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Elmis syriaca (Kuwert, 1890) and *E. zoufali* (Reitter, 1910) (Coleoptera: Elmidae) confirmed as distinct species based on molecular data, morphology and geographical distribution

Manfred A. Jäch [1,*], Michaela Brojer [1], Vlatka Mičetić Stanković [2], Marija Bošnjak [2], Dafna Luz [3], Netta Dorchin [3,4], Yaron Hershkovitz [4], Boris Novaković [5], Ivana Živić [6], Wolfgang Dorfer [7] and Branka Bruvo Mađarić [8,*]

1 Natural History Museum Vienna, Burgring 7, 1010 Wien, Austria; michaela.brojer@nhm-wien.ac.at (M.B.)
2 Croatian Natural History Museum, Demetrova 1, 10000 Zagreb, Croatia; vlatkams@hpm.hr (V.M.S.); marija.bosnjak@hpm.hr (M.B.)
3 School of Zoology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 6997801, Israel; dafnaluz@gmail.com (D.L.); ndorchin@tauex.tau.ac.il (N.D.)
4 Steinhardt Museum of Natural History, Tel Aviv University, Tel Aviv 6997801, Israel; yaronhe@tauex.tau.ac.il (Y.H.)
5 Serbian Environmental Protection Agency, Žabljačka 10a, 11160 Beograd, Serbia; boris.novakovic@sepa.gov.rs (B.N.)
6 Department of Invertebrate Zoology and Entomology, Faculty of Biology, University of Belgrade, Studentski Trg 16, 11000 Beograd, Serbia; ivanas@bio.bg.ac.rs (I.Ž.)
7 Wasserwirtschaftsamt Regensburg, Landshuter Straße 59, 93053 Regensburg, Germany; wolfgang.dorfer@wwa-r.bayern.de (W.D.)
8 Molecular Biology Division, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia
* Correspondence: manfred.jaech@nhm-wien.ac.at (M.A.J.); branka.bruvo.madjaric@irb.hr (B.B.M.)

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Abstract: Molecular data for 19 specimens of *Elmis syriaca syriaca* and *E. s. zoufali* from eight countries have been analysed in order to investigate the taxonomic status and the geographical distribution of these two subspecies. The nominative subspecies was previously thought to be endemic to the Levant (Israel, Lebanon, Syria), while *E. s. zoufali* was regarded as being widespread from the Balkans to eastern Anatolia and Afghanistan. The results of our molecular studies using DNA barcoding and nuclear DNA data reveal that the two taxa are in fact distinct species, which separated around 2 Mya. A distinction based on the external morphological characters of 354 specimens was found to be impossible due to the pronounced variability, especially of the pronotal microsculpture, which had hitherto been used as the main distinguishing feature. The two species can only be distinguished by the aedeagal parameres and by the geographical distribution, which deviates considerably from the concept of previous authors. *Elmis zoufali* is distributed in Romania, the Balkan Peninsula, some Aegean Islands and in western Anatolia, while *E. syriaca* occurs from the Caucasus region southwards to eastern Turkey, Iran (probably also Afghanistan) and the Levant. Geographically, both species are widely separated by the so-called Anatolian Diagonal. *Elmis zoufali* resp. *E. syriaca* are recorded for the first time from Croatia, Romania, Azerbaijan, Georgia, Iran and Turkey. In addition, we examined 13 specimens tentatively identified as *Elmis quadricollis* (Reitter, 1887), a closely related species from Central Asia; we sequenced one specimen from China, which was revealed to be a sister to *E. zoufali* and *E. syriaca*.

Keywords: DNA barcoding; species delimitation; molecular clock; Anatolian Diagonal; morphological variability; distribution; taxonomy; aquatic beetles; *Elmis syriaca/zoufali*; *Elmis quadricollis*; type material

1. Introduction

Molecular data have become indispensable in the frame of modern integrative taxonomy and phylogenetic analyses. Since its introduction in 2003 [1] and the subsequent

establishment of the supporting BOLD Systems (barcode of life database systems [2]), DNA barcoding has proved to be a promising tool for identifying species and delineating highly related taxa or taxa with questionable morphological distinguishing characters, which are particularly common in aquatic beetles [3,4]. In the family Elmidae, recent molecular investigations enabled the clarification of various taxonomic and phylogenetic questions in certain genera [5–9]. DNA barcoding was used in population studies [10–12] and contributed to revisions of species distribution and in descriptions of newly discovered or previously overlooked taxa [7,8,13–20]. Besides the cytochrome C oxidase subunit I (COI) gene (the standard mitochondrial barcoding marker), various nuclear markers have been proposed as alternative or supplementary barcodes for species delineation in Coleoptera (e.g., [21–24]); however, the sole use of molecular data may be misleading, and a combined evidence approach is warranted whenever possible in order to gain insight into taxonomic and systematic relationships (e.g., [3,6,8]).

Elmis zoufali was described as a valid species by Reitter (1910) [25] based on specimens collected in Bosnia and Herzegovina. Since then, it has received very little attention by coleopterists. Berthélemy (1979) [26] downgraded *E. zoufali* to a subspecies of *E. syriaca* (Kuwert, 1890), described from the Levant, and delimited the distribution of the two subspecies as follows: ssp. *syriaca*: Israel, Lebanon, Syria; ssp. *zoufali*: Bosnia, Serbia, Albania, Bulgaria, Aegean Islands, East Anatolia and Afghanistan. The subspecific status proposed by Berthélemy (1979) [26] has been accepted by all subsequent authors (see [27]).

It is important to note that Berthélemy (1979) [26] had a rather limited set of specimens available, and he was therefore not aware of the remarkable morphological variability, which is generally encountered in a number of elmid species.

Over the past 40 years, a large number of specimens of *E. syriaca/zoufali* from numerous countries has been amassed at the Natural History Museum in Vienna, Austria (NMW), enabling a closer examination of the external and aedeagal morphology of these two taxa. Identification based on the distinguishing characters provided by Berthélemy (1979) [26] proved to be unsatisfying because of the populational and geographical variability of the specimens; thus, the authors collected fresh material from eight countries to be used for molecular analysis, which would enable a better evaluation of the diagnostic values of the morphological characters.

2. Material and Methods

2.1. Morphological Examination of Specimens

A total of 109 specimens of *Elmis zoufali*, 245 specimens of *E. syriaca* and 13 specimens tentatively identified as *E. quadricollis* (Reitter, 1887) (hereafter referred to as *E. ? quadricollis*) have been examined morphologically. All these specimens are deposited in the NMW.

