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DNA vs. Morphology in Delineating Species Boundaries of Endemic Mongolian *Eodorcadion* Taxa (Coleoptera: Cerambycidae)

Lech Karpiński [1,2,*], Patrick Gorring [2] and Anthony I. Cognato [2]

[1] Museum and Institute of Zoology, Polish Academy of Sciences, Wilcza 64, 00-679 Warszawa, Poland
[2] Department of Entomology, Michigan State University, 288 Farm Ln, East Lansing, MI 48824, USA
* Correspondence: lechkarpinski@gmail.com or lkarpinski@miiz.waw.pl

Abstract: This paper provides the first DNA sequences and phylogenetic insight into the Central Asian genus *Eodorcadion*. We used four molecular markers (COI, CAD, ITS2, and Histone 3) and investigated COI genetic distances to verify phylogenetic position of closely related taxa endemic to Mongolia of the '*Eodorcadion intermedium* species-group'. Histone3 data are presented for the first time for Cerambycidae species. We also designed new PCR primers for better amplification of the Cerambycidae COI barcode region. Morphology of all targeted taxa was examined by means of scanning electron microscopy. Our study showed that while there were very few nucleotide differences among COI sequences of three closely related taxa, such that they shared one haplotype, two of them were grouped separately in the all-data tree, and all three were morphologically distinguishable. Conversely, there was a clear barcode gap between *E. intermedium intermedium* and all the remaining taxa, including *E. intermedium kozlovi*. Based on the phylogeny, they belong to two different species-groups; thus, *E. kozlovi* is herein restored to specific rank. Using Bayesian analysis, we contrasted the COI-based tree with the one supported by nuclear data and showed that COI-only data are not sufficient to resolve the phylogeny of the recently derived flightless groups such as Dorcadionini. We conclude that the *Eodorcadion intermedium* species-group is a polyphyletic species assemblage, established based on the pattern of the elytral stripes, which may be a case of parallel evolution driven by ecological adaptation.

Keywords: Central Asia; Dorcadionini; homoplasy; parallel evolution; phylogeny; taxonomy

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1. Introduction

The tribe Dorcadionini Swainson, 1840 sensu Danilevsky [1], and Tavakilian and Chevillotte [2], is one of the most speciose and taxonomically challenging groups of Cerambycidae. Although this tribe was recently synonymised with Lamiini Latreille, 1825 by Souza et al. [3], the taxon sampling was limited (three species of Dorcadionini), and the group remained monophyletic; hence, we decided to use the tribal division until further information is gathered. *Eodorcadion* Breuning, 1947 is one of the five genera of Dorcadionini, and it includes approximately 40 species (almost 70 taxa, including subspecies) that are distributed in southern Russia (between the Southern Ural Mts. and the Pacific Ocean), eastern Kazakhstan, the whole territory of Mongolia, and northern China. Currently, the genus is divided into three subgenera: *Eodorcadion* s. str.; *Humerodorcadion* Danilevsky, Kasatkin and Rubenian, 2005; and *Ornatodorcadion* Breuning, 1947 [1,2]. Although *Eodorcadion* has been revised by Danilevsky [4], that tremendous work is based solely on morphological data, with no phylogenetic reconstruction. The taxonomic cohesiveness of most of the species-groups is uncertain, and molecular data seem essential to solve these problems.

According to Danilevsky [5], the '*Eodorcadion intermedium* species-group' includes six taxa of the subgenus *Ornatodorcadion*: *Eodorcadion gorbunovi* Danilevsky, 2004, *Eodorcadion heros* (Jakovlev, 1899), *Eodorcadion intermedium intermedium* (Jakovlev, 1889), *Eodorcadion*

intermedium kozlovi (Suvorov, 1912), *Eodorcadion oryx* (Jakovlev, 1895), and *Eodorcadion zichyi* (Csiki, 1901). This species-group is morphologically nebulous, and some of the taxonomic decisions are apparently mis-guided, as they have been made based on the external morphology, especially the elytral pattern that is a by-product of ecological adaptation and does not necessarily represent phylogenetic relationships. While some of these taxa, such as *E. heros* and *E. zichyi*, appear to be less related to the other species, *Eodorcadion exaratum* (Ménétriés, 1854) should be included in the group, based on its external morphology and geographical range. Moreover, the reduction of *E. kozlovi* to a subspecies of *E. intermedium* by Danilevsky [5] is controversial, as it was not supported by adequate morphological evidence. This species-group is distributed in central and southern Mongolia and northern China, and is ecologically associated with grasses, mainly *Achnatherum* P. Beauv. (Poaceae) species [5].

The fauna of southern Mongolia is unique, due to the inland location of the country, its geological history, and generally pristine habitats. Many of the cerambycid species found there are endemic to this region (including northern China), and their bionomy is poorly studied [6]. Mongolian ecosystems are under enormous pressure due to climate change (occurring at a much greater rate there than the global average), overgrazing (connected with increase in livestock since its privatisation in 1992), and mining [7,8]. The first two factors are the main cause of land degradation that leads to desertification [8]. Therefore, many groups of phytophagous, especially flightless invertebrates, including *Eodorcadion* species, may be at risk due to the potential loss of their habitat and reduction in host plant resources. Given such a poor understanding of the local fauna, there is the risk that some species will go extinct or drastically reduce their range, due to semi-natural processes related to desertification and the transformation of steppes and semi-deserts into desert, before they are discovered and described and their bionomy better understood.

