differentiation. The main criterion for recognising two distinct populations as subspecies, rather than as separate species, is their ability to interbreed without a fitness penalty. In natural conditions, subspecies do not interbreed due to their geographic isolation or sexual selection. A subspecific status should be considered when geographically separate populations of a species exhibit recognisable and predominantly stable phenotypic trait values. In other words, a subspecies is a recognised local variant of a species (Russell et al. 2011).

Conversely, an ecotype status is frequently assigned if a given form occurs throughout the geographic range of a species. Additionally, an ecotype is a variant in which the phenotypic differences are too few, too subtle or too unstable to warrant being classified as a subspecies. No taxonomic rank can be determined due to a lack of sterility barriers at this stage. Ecotypic variations can occur in the same geographic region where distinct types of habitats such as sand dunes and rocky slopes provide ecological niches. In the event that similar niches occur in widely separated places, similar ecotypic forms that are adapted to the effects of a very specific local environment can occur independently (Langerhans et al. 2007; Riesch et al. 2013).

Thus, the main difference between these two terms is that a subspecies can exist across a number of different habitats and would usually be limited to a restricted part of a species' range while ecotypes occur throughout the geographic range of a species in similar ecological niches.

4.3. Recognition of ecotypic variations in *Anoplistes*

As has been justified above, *Anoplistes halodendri* in Mongolia, northern China (Inner Mongolia) and southern Siberia has developed into two distinct ecotypes. Populations of sand and rock ecotypes of *A. halodendri minutus* are found throughout the geographic range of this taxon. The same pattern was documented in the case of lizards of the *Eremias multiocellata*—*E. przewalskii* species complex, which are also found in the region (Orlova et al. 2017). Therefore, the synonymisation that was proposed by Namhaidorzh (1972): *Cerambyx halodendri* Pallas, 1773 = *Cerambyx ephippium* Steven and Dalman, 1817 = *Anoplistes minutus* Hammarström, 1892 = *Asias kozlovi* Semenov and Znoiko, 1934, is partially correct. Despite the results of the COI analysis, it is too early for us to comment on the synonymisation with *Anoplistes halodendri halodendri* (Pallas, 1773) and, in particular, we do not agree with the synonymy of *Anoplistes halodendri ephippium* (Steven and Dalman, 1817), which is mainly distributed in the southern part of European Russia. While the taxonomic position of the latter requires further research (in prep.), the preliminary results based on mitochondrial DNA support its dissimilarity. Although Danilevsky (2019) also recognised this extensive synonymisation as incorrect, he rejected the synonymy of *A. kozlovi* as well, claiming that it is definitely a separate species. The differences between these two taxa that he identified were disproportions in the elytral and pronotal pubescence, pronotal punctation and body size. In the presented study, we clarified that any differences in these traits result from individual variability and cannot serve as stable diagnostic characters. Furthermore, he based his conclusions only on the study of the series of *A. kozlovi* sensu Danilevsky and Smetana (2010) from Arkhangai Province (47.33, 103.68). Representatives of the same population were also examined by us and although they indeed stand out with considerable body size, numerous individuals of this sand ecotype are still smaller than the regular individuals of the rock form.

To conclude, the detailed morphological analysis, in particular regarding the endophallic structures, as well as the lack of clear patterns in the nuclear trees and relatively very low COI genetic distances (especially considering much higher genetic distances for morphologically and ecologically close *A. jacobsoni*) strongly indicate the cohesiveness of *A. h. minutus* including *A. kozlovi* and the need for synonymization.

It is worth mentioning that only one case of ecotypic variation has been documented in the family Cerambycidae to date. The yellow spotted longicorn beetle *Psacothea hilaris* (Pascoe, 1857) (Lamiinae: Lamiini) was divided into several subspecies, which are distributed mainly in Japan (Danilevsky and Smetana 2010). *Psacothea hilaris hilaris* has developed two ecotypes in Honshu, which, besides their elytral pattern, primarily differ in their larval biology, which do or do not diapause (Sakakibara and Kawakami 1992; Sakakibara 1995a,b). Thus, the reported environmental adaptation of *Anoplistes halodendri* is the second documented ecotypic speciation in this beetle family. Considering the entire superfamily Chrysomeloidea, several examples of ecotypes were also documented; mostly, however, in the family Chrysomelidae and for the species of high economic importance: *Leptinotarsa decemlineata* (Say, 1824) (Hsiao 1978), *Galerucella aquatica* (Geoffroy, 1785) (Lohse 1989), *Galerucella lineola* (Fabricius, 1781) (Ikonen et al., 2003), and *Oreina speciosissima* (Scopoli, 1763) (Borer et al. 2011).