The specimens were examined using Leica MZ16 and Wild M-10 stereomicroscopes and an Olympus BH-2 compound microscope. Macroscopic figures are multilayer photographs generated by using a Leica MZ16 stereomicroscope connected to a camera (DFC490), and these were processed and edited applying AutoMontage Pro and Adobe Photoshop 7.0. The microscope multilayer photographs were processed using a Nikon Eclipse 80i microscope connected to a camera (Nikon DS-Fi1).

2.2. DNA Extraction, Amplification and Sequencing

After removal of the genitalia, genomic DNA was extracted from the specimens using a non-destructive method (Table 1). The GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, Taufkirchen, Germany) was used, following the protocol for rodent tail preparation with slight modifications (the specimens were incubated overnight in proteinase K at 55 °C with constant shaking; the final DNA elution was carried out in 100 µL of elution buffer). After DNA extraction, the specimens were briefly rinsed in Milli-Q water and deposited in the NMW; details about the vouchers are listed in the public BOLD datasets DS-NMWELSYZ and DS-CROELSYZ. The DNA samples are kept in the Laboratory for Evolutionary Genetics at the Ruđer Bošković Institute, Zagreb, Croatia (RBI).

Table 1. List of specimens sequenced in this work, BOLD process IDs of COI sequences in BOLD database and NCBI GenBank accession numbers for all examined gene regions.

Species	Country	BIN	BOLD Process ID/GenBank acc. nr. for COI [1,2]	ITS1	H3	CAD
Elmis zoufali	Croatia	BOLD:ADP5230	ELMEU105-18/-	-	-	-
	Serbia		ELMEU101-18/-	-	-	-
	Serbia		**CROEL003-20/OR138318**	**OR142274**	**OR146742**	**OR146727**
	Serbia		**CROEL019-20/OR138316**	**OR142273**	**OR146741**	**OR146726**
	Albania		**NMWEL003-23/OR138311**	**OR142271**	**OR146739**	**OR146724**
	Bulgaria		**NMWEL004-23/OR138310**	**OR142272**	**OR146740**	**OR146725**
	Bulgaria		ELMEU103-18/-	-	-	-
Elmis syriaca	Israel	BOLD:AFC9293	**-/OR187603**	-	-	-
	Israel		**-/OR187604**	-	-	-
	Israel		**-/OR187605**	-	-	-
	Israel		**-/OR187606**	-	-	-
	Israel		**NMWEL007-23/OR138313**	**OR142269**	**OR146737**	**OR146722**
	Israel		**NMWEL008-23/OR138308**	**OR142270**	**OR146738**	**OR146723**
	Georgia		**NMWEL005-23/OR138305**	-	**OR146734**	**OR146719**
	Georgia		**NMWEL006-23/OR138309**	**OR142267**	**OR146735**	**OR146720**
	Georgia		**NMWEL010-23/OR138306**	**OR142268**	**OR146736**	**OR146721**
	Armenia		**NMWEL013-23/OR138312**	-	**OR146733**	**OR146718**
	Azerbaijan		**NMWEL009-23/OR138307**	**OR142266**	**OR146732**	**OR146717**
	Azerbaijan		**NMWEL011-23/OR138315**	**OR142265**	**OR146731**	**OR146716**
Elmis ? quadricollis	China	BOLD:ADL0116	**NMWEL012-23/OR138314**	**OR142275**	-	-
	China		XJDQD253-18/-	-	-	-
Elmis aenea	Croatia	BOLD:AAF0074	CROBF005-19/OR138317	**OR142277**	**OR146743**	**OR146729**
Elmis rioloides	Croatia	BOLD:ABY6700	CROBF017-19/OR138319	**OR142278**	**OR146744**	**OR146730**
Elmis bosnica	Croatia	BOLD:ADR7537	CROBF008-19/OL874462	**OR142276**	-	**OR146728**

[1] BOLD-ID and GenBank accession numbers for the sequences obtained in this work are marked in bold font; [2] Sequence IDs correspond to labels in the phylogenetic tree.

The standard ~658 bp barcoding region of the mitochondrial (mtDNA) COI gene [1] was amplified with the universal primers LCO1490/HCO2198 and under conditions described in [28]. Nuclear genomic regions (nuDNA) spanning the partial internal transcribed spacer 1 (ITS1; ~700 bp), histone 3 (H3; ~300 bp) and carbamoyl–phosphate synthetase 2/aspartate transcarbamylase/dihydroorotase (CAD; ~820 bp) were amplified using primers under PCR conditions described in [29–31], respectively. The amplification mixtures consisted of a 1xDreamTaq™ reaction buffer containing 2 mM $MgCl_2$ (Thermo Fischer Scientific, Waltham, MA, USA), 0.2 mM dNTP mix (Qiagen, Hilden, Germany), 0.5 μM of each primer, 1.0 U DreamTaq polymerase (Thermo Fischer Scientific, Waltham, MA, USA) and 3 μL of DNA in a 20 μL reaction volume. The PCR products were verified on 1% agarose gels, enzymatically purified using the ExoI-rSAP system (NEB, Ipswich, MA, USA) and bidirectionally sequenced in Macrogen Inc. (Amsterdam, The Netherlands), using amplification primers.

2.3. Sequence Data Analyses

The sequences and ab1 files were inspected, edited and pruned in Geneious 8.1.4. (https://www.geneious.com) (accessed on 10 March 2023). For the protein-coding gene regions, the EMBOSS Sixpack tool (https://www.ebi.ac.uk/Tools/st/emboss_sixpack/) (accessed on 10 March 2023) was employed to ensure the continuity of the open reading frames.

For mtDNA COI barcode sequences amplified from our samples, the BOLD identification tool was used for comparison with the public data available in BOLD (http://www.boldsystems.org/index.php/IDS_OpenIdEngine; last accessed on 15 March 2023). In addition, for the nuDNA genomic regions, an NCBI BLAST search was performed to confirm the authenticity of the amplified product, i.e., to assess their similarity with homologous sequences and to rule out possible contaminations in the PCR products. Blastn suite was used with the default search parameters for the program Megablast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome; last accessed on 15 March 2023).