Various studies across a broad range of taxa have shown that DNA barcoding is an important tool that can greatly facilitate and speed up taxonomic procedures. This method can serve automatic species identification by matching new sequences to existing taxa in reference libraries, differentiating closely related or sibling species, and associating different life stages and dimorphic sexes, e.g., refs. [9–15]. However, there are some insect groups and situations where the use of this method may be seriously limited, e.g., hybridising species or maternally-inherited endosymbionts [16]. In *Eodorcadion* (as well as in other similarly distributed flightless beetle groups), some taxa are assumed to be by-products of hybridisation and introgression of previously independently evolving lineages. During the preparation of a comprehensive revision of this genus and barcoding various taxon levels, we observed cases of haplotype sharing in some relatively easily morphologically distinguishable species. On the other hand, some subspecies show a significant difference in COI distances compared to the nominative subspecies, indicating that they should be considered separate species, sometimes even belonging to different species-groups.

Considering the above-mentioned problems, in addition to COI barcoding, we sequenced the most taxonomically challenging taxa of the *Eodorcadion intermedium* species-group and *E. exaratum* for three nuclear genes and verified their morphology to assess evolutionary support for the current taxonomy and the ability of COI data to distinguish a taxonomically challenging group of beetles. The secondary goal of this work was to provide the first molecular phylogeny of *Eodorcadion* and, more broadly, of flightless Central Asian cerambycids. We hypothesised that the current taxonomy, very often based solely on variable morphological characters, does not reflect phylogeny groups, due to the complex evolutionary history of these beetles, which includes ecological adaptation and cases of hybridisation and introgression.

2. Materials and Methods

This study is based on an examination of approx. 90 specimens of the subgenus *Ornatodorcadion*, mainly belonging to the *Eodorcadion intermedium* species-group sensu Danilevsky [5]. The ingroup includes *E. i. intermedium*, *E. i. kozlovi*, *E. oryx*, and *E. exara-*

tum argali (Jakovlev, 1889). *Eodorcadion consentaneum* (Jakovlev, 1899), *Eodorcadion dorcas* (Jakovlev, 1901), and *E. gorbunovi* served as other intraclade species. *Eodorcadion* (*Humerodorcadion*) *humerale impluviatum* (Faldermann, 1833) was used as the closest related outgroup, and *Dorcadion* (*Acutodorcadion*) *alexandris* Pic, 1900 was used to root the trees. We surveyed more broadly before selecting taxa at various levels of relatedness. *Eodorcadion exaratum* includes two subspecies (both distributed in Mongolia), and although we barcoded individuals from a few localities (including two very distant regions: LK0033–38 vs. PSG1825/26 and LK0160/161; Table S1) that differ slightly in morphology, all of them represent the same subspecies—*E. exaratum argali*. Therefore, it should be emphasised that the presented results refer only to this taxon, which hypothetically may be a separate species. The individuals collected in the field were preserved in 96% ethanol. In a few cases, dried specimens, which were collected a few years before sequencing and killed with ethyl acetate, were utilised.

The beetles were examined using an Olympus SZH10 stereo microscope at 7–140× magnification and a Keyence VHX-7000 digital microscope, at the Museum and Institute of Zoology, Polish Academy of Sciences, Poland (MIZ). Additionally, several specimens representing all the ingroup taxa were examined and imaged using a Hitachi S-3400 N SEM at MIZ. 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 or Canon MP-E65 mm f/2.8 1–5× lens. The images were stacked, aligned, and combined using Zerene Stacker v. 1.04 (www.zerenesystems.com) software. All plates were prepared using GIMP v. 2.10.10 (www.gimp.org).

Thirty specimens were barcoded; fourteen of them, representing all the ingroup taxa, were additionally sequenced for three nuclear genes to obtain a more reliable phylogenetic estimate (Table S1). In total, 65 sequences were produced in this study. Part of the laboratory work (barcoding of the specimens with vouchers between LK0020 and LK0081) 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. For the tissue submission, a meso-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 contained two drops of 95% ethanol on a standard 96-well microplate. Twenty one specimens were successfully sequenced for a 597–658 bp DNA barcoding fragment. 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 "Eodorcadion II Central Asia LK" (DS-LKCCAEII; DOI: https://doi.org/10.5883/DS-LKCCAEII). The COI sequences were submitted to GenBank under the accession numbers: OQ955546–OQ955575.

To attain the remaining COI barcodes (nine specimens; vouchers between LK0149 and LK0161 and PSG1825/PSG1826) and the nuclear genes, we used the DNeasy column extraction kit (Qiagen, Hilden, Germany) to extract DNA from ethanol-preserved and dried samples. Usually, whole leg tissue was taken from adult beetles, dried to remove ethanol, and incubated overnight at 55 °C with lysis buffer. DNA was eluted twice into 150 µL (for dried specimens, first into 100 µL, then into 50 µL) with Qiagen buffer AE. Four gene segments were sequenced: ~940 bp of nuclear protein-coding rudimentary (CAD), ~660 bp of mitochondrial protein-coding COI, ~490 bp of nuclear ribosomal internal transcribed spacer 2 (ITS2), and ~330 bp of nuclear histone 3 (H3), totalling ~2500 bp of aligned sequence data. These gene regions have proven useful for taxonomic discrimination in Cerambycidae and Dorcadionini, e.g., refs. [14,17–20]. PCR amplification was carried out using published primers and newly designed primers specific to the Cerambycidae COI barcode region (Table 1). The PCR conditions for CAD followed Karpiński, Gorring, et al. [14]. For the ITS2 and H3 master mix, we used 2 µL of 10x PCR buffer, 2 µL of 25 mM MgCl2, 4 µL of Promega 5x green GoTaq flexi buffer, 0.3 µL of 100 mM dNTP solution, 1.25 µL of each primer (10 µM), 0.2 µL Taq polymerase (5 u/µL, Qiagen Hot Start Taq DNA Polymerase), 1 µL DNA template, and 13 µL of molecular water. For several samples that failed on the first attempt, a second attempt was made by using a higher volume of