4.4. An evolutionary scenario of speciation

The beginnings of the differentiation of *A. halodendri* in the territory of today's Mongolia most likely can be traced back to the period of the intensification of aridification in the region and the formation of the Gobi Desert ~ 24 to 2.6 Ma (Lu et al. 2019). As there were probably no significant geographical barriers for flying insects in the region at that time and considering the almost sympatric distribution of the populations of both ecotypes in the Choiriin Bogd Mountain and some other known localities, it seems rational to assume that parapatric speciation is ongoing here. This model of speciation has already been documented in Cerambycidae for both the flying (Karpiński et al. 2020) and flightless taxa (Nakamine and Takeda 2008). Furthermore, evidence for parapatric speciation in the case of sympatrically distributed ecotypes was provided by Fisher-Reid et al. (2013) in a study on the salamanders of the genus *Plethodon* Tschudi, 1838. Their results support the idea that the spatial segregation of sympatric ecotypes may play an important part in this speciation model. It is worth noting, however, that in the presented case of ecotypic speciation in the genus *Anoplistes*, no switch to a new host plant was observed, unlike in several documented cases in some groups of insects (e.g. Sandoval and Nosil 2005; Nosil et al. 2006).

Given some of the rather stable differences in the phenotype of distant sand form populations, we consider it likely that similar ecotypes arose independently in different parts of the species' range under parallel speciation. Individuals from the sand dunes in the northernmost part of the range differ from those from gravel desert in southern Mongolia and those from the Choiriin Bogd Mountain site in the steppe region (Fig. 7G, H vs I, J vs K, L, respectively)—although all of these populations are somewhat different, some specimens are sometimes indistinguishable from one other. Still, however, the rock ecotype is more homogeneous in phenotype throughout its range. This may lead to the conclusion that some part of the different populations of the rock ecotype had begun to transform in response to the increasing desertification. The first mentioned form remained on the stony hills (Fig. 2) within its entire range, while the latter began to adapt to new habitats simultaneously in many places. It has already been proven that common environmental factors repeatedly lead to the evolution of similar phenotypic forms in independent geographic localities under parallel speciation (Schluter and Nagel 1995; Johannesson 2001; Nosil et al. 2002; Schluter 2009). Multiple origins were supported in several studies, for instance, in the populations of threespine sticklebacks (Rundle et al. 2000, Taylor and McPhail 2000, McKinnon et al. 2004), *Timema* walking sticks (Nosil et al. 2008), *Littorina* marine snails (Butlin et al. 2008), and *Gambusia* mosquito fishes (Langerhans et al. 2007). Moreover, it was shown in these studies that individuals of the same ecotype are more likely to mate, regardless of relatedness as indicated by phylogenetic affinity.

The results of the COI analysis seem to confirm this scenario to a certain extent as the individuals of the rock ecotype from the Choiriin Bogd Mountain population turned out to be identical to the individuals of the very remote Kazakh population that share the same phenotype, while the individuals of the sand ecotype from two different regions in Mongolia, probably due to some assortative mating connecting populations in desertified habitats, were closer with each other (median, 0.18%) than with the neighboring population of the opposite ecotype (median, 0.71%). Still, however, when analysing the genetic distances more carefully, "minutus" revealed a closer relationship to the adjacent individuals of "kozlovi" (median, 0.71%) than to "kozlovi" from the southern populations (median, 0.89%).

Genetic data holistically supports the proposal that ecotypes can sympatrically form and develop a diagnosable morphology as a step towards reproductive isolation. The nuclear data show species level breaks in other taxa of *Anoplistes* yet there is no shared polymorphism in each of the putative ecotypes. The mitochondrial data, which evolve with a smaller effective population size than nuclear genes, indicate some isolation between ecotypes. A continuation of the observed population dynamics will be necessary for evolution beyond this incipient stage in the speciation process.

4.5. Environmental influence

The differences in elytral colour pattern are probably correlated with the soil substrate. It seems opportunistic in more desertified habitats to develop lighter colored (pale orange) elytra with a narrow black stripe (most likely imitating the shadow of a peashrub stem). Colours in this pattern visibly better camouflage the beetle against the sand/gravel substrate and shrub structure, compared to almost entirely black elytra with two red spots, which in turn may work much better in spatially diversified rocky habitats with numerous objects that create varied shadows. Elytral and pronotal colouration resembling grass shadows on the ground is found in many species of the genus *Eodorcadion* (Cerambycidae: Lamiinae) that are widely distributed in this region. Most of the species have black elytra with a few wide stripes formed of white pubescence on each elytron. Such a contrasting colouration seems to be poor camouflage in desert and steppe habitats, however upon careful observation of these beetles, it becomes clear how effectively this pattern resembles shadows of *Stipa* grasses, especially in full sun.