The newly obtained sequences were deposited in the BOLD and NCBI databases. The NCBI GenBank accession numbers for all the examined gene regions and the BOLD process IDs for the COI sequences are listed in Table 1; further details about the sequenced specimens are listed in the public BOLD datasets DS-NMWELSYZ and DS-CROELSYZ. The available public COI sequences with high similarity to our sequences (>98%), as well as three congeneric outgroup sequences (corresponding to *E. aenea* (Müller, 1806), *E. rioloides* (Kuwert, 1890) and *E. bosnica* (Zaitzev, 1908)), were withdrawn from BOLD and used in subsequent phylogenetic analyses (last accessed on 15 March 2023; BOLD IDs are listed in Table 1).

For each separate genomic region, the sequences were aligned with MAFFT version 7, using "Auto" strategy [32] (https://mafft.cbrc.jp/alignment/server/index.html) (accessed on 15 March 2023). NuDNA alignment datasets (ITS1, CAD, H3) were concatenated in BioEdit 7.2.5. [33] and the resulting combined dataset (1632 bp) was used in further analyses. The alignment files are available in Supplementary File S1.

The maximum likelihood (ML) trees for the mtDNA and concatenated nuDNA datasets were calculated in IQ-TREE v.2 [34] on the W-IQ-TREE web server [35] (http://iqtree.cibiv.univie.ac.at/) (accessed on 15 March 2023), making use of the implemented ModelFinder [36] and ultrafast bootstrap (UFBoot) with 1000 replicates [37]. HKY + G was the inferred best-fit model of nucleotide substitution for the COI dataset. For the combined nuDNA dataset, the edge-proportional partition model was used, with three partitions defined by the gene [38,39]. ModelFinder inferred HKY + F, TN + F + I, and TIM3e models for ITS1, CAD and H3 partitions, respectively.

Barcode index numbers (BINs) were assigned to the COI barcoding sequences through the refined single linkage analysis (BIN-RESL) algorithm in BOLD [40], corresponding to the putative species (i.e., molecular operational taxonomic units—MOTUs). The algorithm is based on the existence of a barcoding gap between the putative MOTUs so that the BIN

system is subject to dynamic changes depending on the current taxonomic coverage of the groups in question in BOLD. Uncorrected p-distances within and between the MOTUs were calculated in MEGA 7 [41]; a barcoding gap analysis was performed using the BOLD sequence analysis tools.

In addition, species delimitation methods bPTP (Bayesian Poisson tree process; https://species.h-its.org/ptp/) [42] (accessed on 22 March 2023) and ASAP (assemble species by automatic partitioning; https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) [43] (accessed on 22 March 2023) were performed on the COI dataset in order to validate the species limits defined by BIN-RESL. As the input for bPTP, the inferred ML tree was used. An MCMC analysis was run for 5×10^5 generations, with a thinning of 200 and a burn-in proportion of 0.2; the species delimitation was inferred by the highest Bayesian supported solution. As the input for ASAP, MAFFT alignment of the COI sequences was used; the MOTUs were inferred based on the p-distances under the default program parameters.

The relative lineage divergence age was estimated in BEAST v.1.8.2 [44] based on the BEAUti v.1.8.2. (BEAST package) input file built for the mtDNA COI dataset. An uncorrelated relaxed lognormal molecular clock model was implemented under a Yule process of speciation as a tree prior to the analysis, with the HKY + G substitution model and the COI substitution rate set to 0.0115 substitutions per site per million years (Myr) [45]. Three independent BEAST analyses were performed, with 100 million generations and sampling of trees every 10,000 steps. Tracer v1.6 [46] was used to evaluate the convergence of MCMC runs; a consensus tree with calculated median heights was generated in TreeAnnotator v1.8.2 (BEAST package) after a 20% burn-in. All the resulting phylogenetic trees were edited in FigTree v.1.4.3. (http://tree.bio.ed.ac.uk/software/figtree/) (accessed on 20 April 2023).

3. Results

3.1. Morphology and Distribution

Based on our morphological and molecular studies, *Elmis syriaca* and *E. zoufali* are two distinct species. Together with *E. quadricollis*, described from Uzbekistan, they form the *E. quadricollis* species complex (Berthélemy 1979) [26] characterised by the usually smaller elytral punctures and more uniform elytral disc with the intervals, especially the fifth one, being very weakly or not at all elevated (albeit with a few exceptions, e.g., a male from Ios, Greece, NMW, with a distinctly raised fifth interval; however, intervals 1–4 are equally flat, as in the other specimens), and by the presence of sensorial pores and (more or less distinct) pointed papillae on the mesal face of the paramere. Furthermore, the colour of the integument is usually less black and more dark brown than in other species of the genus. Males are usually (but not always) recognised by the mesal face of the hind tibiae, which is very slightly concave in the posterior half and provided with a longitudinal row of denticles, each with a stiff seta at its apex.

3.1.1. ***Elmis syriaca*** **(Kuwert, 1890)**

Type locality: Not exactly known (Israel or Lebanon). The label data of the lectotype are confusing. According to Berthélemy (1979) [26], one of the labels reads "Syrien [Syria]—Kaifa [possibly referring to Haifa in Israel]", while the second label reads "Baalbeck [possibly referring to Baalbek, a town in Lebanon]".

Type material: Lectotype, female (Muséum national d'Histoire naturelle, Paris, France), designated by Berthélemy (1979: 35) [26]. Although Berthélemy (1979: 35) [26] stated that he had examined the "holotype", this specimen cannot be regarded as holotype because of the International Code of Zoological Nomenclature (ICZN 1999: Art. 73) [47], which rules that a holotype can only be fixed in the original publication, if the author used the word "holotype" or some equivalent expression, or if the nominal species-group taxon is based on a single specimen, either so stated or implied in the original publication (holotype fixed by monotype); however, Kuwert (1890: 50) [48] did not fix a holotype, nor did he mention the number of specimens examined. In the description, he wrote that "...das Thier...hat..." [the animal has], which could imply that he had seen only a single individual; However,

in the same article (p. 49), he used the same wording in the description of "Lareynia rioloides" [*Elmis rioloides*], although he stated in the last line that two specimens had been sent to him. Therefore, it is suggested to regard this specimen as a lectotype (designated by Berthélemy (1979) [26]) as long as there is no evidence that the description was based on a single specimen (ICZN 1999: Art. 74.6 [47]).

Morphological variability (Figure 1): The colouration of the integument is usually chestnut brown to very dark brown (Figure 1B), although entirely black specimens are also found occasionally (Figure 1C).

