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Phylogeography of *Dolichophis* Populations in the Aegean Region (Squamata: Colubridae) with Taxonomic Remarks

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Abstract: In this study, we investigate the phylogeographic patterns of *Dolichophis* species in the Aegean region, aiming to elucidate their genetic diversity and putative historical colonisation routes through mitochondrial and nuclear DNA data. Our findings revealed distinct phylogeographic patterns: *D. caspius* exhibited a higher level of haplotypes within two shallow mitochondrial lineages, contrasting with *D. jugularis*, which displayed lower genetic variability in the area. Additionally, we identified evidence showing possible human-mediated historical translocation of *D. caspius* populations to Karpathos from the Balkans mainland. The mitochondrial variability in *D. jugularis* remained relatively uniform across southwestern Anatolia and Dodecanese, except for Rhodes Island. The evidence from mitochondrial and nuclear data confirming the previously described morphological differentiation of the Rhodes snakes, and thus the name *D. j. zinneri* Cattaneo, 2012, described on the island, could be applied to this isolated population. This result addresses the first genetic view on the long-standing taxonomic uncertainties regarding the subspecies status of Rhodes *D. jugularis*. Our results also raise questions regarding possible historical hybridisations between *D. caspius* and *D. jugularis* in the Dodecanese islands, prompting the need for further investigation using extensive field studies and genomic approaches. Ultimately, the Aegean islands, particularly Kos and Rhodes, seem to be important sites for the evolution of these colubrid snakes and their historical dynamics.

Keywords: *Coluber*; Colubridae; distribution; DNA barcoding; phylogeography; subspecies; taxonomy

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1. Introduction

The Aegean region serves as an outdoor evolutionary laboratory within the Mediterranean biodiversity hotspot, where the present-day biota has undergone remarkable development since the Miocene [1]. Over time, owing to unique local conditions, vertebrates within this region have experienced significant microevolutionary processes and speciation events [2–4]. These include hybridisations, specific adaptations, habitat contractions due to climate change, subsequent recolonisations, and competitions resulting from encounters with similar species that have colonised the area from various sources. Additionally, the later human influence played a role in shaping the Aegean biota, particularly through the introduction of species in different locations, notably the numerous islands found in the Aegean [1].

The diversity of snake fauna in the Aegean region is rich (19 species in 15 genera) but still includes many gaps in our knowledge as to distribution, morphology, or genetics. Numerous studies have been related to the historical biogeography of the Aegean herpetofauna, including colubrid snakes [5–7] that showed contrasting phylogeographic patterns. However, many snake species have never been genetically studied in the area, and their evolutionary history is only hypothesised (e.g., *Eirenis* Jan, 1863; *Hemorrhois* Boie, 1826; *Platyceps* Blyth, 1860).

The two prominent species of the genus *Dolichophis* Gistel, 1868, within the region are also less explored. The geographical range of this genus spans from Central Europe (Hungary) across the Balkans and the Aegean to Anatolia, Ukraine, the Caucasus region, the Middle East, and Central Asia [8]. *Dolichophis caspius* (Gmelin, 1789) and *D. jugularis* (Linnaeus, 1758) meet in the Aegean region and Anatolia [8,9]. The former has a wide distribution encompassing the Balkans and Anatolia's mainland, along with numerous Aegean islands. The latter reaches the region via southern Anatolia and can be located on several Dodecanese islands (i.e., east Aegean islands close to Anatolia). However, their phylogeography in this area and the implications of their range overlap, particularly on the small Greek island of Kos, have not been thoroughly investigated. Nonetheless, this does not imply a complete lack of information on the genetic patterns of these two snake species in the region, albeit primarily focused on *D. caspius* [10,11]. The second species has been studied even less across its entire range [12,13]. The intriguing aspect is that the Aegean region serves as a contact zone for these two snakes and includes numerous islands with varying geological periods of isolation, influencing their phylogeography. Moreover, this area harbours two names linked to the genus *Dolichophis* that have not undergone genetic testing to date, i.e., *Coluber caspius eiselti* Zinner, 1972 (with NHMW 18618:1 designated as a holotype), and *Dolichophis jugularis zinneri* Cattaneo, 2012 (holotype ZFMK 92945). Both taxa come from Rhodes Island [14–18], with the former taxon considered invalid (see [19]) due to its description in an unpublished doctoral thesis of Hermann Zinner [20].

Consequently, we aimed to elucidate these snake populations in the Aegean region using genetic data (mitochondrial and nuclear DNA markers) to delineate their local phylogeography. In addition, we tried to provide pertinent insights into the taxonomy of morphologically distinct populations in Rhodes.

2. Material and Methods

2.1. Sampling

In total, we used 35 new samples of *D. caspius* and 11 samples of *D. jugularis* from the Aegean islands (Agios Efstratios, Karpathos, Kos, Lesbos, Limnos, Rhodes, Samothraki, Serifos, Tinos) and from the related mainland Balkans and Anatolia (Türkiye). The material was obtained either directly during field surveys or from collections of the Aydın Adnan Menderes University, Türkiye (ADU); Zoology Department, Ege University, Izmir, Türkiye (ZDEU); Natural History Museum of Crete, Greece (NHMC); and the Zoological Museum University of Patras, Greece (ZMUP). Detailed information about sample codes, sampling localities, and GenBank accession numbers can be found in Table 1.

2.2. DNA Extraction, Molecular Markers, PCR Amplification and Sequencing

Tissue samples were preserved in 96% ethanol. DNA was extracted using the E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA). Sequences of one mitochondrial (mtDNA) and three nuclear (nuDNA) genes were targeted: the mitochondrial protein-coding segment cytochrome *b* (Cyt *b*), the nuclear protein-coding genes for the brain-derived neurotrophic factor (BDNF), the oocyte maturation factor Mos (C-mos), and recombination activation gene 1 (Rag1). For PCR, we used Red Taq 2X Master Mix 2 mM MgCl. The primers and PCR conditions used for each marker are presented in Table S1. The same primers were also used for sequencing. PCR products were purified with ExoSAP-ITTM

PCR Product Cleanup Reagent (USB Europe GmbH, Staufen, Germany, following manufacturer's protocol). Sequencing was performed by Macrogen Inc. (Amsterdam, Netherlands; http://www.macrogen.com). We performed a BLAST search in GenBank to confirm that the targeted loci and species were amplified. The translation of protein-coding sequences into amino acids was checked using DnaSP 6.00 [21], confirming the absence of stop codons. Then, we combined new sequences with GenBank data (Table 1).

Table 1. The data on *Dolichophis caspius* and *D. jugularis* along with their geographic origins, in addition to GenBank sequences utilised in this study.