DNA template (2 µL) and 12 µL of water. Thermal cycler conditions for ITS2 followed Dascălu et al. [19]. For H3, we used the following PCR conditions: 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 49 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 10 min. The PCR primers were used for sequencing in all cases except CAD, where CD338F and CD668R were used. Sequencing was performed at the Research Technology Support Facility at Michigan State University, East Lansing, MI, USA. Resulting chromatograms were loaded into *Sequencher* software v.5.1 (Gene Codes Corporation, http://www.genecodes.com) to assemble, manually edit, and export consensus gene sequences. These sequences were submitted to GenBank under the accession numbers: OQ914437–OQ914449 (H3), OQ914450–OQ914460 (CAD), and OQ924612–OQ924621 (ITS2). All of the voucher specimens reported in this paper were deposited and kept frozen in the LK's Cerambycid DNA-grade specimen bank at MIZ.

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, and IUPAC ambiguity codes were added at ambiguous or heterozygous sites. After export from *Sequencher*, each gene was aligned using MAFFT v. 7 [21]. Gene matrices were then constructed by importing the aligned sequence files into Mesquite v. 3.7 [22]. Here the protein-coding genes were also translated to check for stop codons. The concatenated dataset of ~2500 bp sites was analysed in PartitionFinder v. 2.1.1 [23], using the greedy search algorithm with AICc as the selection metric, and comparing unlinked and linked branch lengths. Models were reduced to those not combining gamma + I, as that interaction may cause problems [24]. The lowest AICc scheme partitioned the data into nine subsets with six models—TRN+G: COI_pos1, COI_pos3, CAD_pos1, H3_pos1; TRN+I: COI_pos2; TRN: H3_pos3; HKY+I: CAD_pos2, CAD_pos3; JC: H3_pos2; HKY+G: ITS2.

Bayesian analysis for the concatenated COI, CAD, ITS2, and H3 dataset was run with MrBayes v. 3.2.6 [25] on CIPRES Science Gateway v. 3.3 [26], using two runs with four MCMC chains each (one cold). Sequences were partitioned according to the best partitions and model determined with PartitionFinder 2 for nuclear genes. It was run for 10 million generations, with sampling every 1000 generations. Stationarity and convergence were evaluated by deviation of split frequencies < 0.01, potential scale reduction factor values ~1.00, and effective sample size (ESS) > 200 as measured in *Tracer* v. 1.6 (http://beast.community/tracer). The tree files were summarised with the sumt command in MrBayes with a burn-in of 25% to produce a consensus tree. All trees were viewed and manipulated in *FigTree* v. 1.4 (https://github.com/rambaut/figtree). We considered Bayesian posterior probability from MrBayes (PP) 0.95–1 to be strongly supported and 0.85–0.94 to be moderately supported. Nodes with PP < 0.85 were considered to be unsupported. Analyses of the intra-and interspecific COI genetic distances were conducted in Mesquite using a p-distance model.

Table 1. Primer sequences used for PCR amplification.

Gene	Primer Name	Direction	Sequence	Reference
COI	LCO1490	F	GGTCAACAAATCATAAAGATATTGG	[9]
COI	HCO2198	R	TAAACTTCAGGGTGACCAAAAATCA	[9]
COI	bycF	F	TTTCAACWAACCAYAAAGATATTGG	unpublished data
COI	bycR	R	TAAACTTCWGGATGWCCAAAAAATC	unpublished data
CAD	CD338F	F	ATGAARTAYGGYAATCGTGGHCAYAA	[27]
CAD	CD668R	R	ACGACTTCATAYTCNACYTCYTTCCA	[28]
CAD	CD688R	R	TGTATACCTAGAGGATCDACRTTYTCCATRTTRCA	[28]
ITS2	5.8S_cbgp_F	F	TCGATGAARRMCGCAGYDAAHTG	[19]
ITS2	28S_cbgp_R1	R	GATATGYTTAAATTCRGSGGGT	[29]
Histone 3	H3F	F	ATGGCTCGTACCAAGCAGACVGC	[30,31]
Histone 3	H3R	R	ATATCCTTRGGCATRATRGTGAC	[30,31]