Figure 1. Habitus of sequenced male specimens of (**A**) *Elmis zoufali*, Bulgaria, Vidin Prov., Sinagovtsi; (**B**) *E. syriaca*, Georgia, Greater Caucasus, Iori River; (**C**) *E. syriaca*, Israel, Nahal Hermon (Banyas) Reserve. Scale bar = 1 mm.

In body size (pronotal length + elytral length), *Elmis syriaca* varies considerably (1.5–2.2 mm). The smallest specimen, a male from Israel (Golan Heights, Ein Jalabina [=Nahal Jalaboun (ca. 33°2′28″ N 35°39′59″ E, left tributary of upper Jordan river)], NMW), is probably brachypterous, because of the poorly developed humeri; a female from the same locality measures 1.6 mm, and its humeri are hardly larger; and a female from a nearby river (Ein Tina, NMW) with well-developed humeri measures 1.9 mm. The largest specimen examined, a male from Iran (Chahar Mahal & Bakhtiari, NE Shahr-e-Kord, Mike Spring, NMW), is 2.2 mm long.

The micropunctation of the pronotal disc (the area between the sublateral longitudinal carinae) is extremely variable, more so than in any other species of the genus. Specimens from the northern part of the distribution area are, on average, more densely micropunctate than specimens from the south, but the micropunctation is generally variable everywhere, even within the same population. Among a series of 29 specimens from Halfeti (Turkey, Şanlıurfa Prov.), there are specimens with the pronotal disc entirely mi-

cropunctate and specimens in which at least the middle of the disc is glabrous between the macropunctures (Figure 2). In the material from the Caucasus Region, the pronotal disc is usually dull (matte); however, some specimens with at least some small glabrous areas can also be found. In Israel, the majority of the specimens has a very glabrous disc with distinct macropunctures, but specimens with at least a partly micropunctate disc are sometimes encountered (e.g., Golan, En Brakha, one female, NMW). In the specimens from Iran (seven exs., NMW), one is more or less glabrous, one is dull, and the remaining ones are intermediate.

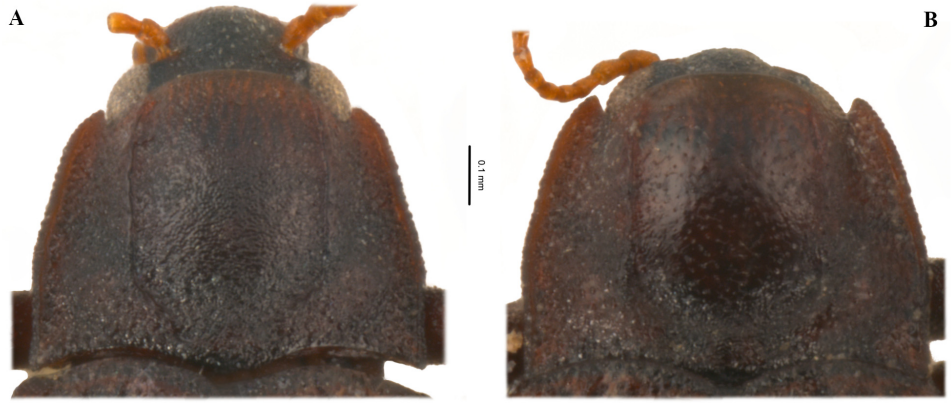

Figure 2. Pronotum of two specimens of *Elmis syriaca* from Turkey (Şanlıurfa Prov., Halfeti) with (**A**) densely micropunctate pronotal disc and (**B**) with largely glabrous pronotal disc.

The teeth on the male tibiae vary slightly in size and number (usually five to seven); however, in females, the hind tibiae may occasionally also be very slightly emarginate posteriorly and provided with small teeth (e.g., specimens from Khashuni River, Armenia, NMW), hampering an unambiguous identification of the sex.

The aedeagal variability is remarkable, as well (Figures 3 and 4). Its length usually varies from 360 to 430 µm (Ø = 400 µm, n = 18), but the aedeagus of the dwarfish specimen from the Golan is only 310 µm long. One specimen from Georgia (Iori River, NMW, Figure 4J) possesses a weakly sclerotised fibula, absent in all the other specimens examined. The sensorial pores on the mesal face of the parameres vary in size, number and distribution; the pointed papillae are often difficult to spot under a compound microscope, as they are never as distinct as the papillae in the *E. aenea* and *E. maugetii* groups. In certain specimens, they seem to be numerous (e.g., Iran, Kerman Prov., Sirch, one exemplar, NMW), while in many others, they are less numerous and/or very small. In any case, further studies are needed to clarify the ultrastructure of the mesal face of the parameres using electron microscopy.

Distribution (Figure 5): According to our studies, this species is more widespread than previously thought. We have examined specimens from the following countries (countries from which we obtained molecular data are marked by an asterisk): Armenia *, Azerbaijan * (first record), Georgia * (first record), Iran (first record) (provinces of Chaharmahal and Bakhtiari, Kerman, Yazd), Israel *, Lebanon, Syria and eastern Turkey (first record) (provinces of Bitlis, Diyarbakır, Şanlıurfa, Van).