Species	Code/Voucher Number (Museum)	Locality	N	E	Country	GenBank Number Cyt *b*/C-mos/BDNF/Rag1	Source
D. jugularis	492 (CUHC)	Palio Pyli, Kos Island	36.8457	27.1885	Greece	PP378899/-/-/-	This study
D. jugularis	493 (CUHC)	Tigaki, Kos Island	36.8888	27.2697	Greece	PP378900/PP378937/PP378942/PP378947	This study
D. jugularis	12199 (I. Strachinis)	Alyki, Kos Island	36.8779	27.1757	Greece	PP378926/-/-/-	This study
D. jugularis	12200 (I. Strachinis)	Zia, Kos Island	36.8522	27.2316	Greece	PP378927/-/-/-	This study
D. jugularis	12204 (I. Strachinis)	Gennadi, Rhodes Island	36.0298	27.9236	Greece	PP378928/PP378940/PP378941/PP378948	This study
D. jugularis	10568 (ADU)	Kızılot, Antalya Province	36.6864	31.6048	Türkiye	PP378913/PP378938/PP378944/PP378949	This study
D. jugularis	10608 (ADU)	Bafa Lake, Muğla Province	37.4859	27.5435	Türkiye	PP378918/-/-/-	This study
D. jugularis	10621 (ADU)	Sabuca Village-Koçarlı, Aydın Province	37.7623	27.6765	Türkiye	PP378920/-/-/-	This study
D. jugularis	10627 (ADU)	Madran Village-Bozdoğan, Aydın Province	37.6744	28.2866	Türkiye	PP378921/-/-/-	This study
D. jugularis	10629 (ADU)	Topçam-Çine, Aydın Province	37.6891	28.0148	Türkiye	PP378922/-/-/-	This study
D. jugularis	10630 (ADU)	Güneyköy-Bozdoğan, Aydın Province	37.7136	28.2114	Türkiye	PP378923/-/-/-	This study
D. jugularis	MVZ 230242	2 km NE Finike, Antalya Province	36.3238	30.1672	Türkiye	AY486917/AY486941/-	[13]
D. jugularis	ZMHRU 2012/50	Kocaaliler, Burdur Province	37.3166	30.6833	Türkiye	AY486917/-/-	[22]
D. jugularis	ZMHRU 2012/51	Kızılseki, Burdur Province	37.2666	30.7500	Türkiye	AY486917/-/-	[22]
D. jugularis	-	-	-	-	Jordan	-/AY376798/-	[12]
D. caspius	216 (ZMUP)	Apollonia	40.6441	23.4561	Greece	PP378890/-/-/-	This study
D. caspius	231 (ZMUP)	Farsala	39.2564	22.3159	Greece	PP378891/-/-/-	This study
D. caspius	418 (ZMUP)	Tinos Island	37.5741	25.1501	Greece	PP378892/-/-/-	This study
D. caspius	427 (ZMUP)	Tinos Island	37.5957	25.1535	Greece	PP378893/-/-/-	This study
D. caspius	430 (ZMUP)	Karpathos Island	35.5235	27.1103	Greece	PP378894/-/-/-	This study
D. caspius	431 (ZMUP)	Karpathos Island	35.5641	27.0993	Greece	PP378895/-/-/-	This study
D. caspius	458 (ZMUP)	Serifos Island	37.1303	24.4876	Greece	PP378896/-/-/-	This study
D. caspius	459 (ZMUP)	Serifos Island	37.1440	24.4485	Greece	PP378897/-/-/-	This study
D. caspius	489 (ZMUP)	Tigaki, Kos Island	36.8756	27.1853	Greece	PP378898/PP378936/PP378943/PP378946	This study
D. caspius	763 (ZMUP)	Loutros	40.8770	26.0468	Greece	PP378901/-/-/-	This study
D. caspius	6146 (CUHC)	Geoponiki	40.3080	23.0940	Greece	PP378902/-/-/-	This study
D. caspius	6618 (CUHC)	Iztok	41.9908	27.5436	Bulgaria	PP378903/-/-/-	This study
D. caspius	7761/80.3.35.51 (NHMC)	Stratoni	40.5141	23.8238	Greece	PP378904/-/-/-	This study
D. caspius	9895 (CUHC)	Metamorfosi	40.5615	21.3069	Greece	PP378905/-/-/-	This study
D. caspius	9908 (CUHC)	Prinos	39.5900	21.6188	Greece	PP378906/-/-/-	This study
D. caspius	9921 (CUHC)	Deskati	39.9230	21.8209	Greece	PP378907/-/-/-	This study
D. caspius	9928 (CUHC)	Svoronos	40.2645	22.4487	Greece	PP378908/-/-/-	This study
D. caspius	9929 (CUHC)	Lofos	40.2526	22.4046	Greece	PP378909/-/-/-	This study
D. caspius	10551/231/2013 (ZDEU)	Çukurca, Domaniç, Kütahya Province	39.7908	29.6895	Türkiye	PP378910/-/-/-	This study
D. caspius	10552/179/2014 (ZDEU)	İhsaniye, Eyüb, İstanbul Province	41.2411	28.8048	Türkiye	PP378911/-/-/-	This study
D. caspius	10557/232/2013 (ZDEU)	Yörükakçayır, Eskişehir Province	39.7438	30.3389	Türkiye	PP378912/-/-/-	This study