3. Results

3.1. Molecular Analysis

Representatives of the *Eodorcadion intermedium* species-group are very similar morphologically (Figure 1). Therefore, we decided to first barcode four taxa included in the targeted group: *E. intermedium intermedium*, *E. intermedium kozlovi* sensu Danilevsky (2004) (hereafter *E. i. kozlovi*, until this issue is clarified later in this section), *E. oryx*, and *E. exaratum*. The dispersion (range) of the interspecific COI distance was relatively large (for example, 0.6–2.3% between *E. exaratum* and *E. i. kozlovi*, and 0.5–1.8% between *E. oryx* and *E. i. kozlovi*), although the values themselves were low (between 0.5% and 2.3%, excluding *E. i. intermedium*) relative to values for 'good' species in Cerambycidae (Table 2). Moreover, the rate of intraspecific variability of the distance (0.0–2.3% in *E. exaratum* and 0.2–2.0% in *E. i. kozlovi*) was comparable to the rate of interspecific variability (0.6–2.3% between these two taxa). In *E. i. intermedium*, the intraspecific variability was similar to other species and ranged between 0.2 and 1.2%. The intraspecific distance was zero only in *E. oryx*; however, only three specimens from a single locality were barcoded, as this species has one of the most restricted ranges in the genus. Considering again the values of the interspecific distance, there was a noticeable increase in the values between *E. i. intermedium* and all the remaining species. The minimum distance was 5.8% (to *E. i. kozlovi*) up to 7.2% (to *E. exaratum*). Therefore, analysing the COI results alone, *E. intermedium* (Jakovlev, 1889) and *E. kozlovi* (Suvorov, 1912) appear to be separate species. On the other hand, based solely on the COI results, *E. exaratum*, *E. oryx*, and *E. kozlovi* could be considered subspecies.

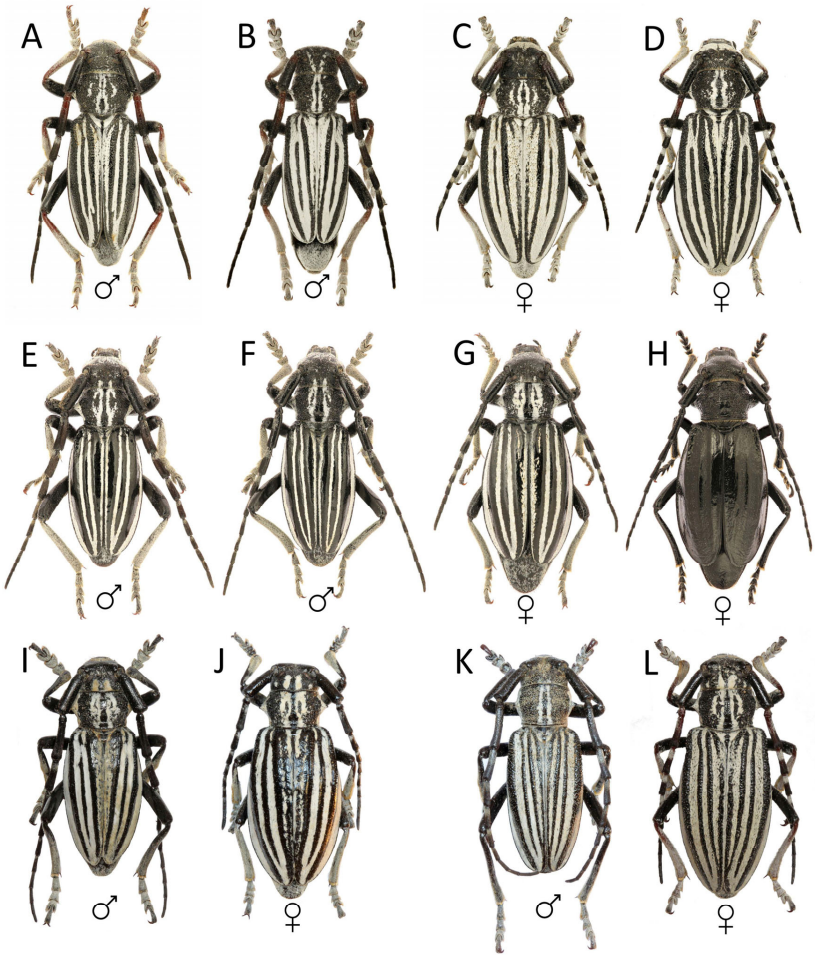

Figure 1. Habitus of the targeted species of the subgenus *Ornatodorcadion*. (**A**–**D**): *E. kozlovi* **stat. res.**; (**E**–**H**): *E. exaratum argali* (with infrequent melanistic females, (**H**)); (**I**,**J**): *E. oryx*; (**K**,**L**): *E. intermedium*. ♂—male; ♀—female.

Table 2. Summary of the intraspecific and interspecific COI genetic distances estimated using a p-distance model.

Intraspecific Distances [%]	No of Spec.	Species		Interspecific Distances [%]			
				1	**2**	**3**	**4**
0.0–2.3	10	*Eodorcadion exaratum*	**1**	-	1.2–2.3	0.6–2.3	6.2–7.2
0.0	3	*Eodorcadion oryx*	**2**	1.2–2.3	-	0.5–1.8	6.2–7.0
0.2–2.0	5	*Eodorcadion kozlovi*	**3**	0.6–2.3	0.5–1.8	-	5.8–7.0
0.2–1.2	6	*Eodorcadion intermedium*	**4**	6.2–7.2	6.2–7.0	5.8–7.0	-

To verify the above-mentioned hypotheses, we sequenced three nuclear loci: CAD, ITS2, and H3. There is no practice of using these more conservative nuclear genes as barcodes like COI. We used this data to produce better-resolved and supported phylogenetic trees and to test the monophyly of the targeted species (see Section 3.3). Histone3 data are presented for the first time for Cerambycidae species.