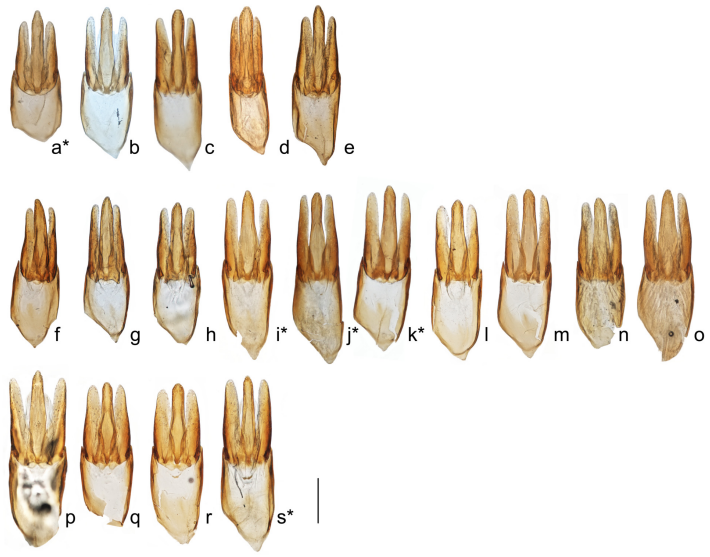

Figure 3. Aedeagi of *Elmis zoufali* (**a**–**e**), *E. syriaca* (**f**–**o**), and *E. ? quadricollis* (**p**–**s**) in ventral view. Sequenced specimens are marked with an asterisk. (**a***) Bulgaria, Vidin Prov., Sinagovtsi; (**b**) Bosnia and Herzegovina, Višegrad; (**c**) Turkey, İzmir Prov., Kozak; (**d**) Turkey, Çanakkale Prov., Behramkale [Assos]; (**e**) Turkey, Konya Prov., nr. Bozkır; (**f**) Turkey, Şanlıurfa Prov., Halfeti; (**g**) Turkey, Diyarbakır Prov., Karacadağ; (**h**) Turkey, Van Prov., Güzeldere; (**i***) Georgia, Lesser Caucasus, nr. Borjomi; (**j***) Georgia, Greater Caucasus, Iori River; (**k***) Israel, Nahal Hermon (Banyas) Reserve; (**l**) Israel, Banyas River; (**m**) Syria, Damascus; (**n**) Iran, Kerman Prov., Sirch; (**o**) Iran, Yazd Prov., Sang Deraz; (**p**) Uzbekistan, Jizzakh Region, Nuratau Mountains; (**q**,**r**) east Kazakhstan, Rakhmanovskoe Lake; (**s***) China, Xinjiang, Lianmuqin Town. Scale bar = 0.1 mm.

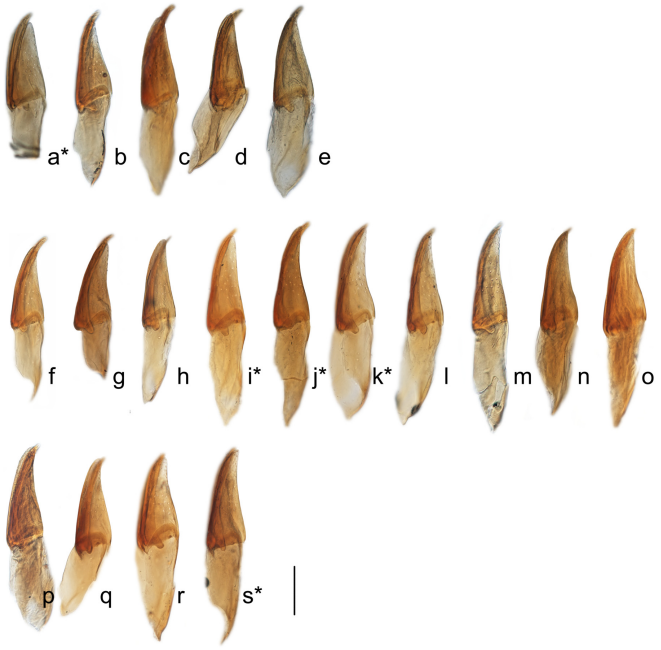

Figure 4. Aedeagi of *Elmis zoufali* (**a**–**e**), *E. syriaca* (**f**–**o**), and *E. ? quadricollis* (**p**–**s**) in lateral view. Sequenced specimens are marked with an asterisk. (**a***) Bulgaria, Vidin Prov., Sinagovtsi; (**b**) Bosnia and Herzegovina, Višegrad; (**c**) Turkey, İzmir Prov., Kozak; (**d**) Turkey, Çanakkale Prov., Behramkale

[Assos]; (**e**) Turkey, Konya Prov., nr. Bozkır; (**f**) Turkey, Şanlıurfa Prov., Halfeti; (**g**) Turkey, Diyarbakır Prov., Karacadağ; (**h**) Turkey, Van Prov., Güzeldere; (**i***) Georgia, Lesser Caucasus, nr. Borjomi; (**j***) Georgia, Greater Caucasus, Iori River; (**k***) Israel, Nahal Hermon (Banyas) Reserve; (**l**) Israel, Banyas River; (**m**) Syria, Damascus; (**n**) Iran, Kerman Prov., Sirch; (**o**) Iran, Yazd Prov., Sang Deraz; (**p**) Uzbekistan, Jizzakh Region, Nuratau Mountains; (**q**,**r**) east Kazakhstan, Rakhmanovskoe Lake; (**s***) China, Xinjiang, Lianmuqin Town. Scale bar = 0.1 mm.

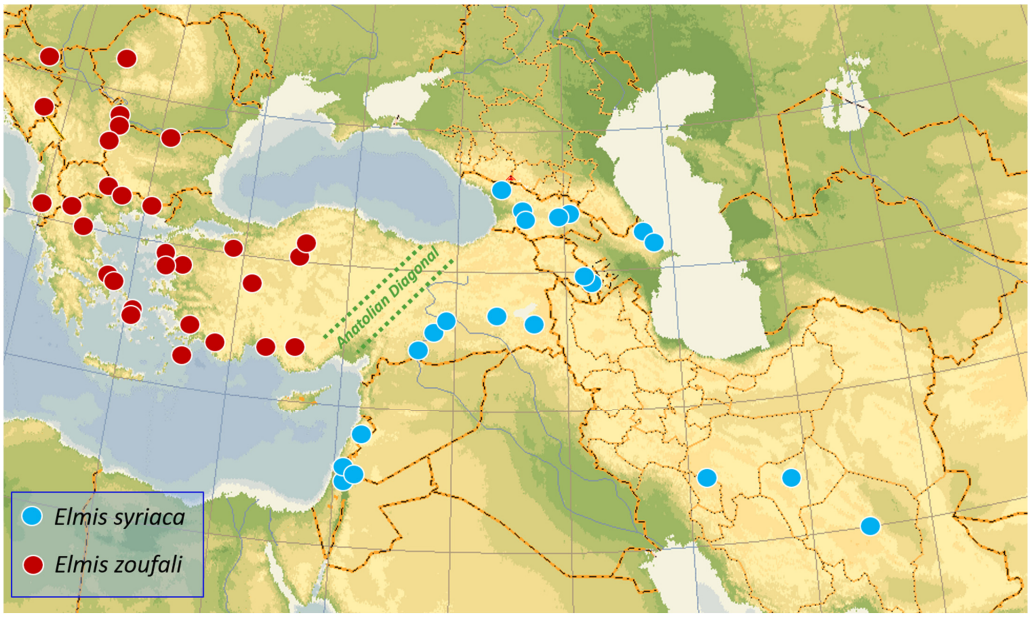

Figure 5. Geographical distribution of *Elmis syriaca* and *E. zoufali*, showing the Anatolian Diagonal. All currently confirmed localities are mapped.