Table 1. *Cont.*

Species	Code/Voucher Number (Museum)	Locality	N	E	Country	GenBank Number Cyt *b*/C-mos/BDNF/Rag1	Source
D. caspius	10573 (ADU)	Between Çalı and Atlas, Bursa Province	40.1506	28.9042	Türkiye	PP378914/-/-/-	This study
D. caspius	10584 (ADU)	Kepez, Aydın Province	37.8611	27.8540	Türkiye	PP378915/PP378939/ PP378945/-	This study
D. caspius	10595 (ADU)	Pamukkale, Denizli Province	37.9296	29.1332	Türkiye	PP378916/-/-/-	This study
D. caspius	10601 (ADU)	Between Simav and Demirci, Kütahya Province	39.1249	28.8580	Türkiye	PP378917/-/-/-	This study
D. caspius	10614 (ADU)	Kuşadası, Aydın Province	37.6866	27.1604	Türkiye	PP378919/-/-/-	This study
D. caspius	12195 (I. Strachinis)	Alykes, Lesbos Island	39.2260	26.2429	Greece	PP378924/-/-/-	This study
D. caspius	12197 (I. Strachinis)	Antimachia, Kos Island	36.8144	27.1086	Greece	PP378925/-/-/-	This study
D. caspius	12205 (I. Strachinis)	Agios Efstratios Island	39.5348	24.9940	Greece	PP378929/-/-/-	This study
D. caspius	12206 (I. Strachinis)	Agios Efstratios Island	39.5396	24.9962	Greece	PP378930/-/-/-	This study
D. caspius	12207 (I. Strachinis)	Kos Island	36.7669	27.0901	Greece	PP378931/-/-/-	This study
D. caspius	12208 (I. Strachinis)	Samothraki Island	40.5087	25.5579	Greece	PP378932/-/-/-	This study
D. caspius	12209 (I. Strachinis)	Samothraki Island	40.4265	25.5311	Greece	PP378933/-/-/-	This study
D. caspius	12211 (I. Strachinis)	Samothraki Island	40.4433	25.4977	Greece	PP378934/-/-/-	This study
D. caspius	12214 (I. Strachinis)	Repanidi, Limnos Island	39.9242	25.3147	Greece	PP378935/-/-/-	This study
D. caspius	NHMW KCC1	Serifos Island	37.1608	24.4810	Greece	AY376739/ AY376797/-/-	[12]
D. caspius	-	Andros Island	37.8467	24.8961	Greece	AY376739/-/-/-	[12]
D. caspius	-	Şile, Istanbul Province	41.1670	29.5923	Türkiye	HM210777/-/-/-	[10]
D. caspius	-	Samos Island	37.7135	26.8166	Greece	HM210778/-/-/-	[10]
D. caspius	-	Samos Island	37.7135	26.8166	Greece	HM210779/-/-/-	[10]
D. caspius	-	Euboea (Evvoia) Island	38.5346	23.8197	Greece	HM210780/-/-/-	[10]
D. caspius	-	Thasos Island	40.6768	24.6439	Greece	HM210782/-/-/-	[10]
D. caspius	-	Prespes	40.7428	21.1597	Greece	HM210782/-/-/-	[10]
D. caspius	-	Veles	41.7081	21.7759	North Macedonia	HM210782/-/-/-	[10]
D. caspius	-	Brest	43.2759	21.7287	Serbia	HM210782/-/-/-	[10]
D. caspius	-	Zlot	44.0119	21.9880	Serbia	HM210782/-/-/-	[10]
D. caspius		Kolets, Haskovo	41.9207	25.5469	Bulgaria	HM210783/-/-/-	[10]
D. caspius	-	Shumen	43.2491	26.9427	Bulgaria	HM210783/-/-/-	[10]
D. caspius	-	Hagieni, Constanta	43.7829	28.4842	Romania	HM210783/-/-/-	[10]
D. caspius	-	Tekirdağ, Tekirdağ Province	40.9845	27.5020	Türkiye	HM210785/-/-/-	[10]
D. caspius	-	Samothraki Island	40.4482	25.5848	Greece	HM210787/-/-/-	[10]
D. schmidti	ZISP 27777	Khumlakh	39.2347	47.0514	Armenia	AY376743/ AY376801/-/-	[12]
D. schmidti	CAS 182953	Mt. Dushak, Mary Province	37.1300	60.0200	Turkmenistan	-/AY486947/-/-	[13]
D. andreanus	ICSTZM.7H.1154	Darreh Shahr, Ilam Province	33.1444	47.3828	Iran	MN531565/-/-/-	[23]
E. collaris	ZISP 27859	Arax Fluss, n. Megri	38.8852	46.2589	Armenia	AY376766/-/-/-	[12]

Abbreviations: ADU—Aydın Adnan Menderes University, Aydın, Türkiye; CAS—California Academy of Sciences in San Francisco, California, USA; CUHC—Comenius University Herpetological Collection, Bratislava, Slovakia; ICSTZM—International Center for Science and Technology Zoological Museum, Kerman, Iran; MVZ—Museum of Vertebrate Zoology, Berkeley, California, USA; NHMC—Natural History Museum of Crete, Iraklio, Greece; NHMW—Natural History Museum Vienna, Austria; ZDEU—Zoology Department, Ege University, Izmir, Türkiye; ZISP—Zoological Institute of St. Petersburg, Russia; ZMHRU—Zoology Museum of Harran University, Şanlıurfa, Türkiye; ZMUP—Zoological Museum University of Patras, Greece.

2.3. Sequence Alignment and Data Analysis

The sequences were aligned and edited manually using Geneious Prime 2023.1.2 (Biomatters, Auckland, New Zealand). The default Geneious alignment algorithm was used. The phylogenetic relationships of 56 sequences of *D. caspius* and *D. jugularis* together with three outgroup sequences [*D. andreanus* (Werner, 1917), *D. schmidti* (Nikolsky, 1909) and *E. collaris* (Menetries, 1832)] of the final alignment were assessed using maximum likelihood (ML) in RaxML 8.0.0 [24]. The best-fit model of sequence evolution (GTR + G with three subsets) was selected using Partitionfinder 2 [25]. The ML clade support was assessed by 1000 bootstrap pseudoreplicates. This mitochondrial dataset also includes sequences from published studies where the same haplotypes, present in different locations, were used (Table 1).

Mitochondrial haplotype networks were examined and drawn using PopArt [26] (http://www.popart.otago.ac.nz) and the implemented parsimony network algorithm of [27], with a 95% connection limit. Independent networks were considered distinct evolutionarily significant units, following Fraser and Bernatchez [28]. The alignment was 1114 bp long in *D. caspius*, and 1083 bp long in *D. jugularis*. DnaSP 6.00 [21] was used to estimate the number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), and uncorrected p distances in mitochondrial datasets.

To access the genealogical relationships between selected taxa/populations of *Dolichophis* in the Aegean region, we separately conducted an allele network analysis on three nuDNA markers, C-mos, BDNF, and Rag1. For comparison, we used previously generated sequences of C-mos AY376797 (*D. caspius*); AY376798 and AY486941 (both *D. jugularis*); AY376801 and AY486947 (*D. schmidti*) from Nagy et al. [12,13]; and our new sequences from Kos Island (sympatric distribution of *D. caspius* and *D. jugularis*; samples 489 and 493), Rhodes (*D. j. zinneri*; sample 12204), and continental Anatolia (samples 10568 and 10584) (due to different quality of resulting sequences, not all of them were equally represented in nuclear datasets; see Table 1). The resulting sequence alignments (567 bp, 661 bp, and 899 bp, respectively) with more than one heterozygous site were resolved in PHASE 2.1.1 [29] for which the input data were prepared in SeqPHASE [30]. PHASE was run under default settings except for the probability threshold, which was set to 0.9. Allele networks of both analysed markers were examined and drawn using PopArt [26] (http://www.popart.otago.ac.nz) and the implemented parsimony network algorithm of TCS with a 95% connection limit [27].