3.2. Morphology

Our morphological investigation revealed some previously unknown diagnostic characters among the targeted taxa. The most important traits are located on the pronotum and elytra and are mainly related to the punctation, sculpture, and granulation (Figure 2). *Eodorcadion intermedium intermedium* (Figure 1K,L) represents a distinct COI group (Table 2, Figure 3), but it was originally considered to belong to the *Eodorcadion intermedium* species-group (or all the remaining taxa as closely related to it) because its elytral pattern is similar to the rest of the taxa included, especially to *E. i. kozlovi* (Figure 1A–D). It was the main reason for reducing the latter to a subspecific rank. Indeed, *E. i. intermedium* seems to be morphologically very close to *E. i. kozlovi*; however, it differs in its pronotum, which is more wide and flat, with more rugose sculpture and deeper punctation (Figure 2D vs. C). *Eodorcadion i. intermedium* has elytra with distinctly deepest longitudinal furrows, where the pubescence (white stripes) is located; there is a clear distinction on the elytral surface between the longitudinal areas with pubescence and the nodular protuberant stripes (Figure 2H). Similar protuberances are present in *E. i. kozlovi*, but they are much less convex and formed by separate, distinct papules, not simply by rugose sculpture (Figure 2G,K vs. H,L, respectively). Moreover, *E. i. kozlovi* has only three, not four, protuberant stripes, and the middle one is usually separated in the upper part of the elytra (Figure 2G). Such structures are absent in *E. exaratum* (Figure 1E–H) and *E. oryx* (Figure 1I,J)—the surface of their elytra between pubescent stripes is smooth and nearly flat (Figure 2E,I and F,J, respectively). *Eodorcadion i. intermedium* is also usually characterised by a clearly larger body size. There are also some further diagnostic features on the ventral side of the body: both the prosternal and mesosternal processes and the last visible sternum have a different shape/structure in *E. i. intermedium* and *E. i. kozlovi*. However, as ventral morphology is rarely reported in the existing literature, it requires additional study for all *Eodorcadion* taxa. Considering the described morphological differences and significant COI sequence distance, we hereby restore *E. kozlovi* to a specific rank.

In the COI data, *E. kozlovi* **stat. res.** is almost identical to *E. oryx*; however, they are easily distinguishable morphologically. In fact, *E. oryx* is more similar to *E. exaratum*; both *E. exaratum* and *E. oryx* are characterised by rounded and very smooth elytra, without protuberant stripes (Figure 2I and J, respectively). Furthermore, the antennae and legs of these two species never have reddish coloured sections, which are found in most individuals of *E. kozlovi* **stat. res.** and some of *E. intermedium* (Figure 1). Regarding *E. exaratum*, this species was not included in the *Eodorcadion intermedium* species-group; however, based on the COI data and morphology, it is clearly closely related. It is the smallest species of the group and is also characterised by the smoothest and most shiny elytra, due to the

narrowest stripes of white pubescence (Figure 2E vs. F), which makes its habitus clearly darker. Furthermore, males have the largest pronotal tubercles. Among all the targeted species, melanistic (or rather, with hairless elytra) forms are only known in females of *E. exaratum* (Figure 1H), although they are very rare (approx. one in a hundred individuals).