3.1.2. *Elmis zoufali* (Reitter, 1910)—species propria

Type locality: Višegrad (Bosnia and Herzegovina).

Type material: Berthélemy (1979: 36) [26] stated that he had studied the holotype and the paratypes ("l'holotype et des paratypes") deposited in the Hungarian Natural History Museum, Budapest, Hungary (Reitter collection); however, Berthélemy (1979) [26] was obviously not aware that no holotype had been designated in the original description (Reitter 1910, [25]), and no lectotype had subsequently been designated. Therefore, the specimens studied by Berthélemy (1979: 36) [26] must be regarded as syntypes (ICZN 1999: Art. 73.2) [47], rather than as holotype and paratypes. It is well known among coleopterists that in the Hungarian Natural History Museum in Budapest, holotype or paratype labels were arbitrarily and illegitimately attached to many syntypes in the past. According to the ICZN (1999: Art. 74.5 "Lectotype designations before 2000") [47], the statement by Berthélemy (1979: 36) [26] cannot be regarded as a lectotype designation because "when the original work (Reitter 1910 [25]) reveals that the taxon had been based on more than one specimen, a subsequent use of the term "holotype" does not constitute a valid lectotype designation unless the author, when wrongly using that term, explicitly indicated that he or she was selecting from the type series that particular specimen to serve as the name-bearing type". It should be also kept in mind that two of the syntypes (one male and one female) from the type locality in fact belong to *Elmis maugettii* Latreille, 1802 (see [26]).

Morphological variability (Figure 1): *Elmis zoufali* is slightly less variable than *E. syriaca*. Colouration (Figure 1A): more or less as in *E. syriaca*; black specimens are rarely encountered.

Body length (pronotal length + elytral length): 1.6–2.1 mm.

Micropunctation: In most specimens throughout the distribution area, the pronotal disc is densely micropunctate and dull, but some specimens with at least some small

glabrous areas (usually in the middle of the disc) can also be found; specimens with an entirely glabrous disc were not encountered.

The male tibial teeth are more or less as in *E. syriaca*; sometimes, they are very poorly developed (e.g., one specimen from Ios, Greece, NMW).

The length of the aedeagus (Figures 3 and 4) varies from 360 to 410 µm (Ø = 390 µm, *n* = 8). As in *E. syriaca*, the pointed papillae on the mesal face of the parameres vary greatly in size and number. In his very short diagnosis of *E. zoufali*, Olmi (1976: 238) noted that this species lacks papillae on the mesal face of the parameres.

Distribution (Figure 5): According to our studies, this species is less widespread than suggested by Berthélemy (1979) [26]. We have examined specimens from the following countries (countries from which we obtained molecular data are marked with an asterisk): Albania*, Bosnia and Herzegovina, Bulgaria*, Croatia* (first record), Greece, Romania (first record), Serbia* and western Turkey (provinces of Afyon, Ankara, Antalya, Bilecik, Çanakkale, İzmir, Konya, Muğla).

3.2. Identification, Delimitation and Divergence Time Calibration Based on Molecular Data

For sequenced nuDNA gene regions, a BLAST search resulted in positive hits with corresponding sequences from the *Elmis aenea* genome assembly (Accession PRJEB58378), with >80%, >94% and >95% sequence similarity in ITS1, CAD and H3, respectively.

The BOLD identification engine returned positive hits for the COI sequences of the newly sequenced specimens from Serbia, Bulgaria and Albania, with >99% similarity to three unidentified specimens (*Elmis* sp.) from Croatia, Serbia and Bulgaria. Additionally, our specimen of *E. ? quadricollis* from China matched an unidentified specimen (*Elmis* sp.) that was also collected in China. On the other hand, sequences of the specimens from Israel, Armenia, Georgia and Azerbaijan had no positive matches in the public BOLD database.

In accordance with the BOLD-ID results, the BIN-RESL algorithm assigned our newly sequenced specimens into three separate BINs. Barcoding COI sequences of the specimens from Serbia, Bulgaria and Albania were assigned to an already existing BIN with the three aforementioned *Elmis* sp. From Croatia, Serbia and Bulgaria, whereas for the sequences of the specimens from Israel, Armenia, Georgia and Azerbaijan, a new BIN was created in BOLD. These two BINs are mutual nearest neighbors (NN) in BOLD. A specimen of *E. ? quadricollis* was assigned to a BIN with the aforementioned *Elmis* sp. specimen from China. The results are summarised in Table 1 and Figure 6.

Uncorrected p-distances within and between the BINs are presented in Supplementary Table S1. Between the two nearest-neighbor BINs with specimens of *E. syriaca* and *E. zoufali*, an average distance of 3.53% is recorded, while the average distance between these two BINs and the one with *E. ? quadricollis* specimens is around 4%. Within the BINs, p-distances range from 0.00 to 0.96%. The highest distance is in the *E. syriaca* BIN, where the specimens from the Caucasus Region and Israel form two clearly defined subclades. A barcoding gap analysis in BOLD showed that there is no overlap of intra- and interspecific distances, confirming a clear barcoding gap for these three BINs. As expected, uncorrected p-distances for the nuDNA gene regions between the same specimen groups are generally much lower (three to four times) than for the mtDNA, with the exception of ITS1, which shows p-distances comparable to those of COI (Supplementary Table S1).

The results of the species delimitation methods bPTP and ASAP are completely congruent with BIN-RESL, delimiting the newly sequenced specimens into three clearly separate lineages corresponding to the MOTUs assigned for *E. syriaca*, *E. zoufali* and *E. ? quadricollis* (Figure 6). The same three lineages were also obtained with high support in the ML tree for the nuDNA dataset (presented in Supplementary Figure S1).

Molecular dating presented in the ultrametric Bayesian phylogenetic tree in Figure 6 suggests that *E. zoufali* and *E. syriaca* have been separated by about 2 Mya, during the Pleistocene. The divergence of their last common ancestor and the ancestor of their sister species, *E. ? quadricollis* (represented by the two specimens from China) is estimated at about 2.6 Mya, around the beginning of the Pleistocene. Similar divergence ages are

calculated for the *E. aenea* group (*E. aenea*, *E. rioloides*, *E. bosnica*), where the species separated in a period of 1.6–3.3 Mya.