3. Results

In the current study, we present a phylogeography (mitochondrial and nuclear DNA) of two species within the *Dolichophis* genus from the Aegean region where they show partially sympatric distribution (Figure 1). Our sampling in the Aegean and surrounding areas is comprehensive, totally encompassing 43 mitochondrial sequences for *D. caspius* and 12 for *D. jugularis,* representing 50 and 14 localities, respectively (Table 1, Figure 1). Notably, we show the first genetic insights into Rhodes populations, historically associated with the description of two names within the genus. Additionally, for the first time, we conducted mitotyping on populations across various Aegean islands where the genus is present. These islands include Agios Efstratios, Karpathos, Kos, Limnos, Rhodes, Samothraki, Thassos, and Tinos, representing the Dodecanese, Cyclades (Central Aegean), and North Aegean archipelagos of the Aegean Sea. It is worth noting that our sampling did not cover the Sporades and several small islands in the Dodecanese where the genus is known to occur too (Figures 1–3). Furthermore, we also provide a first genetic insight into the populations on Kos Island, the only Aegean island where two species of the genus currently coexist.

3.1. Mitochondrial DNA Data

From our analysed dataset, the maximum likelihood (ML) tree revealed four distinct, well-supported (79–100) clades within the *Dolichophis* genus, aligning with the current species taxonomy, i.e., *D. andreanus*, *D. caspius*, *D. jugularis*, and *D. schmidti* (Figure 1B). Our examination of the populations of the two focal species, *D. caspius* and *D. jugularis*, in the Aegean area and surrounding regions, unveiled low levels of intraspecific diversity. Notably, the genetic distance between these two species in the area was calculated as 12.0%, with average intraspecific distances of 0.76% for *D. caspius* and 0.26% for *D. jugularis*.

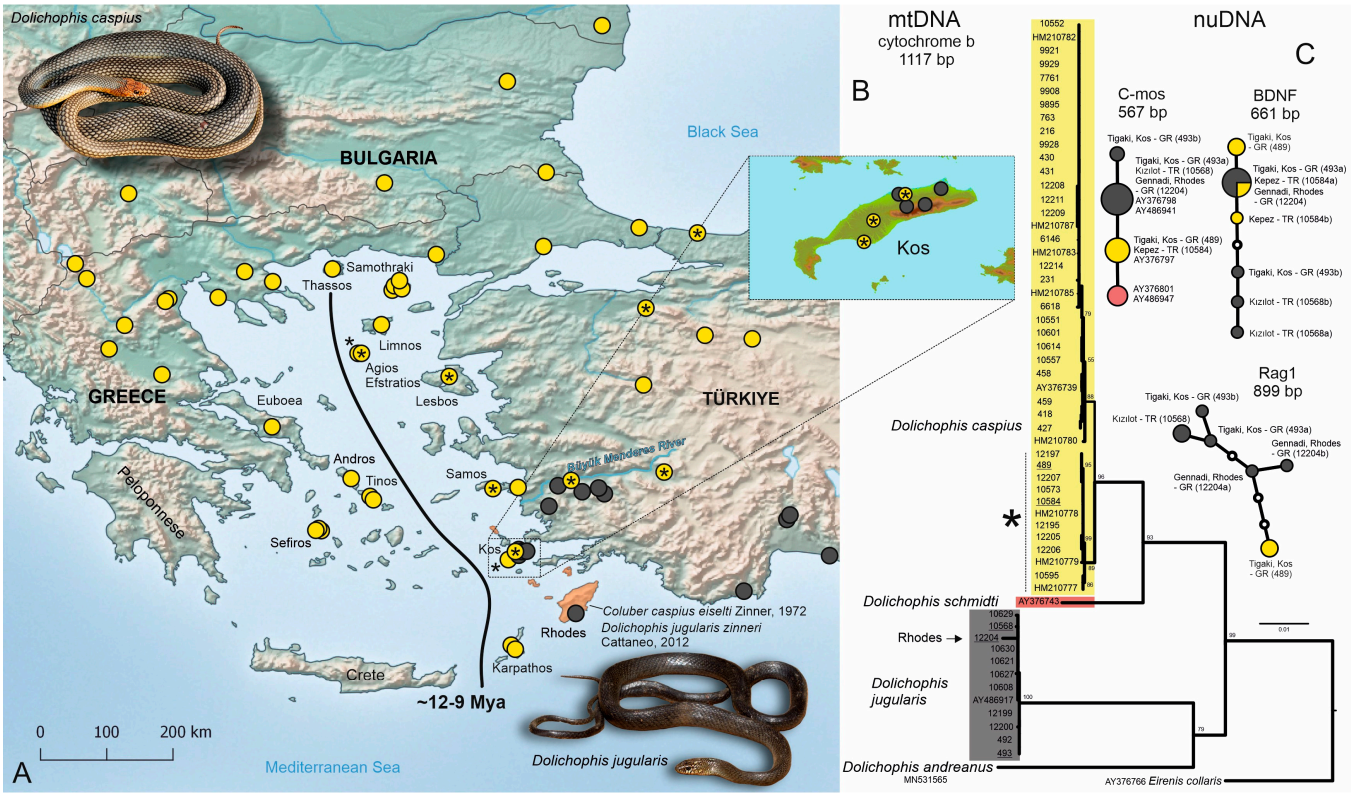

Figure 1. The genotyped localities of *Dolichophis caspius* and *D. jugularis* in the Aegean region (**A**) together with the mitochondrial tree (**B**). Colourations on the map and the tree correspond to taxa. Underlined sequence codes in the mtDNA tree mark those used for nuDNA analysis. The orange shading of Rhodes and surrounding islands marks the range of *D. jugularis zinneri* according to Cattaneo [16]. The asterisk indicates the distribution of the eastern Aegean–Anatolian lineage of *D. caspius*. The line in the middle of the Aegean Sea represents the early formation of the Aegean barrier. Nuclear allele networks of the phased sequences of C-mos, BDNF, and Rag1 genes (**C**) presenting relationships between Rhodes (12204) and selected populations and taxa. Species colours in networks follow those used in the map (**A**) and tree (**B**). Circle sizes are proportional to the number of individuals that share a given allele. A small empty circle in networks indicates a missing or hypothetical allele. Different alleles of a single heterozygous sequence is coded as a and b, and without division, they represent homozygous sequences. Refer to Table 1 for locality details and sample codes used in the tree and networks. Inset photographs: Daniel Jablonski (*D. caspius* from Bulgaria) and Ilias Strachinis (*D. jugularis* from Rhodes). The map was generated using QGIS 3.28, available at https://qgis.org/.

Regarding their intraspecific diversity, *D. caspius* exhibited two well-recognised and well-supported but shallow mitochondrial lineages, with a genetic distance of 1.6% between them (refer to Figure 1B). These two *D. caspius* lineages are roughly distributed on the east and west sides of the Aegean Sea, respectively. The widely distributed lineage (π = 0.20%) represents populations from the west Aegean and continental Balkans, alongside the islands of Thassos, Samothraki, Limnos, and Karpathos, but also Anatolia. The second lineage, the eastern Aegean–Anatolian (π = 0.14%; denoted by * in Figure 1A,B), is situated on the east side of the Aegean and was recorded in the Black Sea coast, east of the Bosporus, in Bursa, along the Büyük Menderes River in western Anatolia, and on Greek islands, i.e., Kos, Samos, Lesbos, and Agios Efstratios.