It seems that the two discussed ecological forms of *Anoplistes* have not yet developed barriers to gene flow (reproductive isolation) as we observed a few mating couples in their contact zone. There are also no differences in endophallic structures, which makes us think that mating may be successful and not futile as is often the case between co-occurring anthophilous cerambycid species. Individuals from two adjacent habitats can meet and occasionally mate, however the total number of such mixed couples constitutes only a small percentage of the populations. Moreover, such a situation is only possible where these distinct habitats border each other, while the greater part of the species' area is either flat sandy/gravelly plains or grassy steppes with rocky hills and the mountain regions. Furthermore, in addition to the above-mentioned fact that individuals of different ecotypes are less likely to mate, it seems reasonable that "alien" individuals die faster in the ecotone, and especially in the opposite habitat, as they are more easily spotted by predators, making these copulations even more sporadic and any offspring less fit. This is one of the basic mechanisms in parapatric speciation. In this mode, divergence may happen because of reduced gene flow within the population and varying selective pressures across the population's range.

4.6. Analysis of relationships among closely related genera of Trachyderini

While *Anoplistes* was recovered as monophyletic in both the BI and ML analyses for all three genes combined with very strong or strong support (respectively), interestingly, *Amarysius* and *Purpuricenus* were rendered as paraphyletic. Although the representation of these genera (mainly for *Purpuricenus* as *Amarysius* includes only five species) might not be comprehensive enough to judge on divisions within this tribe, our results are consistent with those of the latest study in the subfamily Cerambycinae (Lee and Lee 2020). In our analyses, *Amarysius altajensis* was placed (albeit with weak or moderate support) in one clade with all of the *Anoplistes* taxa, which is in the position of a sister group to *Amarysius sanguinipennis*. Low interspecific differences in the endophallic structures in this group indicate that the clades of *Anoplistes* and *Amarysius* are evolutionarily relatively young.

Within *Purpuricenus*, European *P. kaehleri* was placed (with very strong support) into one clade with the Asian genera *Anoplistes* and *Amarysius* as a sister group to the clade of the Asian *Purpuricenus* (*P. sideriger* and *P. temminckii*), which in turn is in the position of a sister group to European *P. desfontainii*. In the aforementioned study, *Purpuricenus lituratus* Ganglbauer, 1886 (not tested by us) revealed the paraphyly of the genus (Lee and Lee 2020).

Surprisingly, *A. jacobsoni* revealed a strong divergence and long genetic separation from the morphologically very similar *A. halodendri*, though it was even considered to be a synonym of the latter by Kostin (1974). Therefore, its species status has been confirmed and the possibility of convergent evolution in morphological traits needs to be carefully examined, especially since the *A. jacobsoni* clade was the sister group to the clade of *A. halodendri* and *A. galusoi*. The latter species differs significantly in its morphology and bionomy and seems to belong to a different lineage with distinct ecological associations with another group of host plants—*Ephedra* L. (Ephedraceae). Further taxonomic work on the phylogeny of this genus is needed and requires the use of both morphological and molecular approaches.

5. Authors' contributions

L.Ka. concepted the work, wrote the main part of the manuscript, obtained SEM images, participated in field research, and managed DNA extraction; W.T.S. wrote some parts of the Material and methods section, performed the measurements, built the figures, and participated in field research; P.G. conducted lab analysis of two nuclear genes, performed the phylogenetic analyses, wrote portions of the paper, and improved the manuscript linguistically; L.Kr. prepared stacked images of the habitus, and participated in field research; D.G.K. prepared the endophallus section of the manuscript and the images of endophallic structures; all the authors meticulously reviewed the text.

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Supplementary material 1

File 1

Authors: Karpínski L, Gorring P, Kruszelnicki L, Kasatkin DG, Szczepański WT (2021)

Data type: .pdf

Explanation note: File 1: KarpińskiEtAl-Anoplistes-MG-Supplement — **Fig. S1.** Length ratios of antennomere 1 to the remaining antennomeres. — **Fig. S2.** Variability of the median lobes in the *Anoplistes halodendri* species-group. **A**–**H**, *Anoplistes halodendri minutus*; **A**–**D**, rock ecotype; **E**–**H**, sand ecotype – *Anoplistes kozlovi* sensu Danilevsky and Smetana (2010); **I**–**L**, *Anoplistes jacobsoni*. — **Fig. S3.** Phylogenetic tree obtained by the Bayesian analysis based on the COI gene sequences. Bayesian posterior probabilities are indicated adjacent to main nodes. *Anoplistes* clades are marked by colors: purple—*A. galusoi*, blue—*A. jacobsoni*, red—*A. halodendri*. — **Fig. S4.** Phylogenetic tree obtained by the Bayesian analysis of the combined nuclear data matrix (AK and CAD). Bayesian posterior probabilities are indicated adjacent to main nodes. *Anoplistes* clades are marked by colors: purple—*A. galusoi*, blue—*A. jacobsoni*, red—*A. halodendri*. — **Table S1.** List of all sequenced *Anoplistes* specimens and their GenBank accession numbers, with individual elytral patterns and geographical data. — **Table S2.** Genetic divergence between the COI sequences (in %) estimated using the Maximum Composite Likelihood model implemented in MEGA7.

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