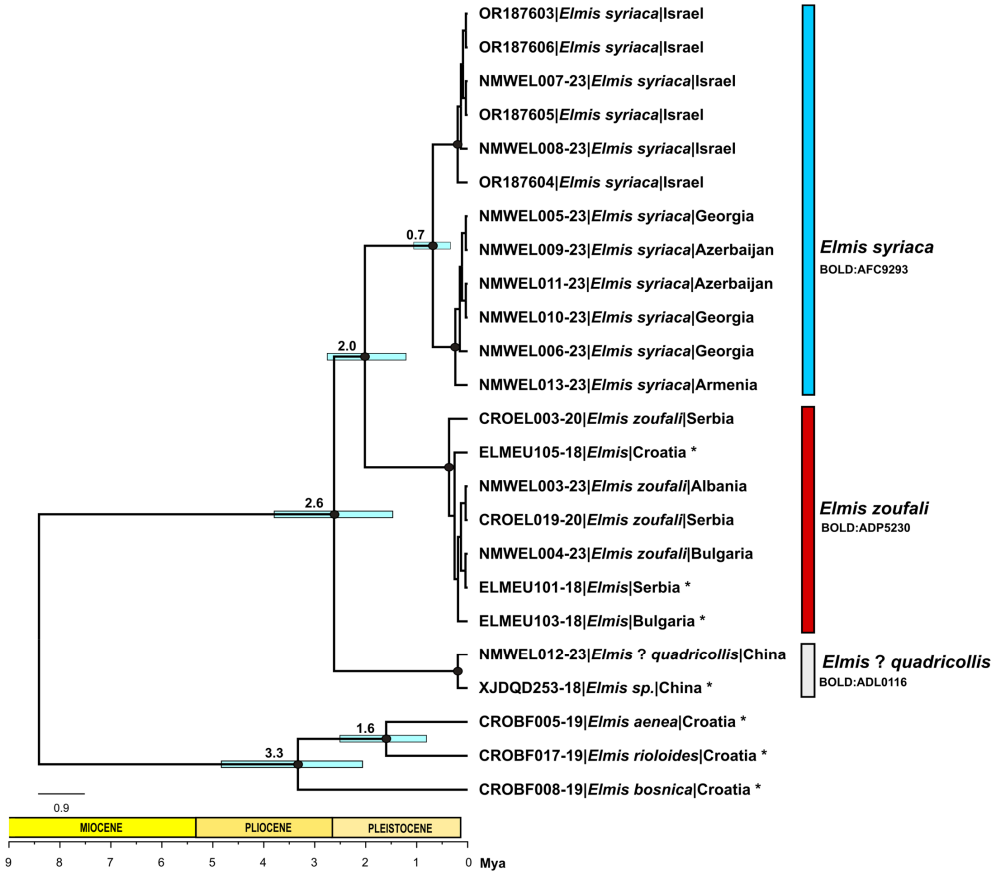

Figure 6. Ultrametric time calibrated tree obtained with BEAST for COI data. Light blue bars represent 95% HPD intervals for node ages in Mya. Black dots denote >0.95 pp Bayesian and >90% bootstrap ML node support; BIN-RESL, bPTP and ASAP groups are marked as coloured vertical bars. Asterisks denote sequences retrieved from BOLD.

4. Discussion

Thirteen species of *Elmis* Latreille, 1802 are currently known. Most of them occur in the West Palearctic Region [27]. Due to the pronounced morphological variability, the identification of the species is often difficult, but molecular methods are usually useful to clarify taxonomic questions satisfactorily [16]. Based on the combined molecular, morphological and distributional data for *E. zoufali* and *E. syriaca*, we herein determine that these two taxa represent different species rather than subspecies. Our conclusion is based on several lines of evidence and analyzed in comparison to other closely related groups of organisms, as follows. Firstly, the genetic distances in the examined mitochondrial and nuclear DNA regions are similar to values found for various closely related species in Coleoptera (e.g., [22,49–52]), including for the sister species *E. aenea* and *E. rioloides* (as shown here). This finding is further corroborated through several species delimitation methods, based on different algorithms, all of which retrieve *E. syriaca* and *E. zoufali* as separate MOTUs with high support.

As of August 2023, there are ten valid BINs in BOLD for the genus *Elmis*. In eight of them, there are specimens identified as seven currently recognised species, whereas in two BINs, the specimens are designated as *Elmis* sp. One of these two unidentified BINs with specimens from Croatia, Bulgaria and Serbia corresponds to our specimens of *E. zoufali*. This match allows us to attribute the respective Croatian specimen from BOLD to *E. zoufali.* This finding thereby provides the first record of *E. zoufali* for this country, which extends

its distribution markedly to the northwest. In the future, we plan to pay special attention to elmid research in northeastern Croatia in order to define the northwestern limit of the distribution of *E. zoufali* and to describe its ecological requirements.

Numerous recent papers describe molecular divergence time dating of various water beetle taxa (e.g., [3,9,53–56]). Reliable geological or fossil records often serve as calibration points, but in their absence, the molecular clock for related animal groups may also be used with considerable reliability. In our analyses, we applied the substitution rate of 0.0115 substitutions per site per million years for insect COI [45], which was extensively used to date the divergence time in various insect groups, including aquatic beetles (e.g., [57–60]). Our results suggest that *E. zoufali* and *E. syriaca* separated during the Pleistocene, and that their separation from *E. ? quadricollis* also occurred during that epoch. It should be noted that similar separation times were calculated here for the *E. aenea* group of three clearly defined species (*E. aenea*, *E. rioloides* and *E. bosnica*).

The allopatric distribution areas of *E. zoufali* and *E. syriaca* lie west and east of the East Anatolian Fault (EAF), which separates the Anatolian Plate from the Arabian Plate. The area immediately west of the EAF has been termed the Anatolian Diagonal (AD) by biogeographers [61]. It is ca. 850 km long and runs from the northeast of Turkey (Erzincan) in a southwestern direction to the Cyprus Arc at the Mediterranean Coast (Figure 1, e.g., [62–65]). From a neotectonic point of view (e.g., [65]), the AD is a NE–SW trending left-lateral strike–slip shear zone between Erzincan and the Cyprus Arc. The formation of the AD is suggested to have been impacted by Cenozoic geological events in the area over millions of years [61]. As described in [66], during the Paleogene period, India collided with Asia, and Africa with Eurasia, starting the formation of the Irano–Anatolian Plate. The Tethys Ocean was closing, and its descendants, the Paratethys and the Mediterranean, started to shape. During the Neogene, tectonic movements began along the EAF and the North Anatolian Fault in the Middle Miocene, and in the Late Miocene and Pliocene, uplift affected Anatolia, with continued movements during the Pleistocene (e.g., [64]). Various Anatolian landscapes have been formed by tectonic movements, geomorphological processes, volcanic activity and karst development (e.g., [64]). According to the geomorphological regions of Turkey defined by Kuzucuoğlu et al. (2019) [64], *E. zoufali* occurs in western Anatolia (Aegean) and the Mediterranean Anatolia, whereas *E. syriaca* is confined to the southeastern Anatolian region (Figure 5).