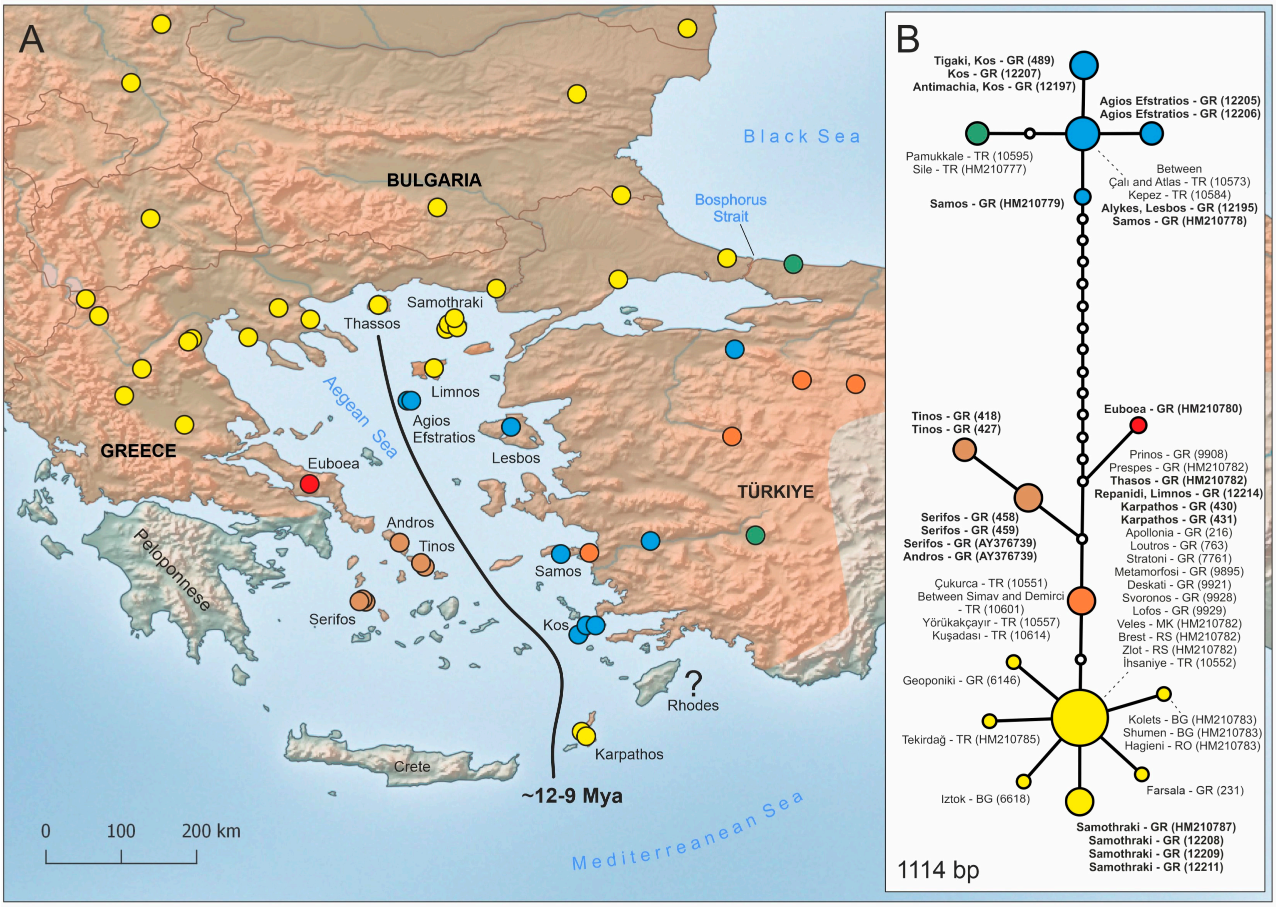

Figure 2. The distribution of mitochondrial haplotypes of *Dolichophis caspius* in the Aegean area (**A**) and the haplotype network (**B**) defining haplogroups by colouration. The names in bold of the haplotype network highlight the Aegean islands. Refer to Table 1 for locality details and sample codes used in the network. The line in the middle of the Aegean Sea represents the early formation of the Aegean barrier. The question mark indicates a place of unclear or possible occurrence of the species on Rhodes Island. The distribution range of the species in the studied area is highlighted in orange. The map was generated using QGIS 3.28, available at https://qgis.org/.

The mitochondrial clade representing populations of *D. jugularis* exhibited no significant phylogenetic substructure. Our sampling encompassed populations from continental western Anatolia (the restricted type locality of the species) and two islands, Kos and Rhodes (the island with the type locality of *D. j. zinneri*). In examining intraspecific mitochondrial diversity among local populations from both the continent and islands, we identified low distances (but see Rhodes and nuclear DNA data) and only at the haplotype level.

Although the number of mitochondrial haplotypes differs between the studied *D. caspius* and *D. jugularis*, with 16 haplotypes and seven haplotypes, respectively, the haplotype variability (*Hd*) has similar values, i.e., 0.88 and 0.86, respectively. However, it is essential to acknowledge that *D. caspius* is a widely distributed species in the studied area, with a higher number of analysed samples, which could potentially impact the observed level of haplotypes. Nevertheless, within the dataset of *D. caspius*, we identified six mitochondrial haplogroups differentiated from each other by a range of two to 13 mutation steps (Figure 2). These haplogroups form a west-to-east division pattern, with the yellow haplogroup being widespread from central Greece to the European part of Türkiye, including islands such as Thassos, Samothraki, and surprisingly Karpathos. Two other groups, red and brown, were detected on the islands of the Cyclades and Euboea (Evvoia), also situated in the west part of the Aegean, while the remaining haplogroups (blue, green, orange) were observed in the east part of the Aegean and Anatolia. The two primary lineages of *D. caspius* converge with their haplotypes near the Bosporus strait, between Limnos and Agios Efstratios islands, as well as near Samos and Anatolia (Figure 2).