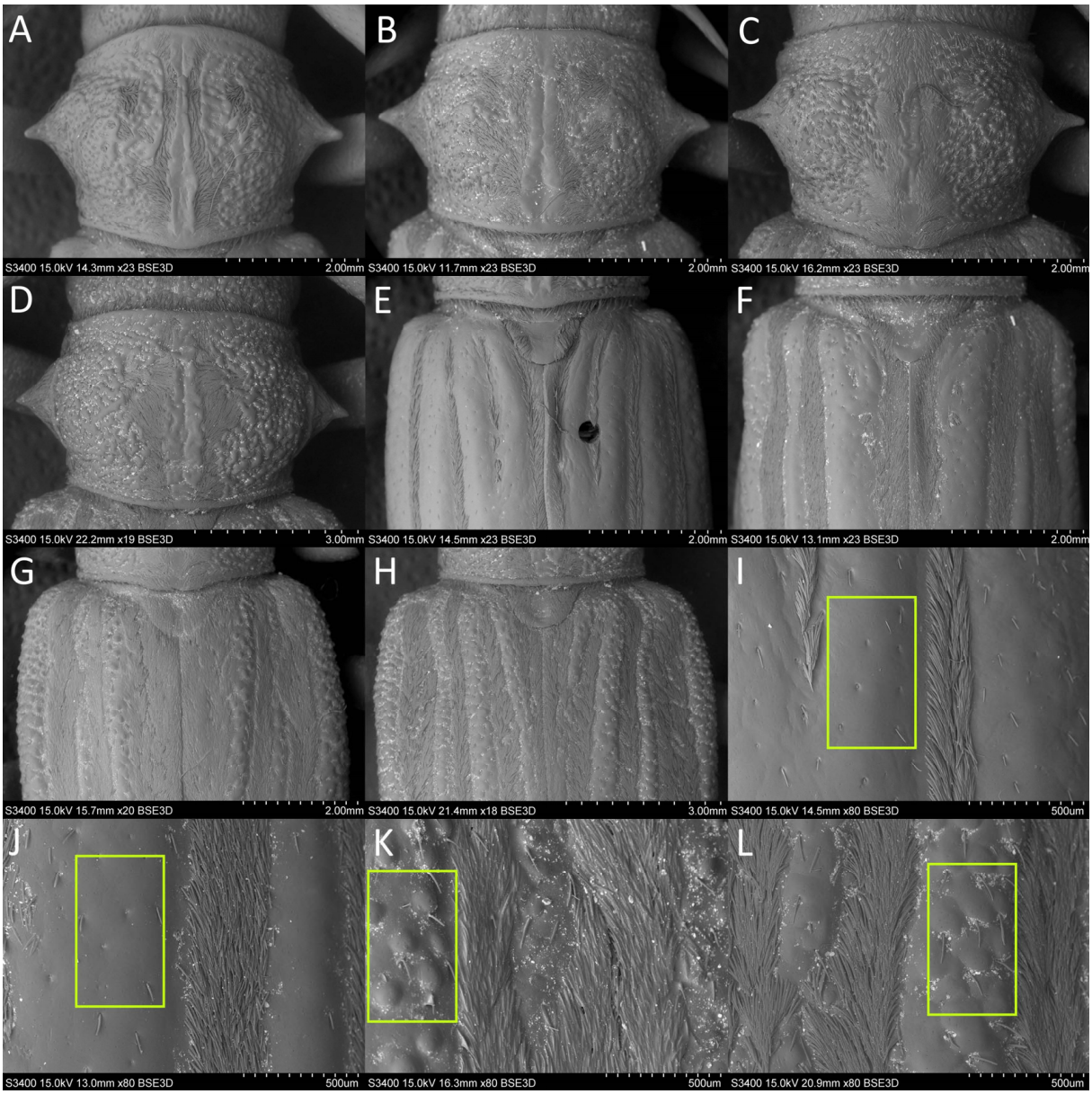

Figure 2. SEM images of pronota (**A**–**D**), basal part of elytra (**E**–**H**), and elytral sculpture (**I**–**L**) of the targeted species of the subgenus *Ornatodorcadion*. (**A**,**E**,**I**): *E. exaratum argali*; (**B**,**F**,**J**): *E. oryx*; (**C**,**G**,**K**): *E. kozlovi* **stat. res.**; (**D**,**H**,**L**): *E. intermedium*. Fragments of elytra marked in yellow indicate differences in their microsculpture (close up onto basal part of right elytron, close to medial suture).

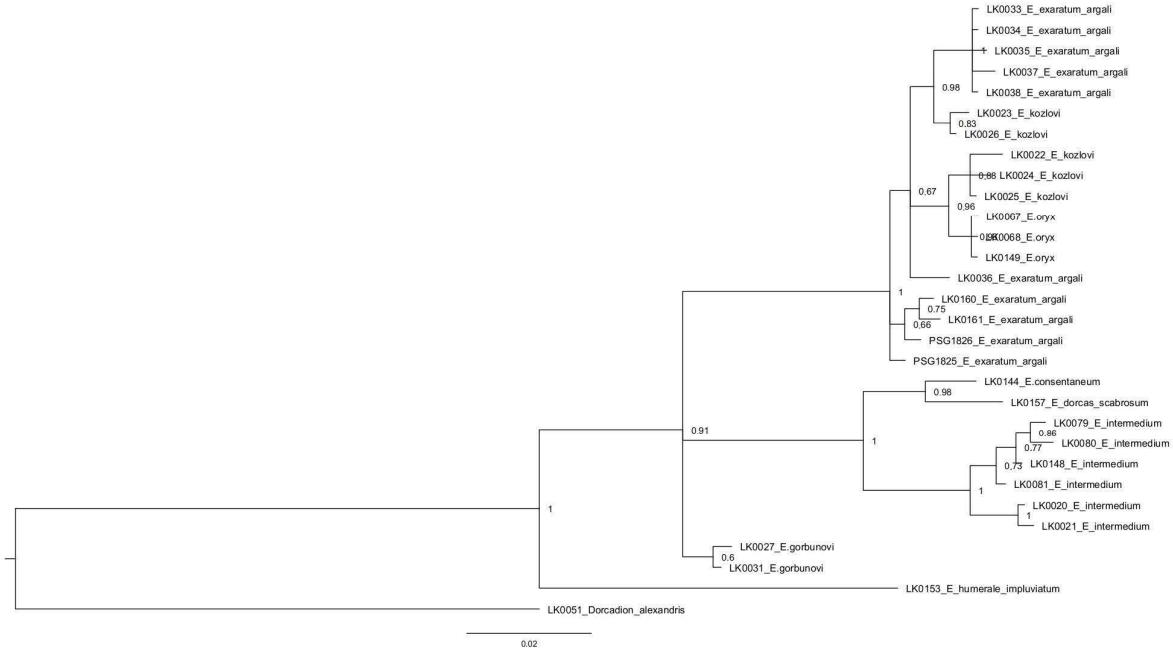

Figure 3. Bayesian phylogeny of the representatives of the *Eodorcadion intermedium* species-group and some other species of the subgenus *Ornatodorcadion,* based on the COI sequences.