The Cenozoic climate was marked by oscillations; after the middle Miocene climatic optimum (17–15 Mya), the climate was gradually cooling (e.g., [67]). The Plio-Pleistocene period was marked by a global climate shift with the onset of the northern hemisphere glaciation (e.g., [68–70]). In the Pliocene, the wet and humid conditions changed to the colder and more arid conditions of the Pleistocene, with an impact on the landscapes as open vegetation started to spread. In the Pleistocene, Anatolia served as a refuge during the last glacial and interglacial cycles, and the AD may have acted as an environmental barrier at that time, limiting the dispersal for many taxa and promoting speciation (e.g., [62,63,71–79]).

The distribution data presented herein corroborate the species status of *E. zoufali* and *E. syriaca* by showing that these two taxa are allopatric and thus not able to interbreed. In 1981, MAJ collected numerous elmids, including four undescribed species, in the Turkish provinces of Mersin and Niğde, which are inside the area of the AD. He found four species of *Elmis* (*E. bosnica*; *E. maugetii* Latreille, 1802; *E. rioloides*; and *E. robusta* Jäch, 1984), but there was no trace of *E. syriaca* or *E. zoufali* (see [80]). Bearing in mind the good dispersal abilities of *E. zoufali* and *E. syriaca*, comparable to other *Elmis* species found in that area, as well as their similar ecological requirements, it is quite difficult to explain the fact that *E. syriaca* and *E. zoufali* are absent in this region. Future efforts should be focused on more intensive collecting of elmids in the area of the AD, which may promote the understanding of the distribution of these two taxa.

In addition, we examined the specimens tentatively identified as *E. ? quadricollis*. The single sequenced specimen from the Xinjiang (China) groups in the BIN with another specimen from the same autonomous region were submitted to BOLD as *Elmis* sp. It

should be kept in mind that the identity of *Elmis quadricollis*, described by Reitter (1887) [81] from Central Asia (Uzbekistan, Tashkent) has not been clarified satisfactorily. According to Berthélemy (1979: 31, 33) [26], who has designated the male lectotype (erroneously regarded as the "type unique", p. 31, resp. as "holotype", p. 33), *E. quadricollis* and *E. syriaca* form a monophyletic unit. According to the illustrations of the lectotype provided by Berthélemy (1979: Figures 76–78, 85) [26], the aedeagus of *E. quadricollis* is similar to that of *E. syriaca*, but the parameres (in the lateral view) are very slender and the ventral margin is not concave. We have examined the aedeagi of all Central Asian males of the *E. quadricollis* group available to us: Uzbekistan (Nuratau Mountains, one male, NMW, Figures 3p and 4p); Kazakhstan (east Kazakhstan region, Rakhmanovskoe Lake, two males, NMW, Figures 3q–r and 4q–r; Almaty region, Jarkent, two males, NMW); and China (Xinjiang, Lianmuqin Town, one male, NMW, Figures 3s and 4s). All these aedeagi agree in general characters with those of *E. syriaca*. None of the parameres were found to be straight in the lateral view. The only specimen from Kyrgyzstan (Naryn) deposited in the NMW is, unfortunately, a female. We have sequenced the single male from China (Xinjiang, Lianmuqin Town), which was surprisingly found to represent a different species, clearly distinct from *E. syriaca*. In comparison to *E. syriaca*, the elytra of the Central Asian specimens are, on average, more acuminate apically, but significant aedeagal differences could not be detected at this time.

Until we do acquire molecular data of specimens from Uzbekistan, we cannot draw any final conclusions. Indeed, more material is necessary to enable a more detailed morphological analysis, and more specimens from all parts of Central Asia that can be sequenced are needed to clarify the identity of *E. quadricollis*.

For completeness, we must point out the existence of another nominal taxon in the *Elmis quadricollis* group: *E. lindbergi* Janssens, 1959, described from Afghanistan (Nimruz Prov., Delaram). It has been synonymised with *E. zoufali* by Olmi (1976: 240) [82] because of the dull pronotal disc. Later, *E. lindbergi* was placed in synonymy with *E. quadricollis* by Jäch et al. (2016: 49) [27] because of the geographical proximity of Afghanistan to Uzbekistan; however, in view of the revised distribution data presented herein, the type locality of *E. lindbergi* is geographically closer to the easternmost records of *E. syriaca* than to Tashkent in Uzbekistan.

5. Conclusions

Based on our analyses, the original specific status of *Elmis zoufali* is reinstated herein.

The only author who had hitherto compared *E. syriaca* and *E. zoufali* was Berthélemy (1979) [26], who was misled by the variability of the micropunctation of the pronotal disc and, therefore, erroneously misinterpreted the real distribution of *E. syriaca* and *E. zoufali* and finally decided to regard these two taxa as subspecies (*E. s. syriaca* with glabrous pronotum, *E. s. zoufali* with dull pronotum). Clearly, this mistake was largely based on the fact that he had not studied enough specimens and therefore did not realise that the western populations of the *E. syriaca/zoufali* complex differ from the eastern ones significantly in the lateral view of the parameres (width and shape of the ventral margin), although in his illustrations (Berthélemy 1979: Figures 79, 83) [26], this character is well recognizable. In the 1970s, the elmid fauna of Turkey was virtually unknown, which furthermore prevented Berthélemy from realizing that the western and eastern populations were geographically separated by a rather wide gap (of ca. 700 km) in central Anatolia, suggesting genetic isolation, assumed by our molecular study to exist since about two million years ago.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15090994/s1, File S1: alignment files in Fasta format; Figure S1: ML tree for the concatenated nuDNA dataset; Table S1: Uncorrected p-distances for mtDNA and nuDNA gene regions.

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Data Availability Statement: The data presented in this study are openly available in BOLD (public datasets DS-NMWELSYZ and DS-CROELSYZ) and NCBI GenBank (acc. nrs. OL874462, OR138305-OR138319, OR142265-OR142278, OR146716-OR146744, OR187603-OR187606).

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