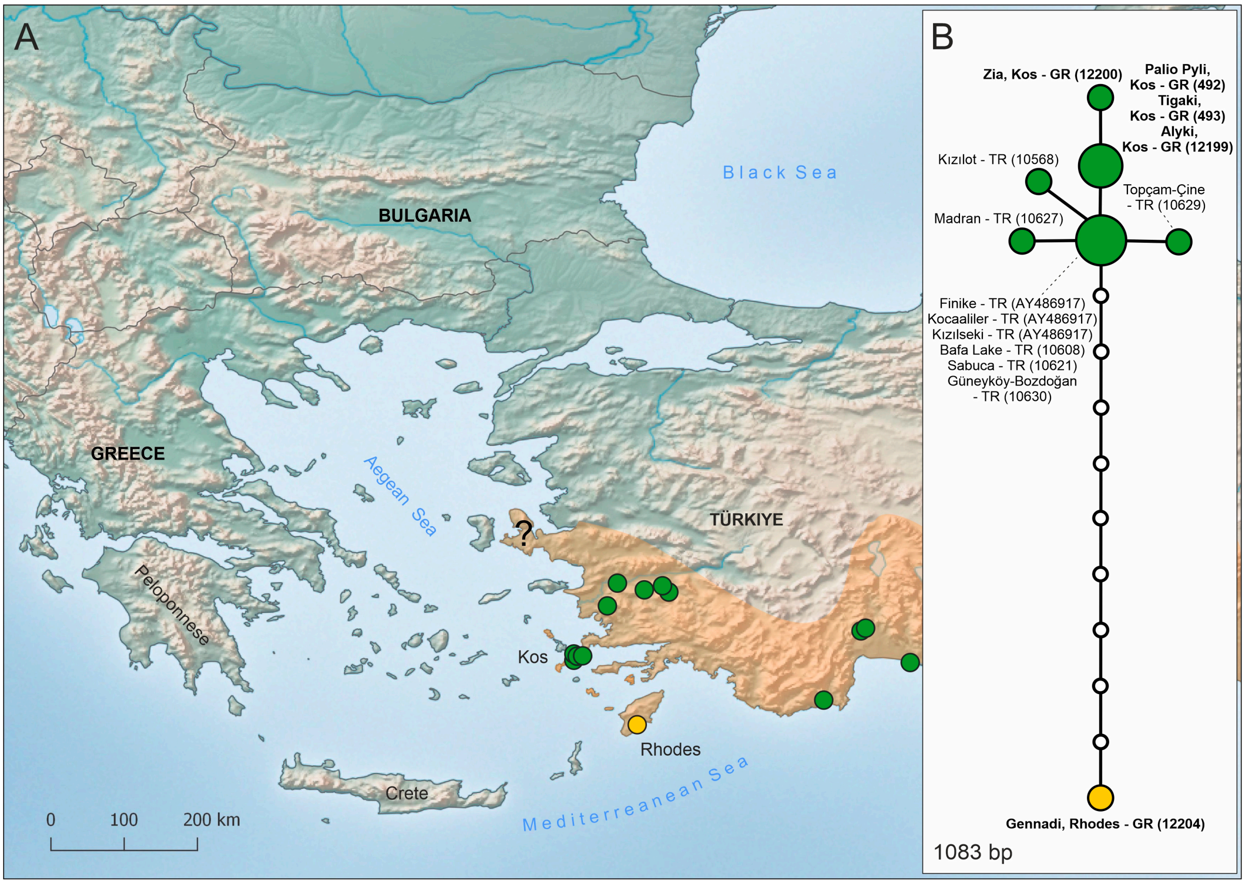

Figure 3. The distribution of mitochondrial haplotypes of *Dolichophis jugularis* in the Aegean area (**A**) and the haplotype network (**B**) defining haplogroups by colouration. The names in bold of the haplotype network highlight the Aegean islands. Refer to Table 1 for locality details and sample codes used in the network. The question mark indicates a place of possible occurrence of the species in the westernmost Anatolia [8]. The distribution range of the species in the studied area is highlighted in orange. The map was generated using QGIS 3.28, available at https://qgis.org/.

Conversely, the haplotype network of *D. jugularis* demonstrated a clear and straightforward division into green and yellow haplogroups, as defined here (Figure 3). Mitochondrial sequences were grouped into a green group from continental western and southern Anatolia and Kos Island (with a maximum of two mutation steps between them) and a yellow haplogroup that includes the population on Rhodes Island (representing *D. j. zinneri*), which was distanced by 10 to 12 mutation steps from other haplotypes of *D. jugularis* (Figure 3). Notably, populations from the green haplogroup represent the region defined as the type locality of *D. jugularis* (Figure 3).

3.2. Nuclear DNA Data

The network analysis of C-mos and BDNF identified four and five alleles, respectively. In C-mos, alleles corresponded to the different analysed species (*D. caspius* and *D. schmidti* alleles showed one and two mutation steps from *D. jugularis*, respectively), except *D. jugularis,* where a heterozygotic sequence from Kos Island (493) was identified (Figure 1C). Sample 12204, which represents the Rhodes population of *D. jugularis,* shares the main C-mos allele with populations of the species from Kos Island (Greece), mainland Türkiye, and even Jordan. Due to the absence of a *D. schmidti* BDNF sequence, we compared only *D. caspius* and *D. jugularis* using this marker. We identified three alleles for *D. caspius* (two of them unique to the species) and four alleles for *D. jugularis* (three unique to the species). The two species share one allele representing geographically close populations from Kos (allele 493a), Rhodes (12204), and western Anatolia (Kepez; 10584a), where both species have come in recent or possible historical contact. The Rhodes population is distanced from the mainland population from Kızılot, Antalya, Türkiye (allele 10568), by three mutation steps and by two from the allele 493b from Kos (Figure 1C). The same allele diversity was recorded in Rag1 (six alleles in two species), where *D. jugularis* individuals from Rhodes (12204) and Kos (493) were heterozygotic and individual from Kızılot (10568) and *D. caspius* from Kos (489) were homozygotic. Notably, alleles from Rhodes were genetically equally distant to *D. caspius* (three to four mutation steps) from Kos and to *D. jugularis* from the same island (Figure 1C).

4. Discussion

4.1. In Situ Evolution and Natural Dispersal

While snakes belonging to the genus *Dolichophis* are recognised as common and sizable reptiles that play significant ecological roles [31], scientific attention toward their biogeography and diversification remains relatively limited [10,11,13,20]. This is a surprise considering the taxonomic interest surrounding these snakes that were historically ranked among different genera [32]. Several taxonomic names within snakes belonging to the *Dolichophis* genus have been described [15,20,33,34], hinting at potential speciation events, yet they have not been thoroughly assessed using contemporary molecular taxonomic methods. This study aims to partially address this research gap in the Aegean area where two species form a contact zone.

Generally, the species-level phylogeography presented here does not bring surprising results compared to the paradigm of phylogeographic patterns and colonisations of the biota and snakes in the Aegean region [1,5]. For both studied *Dolichophis* species, diversification has occurred relatively recently, much after the initial formation of the Aegean Sea 9–12 Mya [1]. Specifically, the low values of the estimated intraspecific p-distances point to Pleistocene diversifications, as already discussed in Nagy et al. [10] for *D. caspius*. It is well known that the broader Aegean region is mostly inhabited by *D. caspius* (sister species to *D. schmidti*; [23]) with a different level of mitochondrial diversity [10]. Although we lack a well-supported biogeographic hypothesis, the origin of the species is expected to be in the Balkans [11] with subsequent colonisations and divergence through space and time. This species was able to colonise most of the current range in the southern Balkans and western Anatolia, including the Aegean islands and further to the east through the steppes of Eurasia [8]. Surprisingly, from the Balkan–Aegean perspective, the species is not present in the Peloponnese peninsula (Figure 2), which is not well explained in the literature but may be connected to high reptile competition in the peninsula or its specific ecological and topographic conditions. In any case, the diversification history of *D. caspius* is so recent that the Peloponnese was geologically separated from the remaining Balkan mainland when the species reached this specific sea barrier.