3.3. Phylogenetic Analysis

In the Bayesian COI tree (Figure 3), there were polytomies at different levels, and the ranks of *E. exaratum*, *E. kozlovi* **stat. res.**, and *E. oryx* are not clear, in particular for the last two species. Although most specimens of *E. exaratum* were grouped separately from the two remaining taxa, two specimens of *E. kozlovi* **stat. res.** were placed in one clade with some *E. exaratum*, with strong support (PP 0.98). Three specimens of *E. kozlovi* **stat. res.** were grouped together with all specimens of *E. oryx* (collected in a single locality), with strong support (PP 0.96). Even though the subgenus *Ornatodorcadion* has not been resolved, *E. intermedium* likely belongs to a different species-group and is more closely related to *E. consentaneum* and *E. dorcas*, which do not belong to the *Eodorcadion intermedium* species-group. These two species differ morphologically from the representatives of the aforementioned group, including different elytral punctation, larger body size, and lack of forms which are characteristic for this group, such as fully developed white elytral stripes, at least in males. This was another reason for separating *E. intermedium* and *E. kozlovi*.

Analysing the Bayesian all-data tree (Figure 4), almost all nodes were resolved, including for the subgenus *Ornatodorcadion*; polytomies only occurred at the terminal nodes, where individuals with identical or very similar DNA sequences, collected at the same sites, are present. The first difference compared to the COI-based topology is that there is strong support (PP 1) for the separation of the species-groups that include *E. exaratum*, *E. kozlovi* **stat. res.** And *E. oryx*, and *E. intermedium*. Similarly to the COI tree, *E. intermedium* was grouped with *E. consentaneum* and *E. dorcas,* with strong support (PP 1), and the two latter species are more closely related to each other (PP 1). There was also strong support (PP 1) for the clade of *E. exaratum*, *E. kozlovi* **stat. res.**, and *E. oryx*; however, unlike in the COI tree, all specimens of *E. exaratum* formed a distinct clade, albeit with moderate support (PP 0.87). Specimens of *E. exaratum* from two distant areas have been sequenced. However, since they have not been grouped according to their region, this eliminates the possibility of a new subspecies. The relationship between *E. kozlovi* **stat. res.** and *E. oryx* has still not been completely explained by the molecular data, as all specimens of *E. oryx* were placed inside the *E. kozlovi* clade. Except for this issue, the all-data inference corresponds to morphology and appears to present evolutionary and taxonomic structure in this group.

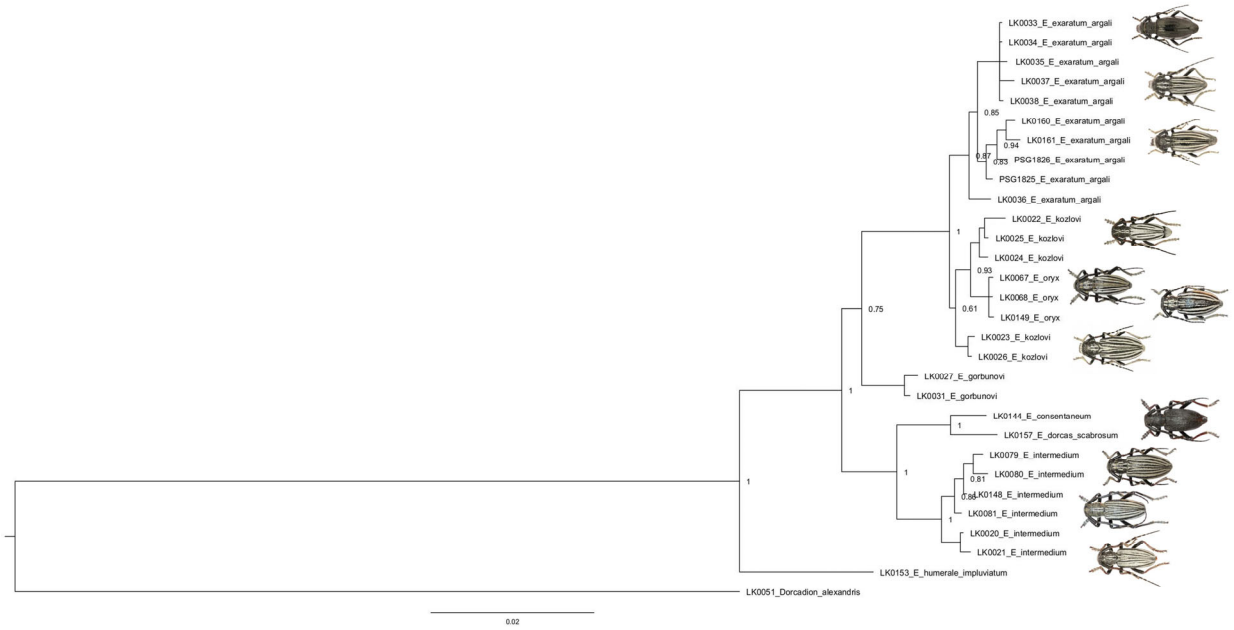

Figure 4. Bayesian phylogeny of the representatives of the *Eodorcadion intermedium* species-group and some other species of the subgenus *Ornatodorcadion,* based on the concatenated sequences of four loci (COI, CAD, ITS2, and Histone3).

4. Discussion

4.1. Molecular Insight into Dorcadionini

Despite such great diversity and numerous taxonomic issues requiring the use of molecular data, until very recently, there were almost no publicly available DNA sequences for Dorcadionini, even considering COI barcodes for European taxa, where many other beetle groups have been barcoded, refs. [32,33]. For instance, taxa of this tribe account for approx. 40% of the cerambycid fauna in Europe [34], but in 2021, a COI barcode was available for only two species. Recently, Dascălu et al. [19] published DNA sequences (mainly of COI but also some of 28S and ITS2) for twenty species of *Dorcadion* Dalman, 1817 (subgenera *Carinatodorcadion* Breuning, 1943 and *Cribridorcadion* Pic, 1901), two of *Neodorcadion* Ganglbauer, 1884, and two of *Iberodorcadion* Breuning, 1943. However, that paper focused only on European (almost exclusively Romanian) taxa, and until now, there have been no molecular data for *Eodorcadion* or any other Dorcadionini from Asia. The molecular contribution made by Dascălu et al. [19] was an important step forward in evaluating the utility of new genes and outlining taxonomic problems.

According to Dascălu et al. [19], Dorcadionini, as a flightless group, can serve as a good model for testing DNA barcoding success and molecular species delimitation. The authors found that the mitochondrial variation was mostly in accordance with the traditional taxonomy, except for one case of haplotype sharing between three morphologically similar species (*Dorcadion murrayi* Küster, 1847; *Dorcadion axillare* Küster, 1847; and *Dorcadion pusillum* Küster, 1847 (interestingly described by one author at the same time)), which suggests hybridisation events. This is very similar to our results regarding *E. exaratum*, *E. kozlovi* **stat. res.**, and *E. oryx*—these three morphologically distinguishable species shared one haplotype in the COI-based tree. However, when data from the nuclear genes were added, *E. exaratum* was separated, similar to the results of Dascălu et al. [19]—in their 28S/ITS2 tree, all three problematic species were separated. This indicates that using COI-only data is not sufficient for such taxonomically challenging and recently derived flightless groups. These authors hypothesised that introgression was responsible for part of the great morphological variability in *Dorcadion*. Presumably, this is also the case for *Eodorcadion*; however, this problem requires further investigation, using additional taxa, genes, genomic data, and more advanced molecular tools, such as allele phasing.

Regarding the interspecific divergence in COI distance, Dascălu et al. [19] observed low values, ranging from 0.62 to 2.38%, in four species. It was very similar to our study for *E. exaratum*, *E. kozlovi* **stat. res.**, and *E. oryx*. Although the range of interspecific distance was considerable (e.g., 0.6–2.3% between *E. exaratum* and *E. kozlovi* **stat. res.**), the values themselves were low, ranging from 0.5 to 2.3%. Overall, the range and values of intraspecific variability in this group are comparable to those of interspecific variability. However, it should be noted that the distance between these three species and *E. intermedium* was much higher and averaged 6.5%. In other cerambycid genera, the reported values for morphologically distinct species of the same genus were either slightly lower in *Anoplistes* Audinet-Serville, 1833 (mean interspecific distances around 5%; ref. [14]) or much higher in *Turanium* Baeckmann, 1922 (approx. 11–14%; ref. [15]).

4.2. Morphological Turmoil as a Result of Ecological Adaptation

The *Eodorcadion intermedium* species-group was proposed by Danilevsky [5] for six taxa of the subgenus *Ornatodorcadion*: *E. gorbunovi*, *E. heros*, *E. intermedium*, *E. kozlovi* **stat. res.**, *E. oryx*, and *E. zichyi*. It appears that this group was established mainly based on one trait—pattern of elytral stripes. However, it is puzzling why at least two more species, with a very similar elytral pattern and distribution in the same region of Mongolia, were not included: *E. exaratum* and *Eodorcadion novitzkyi* (Suvorov, 1909). In addition, a year later, the same author [35], based on the morphology of endophallic structures, presented (p. 132) these two species (in particular *E. exaratum*) as very similar to *E. kozlovi* **stat. res.** and *E. zichyi*. Unfortunately, *E. intermedium intermedium* sensu Danilevsky [5] has not been examined, so it was not possible to compare its endophallus with *E. kozlovi* **stat. res.** Our results clearly show that *E. exaratum* is closely related to *E. kozlovi* **stat. res.** and *E. oryx,* but also that *E. intermedium* belongs to a different species-group. Therefore, the *Eodorcadion intermedium* species-group should now refer to another species-group that includes *E. intermedium* along with *E. consentaneum* and *E. dorcas*, and likely a few more species. However, we propose to discontinue the use of this term, as it is misleading and may only introduce more confusion to this already taxonomically difficult genus. Although endophallic morphology seems to be indeed very informative, it is worth noting that Dascălu et al. [19] reported that hybridisation is possible even between distantly related *Dorcadion* species.

We hypothesise that the current taxonomy of *Eodorcadion*, sometimes based solely on variable morphological characters (endophallic structures of numerous taxa have not been studied in the previously mentioned paper), is unrealistic due to the complex evolutionary history of these beetles, which includes ecological adaptation and cases of hybridisation and introgression. For instance, the similarity in pattern of elytral stripes appears to be a case of homoplasy, clearly related to the environmental conditions. It effectively camouflages these beetles with the substrate and shadows cast by blades of various grass species such as *Achnatherum*, which is the host plant for many eodorcadions. Hence, a similar elytral pattern may occur in different species-groups within the entire genus. Interestingly, while in many species both sexes show such an elytral pattern, in some, males are always black but females are either black (in this case androchromic) or striped (autochromic); however, there are also species with only striped males and two forms in females. This may be evidence of parallel evolution driven by ecological adaptation, although this issue requires further study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15050662/s1. Table S1 List of all sequenced specimens of *Eodorcadion*, their BOLD/GenBank accession numbers, sampling details, and the data on sequenced and analysed molecular markers.

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