On the other hand, due to the distribution, phylogenetic relationship (sister species to *D. andreanus*; [23]), and genetic diversity, the ancestral area of *D. jugularis* is probably in Anatolia or the Middle East from where the species colonised the western areas, reaching the Aegean region on the western coast of Anatolia (the north-western border is probably the Büyük Menderes River; see details in Cattaneo [16]) and several Aegean islands. In this area, the species has contact and possible hybrid zones (see our nuclear evidence Figure 1C) with *D. caspius* that are so far understudied. Similar distribution patterns where Anatolian–Middle Eastern species reach the south-western coast of Anatolia and neighbouring islands are observed in other reptile genera such as *Blanus* Wagler, 1830; *Anatololacerta* Arnold, Arribas, Carranza, 2007; or *Hemorrhois* (Linnaeus, 1758) [8,35,36]. An interesting parallel example of colonisation and contact zone in the eastern Aegean can be observed between the mostly Balkan species *Zamenis situla* (Linnaeus, 1758) and the Anatolian–Middle Eastern *Z. hohenackeri* (Strauch, 1873) [37], although the former species is missing on local islands (but see the discussion regarding Rhodes in Cattaneo et al. [18]).

We can observe more details on the intraspecific level of genetic differentiation for both species. Nagy et al. [10] recognise two main mitochondrial lineages for *D. caspius* (west and east) that are separated by the Aegean Sea and Bosporus. Using a genomic approach, Mahtani-Williams et al. [11] recognised eight clusters, three of which can be associated with the Aegean region or the Balkans in general. Both studies conclude that this genetic diversity is probably a reflection of former glacial refugia. Our data support the previous conclusions, but detailed sampling on both species better highlights the phylogeographic structure related to the historical development of the Aegean region and island isolations. First, we recognised unique haplotypes in the west side of the Aegean Sea, in the Cyclades islands, and Euboea that were not found in other parts of the studied area. Additionally, our results revealed that *D. caspius* dispersed from the Balkan Peninsula to Anatolia at least twice, since we found a previously undiscovered mitochondrial haplogroup in Anatolia that is nested in the west lineage. This has come into secondary contact with the east lineage in west Türkiye and some of the adjoining islands. We can assume that these two mitochondrial clades introgress, which would explain the population structure patterns based on the genome-wide SNPs of Mahtani-Williams et al. [11]. This pattern of multiple dispersals can be explained by the wider and faster colonisation to the east that was conducted by the most widespread lineage containing red, brown, orange, and yellow haplogroups (Figure 2). On the other hand, the lineage of *D. caspius* including blue and green haplotypes (Figure 2) colonised areas in western Anatolia and eastern Aegean islands, where it displays certain levels of local genetic diversity (see unique haplotypes for Kos or Agios Efstratios islands; Figure 2).

Unique mitochondrial haplotypes from islands (Kos, Rhodes) were also found in *D. jugularis*; however, the overall genetic variation in this species is lower in the Aegean region (but see *D. j. zinneri* below). Nevertheless, these data represent the first broader genetic insight into the phylogeography of *D. jugularis*. For further conclusions, more genetic data from the rest of the species range are needed.

4.2. Human Mediated Introductions

Unexpectedly, our genetic analysis revealed an intriguing result for the *D. caspius* populations from Karpathos Island. Mitochondrial sequences from this island exhibited a close genetic affinity with the haplotype observed in the continental Balkans, Thassos, and Limnos islands. This genetic correspondence suggests that the Karpathos population might have an artificial origin, possibly resulting from historical introductions. This scenario mirrors past instances of human-mediated and accidental amphibian and reptile introductions in the Aegean region and the broader Mediterranean area, a phenomenon documented in various snake species such as *Eryx jaculus* (Linnaeus, 1758), *Telescopus fallax* Fleischmann, 1831, *Hierophis viridiflavus* (Lacépède, 1789) (under the name *Coluber gemonensis gyaronensis* Mertens, 1968), and *Zamenis situla* (Linnaeus, 1758) [38–41]. Prior to the utilisation of molecular data, such introductions, particularly concerning snakes, remained largely unnoticed and sometimes led to wrong taxonomic conclusions [42] or uncertain biogeographic hypotheses. Exceptions to this trend are introductions that have been historically well documented [43].

The genus *Dolichophis* partly exemplifies a well-documented case of introductions. In 1960, populations of this genus were deliberately introduced to Cyprus from continental Anatolia to mitigate rodent populations on the island [44,45]. Although contentious, such introductions represent contemporary approaches to addressing agricultural pest-related issues. Similarly, populations of *D. caspius* in the Dalmatian islands of Croatia are believed to have an introduced origin [46], an inference substantiated by genomic investigations revealing a human-mediated origin [11]. These instances underscore the complex interplay between human intervention and wildlife distribution, especially in island ecosystems characterised by high levels of endemism, such as Cyprus and Karpathos. This is why we must carefully evaluate the local biota, at least by population genotyping. The phylogeographic pattern of other populations does not suggest further historical introductions, although populations of *D. caspius* in Anatolia display certain mixed patterns of mitochondrial data that can be considered as natural dispersion in several waves or a combination with human-mediated introduction (see green haplotype at the Black Sea coast; Figure 2).

4.3. Dolichophis jugularis and Island Endemism

Rhodes Island, despite its size and isolation from the Anatolian mainland, is not generally regarded as a sole source of endemism. While the island does harbour certain locally endemic herpetofauna [e.g., *Pelophylax cerigensis* (Beerli, Hotz, Tunner, Heppich, Uzzell, 1994)], these species are not exclusive to Rhodes and in some cases, they have limited or mixed patterns of distribution there. These endemics have centres of distribution in closely neighbouring islands within the Dodecanese, notably Karpathos. Due to its prolonged isolation within the Aegean region [47], Karpathos is considered a notable example of local endemism, hosting species such as *Lyciasalamandra helverseni* (Pieper, 1963) or *Mediodactylus oertzeni* (Boettger, 1888) found throughout the whole island.

Consequently, the geographic distance of Rhodes from the mainland (20 km), its strong chorotype affinity to Anatolian herpetofauna, and the inherent complexities in diagnosis and taxonomy have historically led to uncertainties regarding two names related to the genus *Dolichophis* originating in Rhodes: *Coluber caspius eiselti* Zinner, 1972 (specimens NHMW 18618:1, 3; Figures 4 and 5 in this study), and *D. jugularis zinneri* Cattaneo, 2012 (holotype ZFMK 92945; see [15] (Figures 1 and 2) and Figure 6 in this study). The former is considered invalid due to the unpublished status of the Zinner thesis (see the discussion in [14,19,20]), while the latter's validity is in doubt due to the aforementioned reasons and the lack of available genetic data from the island. The taxonomic classifications by Zinner [20] and Cattaneo [15] primarily relied on morphological characteristics that can be somewhat plastic, particularly concerning patterns, colouration, and observed behavioural differences in Rhodes populations in comparison to others. *Dolichophis j. zinneri* stands out for several distinct morphological features compared to its counterparts. Specifically, it exhibits a wider head with a shorter and pointed shape and consistently displays yellow colouration in certain areas such as the supralabials, neck sides, and throat, contrasting with the red salmon hues observed in *D. j. jugularis*. Additionally, according to Cattaneo [15], this subspecies displays less intense dorsal dark colouration, which tends to darken anterior–posteriorly. Cattaneo [15] also mentions a high proportion of individuals displaying a yellow-bellied phenotype, a trait less common in the nominate subspecies, which typically exhibits red-bellied phenotypes with a lower count of ventral scales (usually fewer than 200). Furthermore, the ventral colouration—either red or yellow but never black—serves as a distinguishing characteristic between *D. j. zinneri* and other populations. These characteristics can be observed in specimens known under the name *C. caspius eiselti* (NHMW 18618:1, 3; Figures 4 and 5). According to Cattaneo [15,16], *D. j. zinneri* is reported to be present on the islands of Halki, Rhodes, Tilos, and Symi, which needs to be confirmed by further genetic screening.

Despite our sequencing efforts being limited to just one specimen from Rhodes, our study provides the first genetic evidence to address this taxonomic challenge. The mitochondrial distinction (although not strong; Figures 1 and 2) and the character of networks of *D. jugularis* from Rhodes, particularly within nuclear DNA markers, suggest a genetic affinity to the island. The allele difference (alongside the geographic isolation) between the Kızılot population of *D. jugularis* in western Türkiye (representing a population of nominotypical subspecies; Figure 3) and Rhodes Island indicates that the subspecies status of *D. j. zinneri* is likely justified. This suggests independent evolution within Rhodes rather than recent colonisation. Our findings may thus help to clarify more than 50 years of uncertainty regarding the distinct phenotype of *D. jugularis* from Rhodes. Based on the information provided in our results, as well as considering morphology, and noting that a name for the Rhodes population already exists, the designation *D. j. zinneri* can be applied to differentiate these snakes. Nevertheless, we strongly advocate for additional research and more comprehensive data collection across the Dodecanese to obtain further evidence supporting or refuting subspecies status [48].

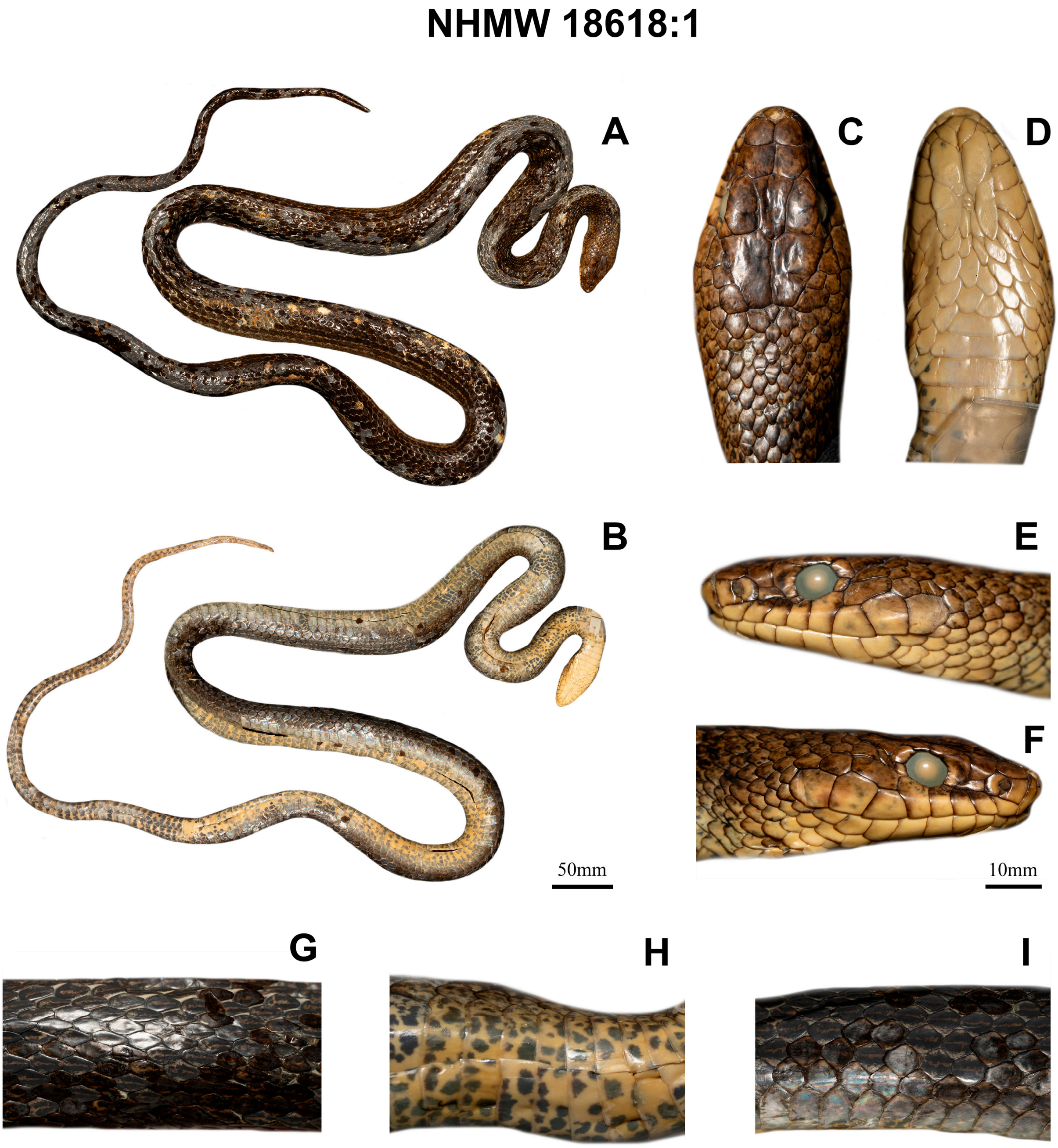

Figure 4. The adult specimen NHMW 18618:1 (holotype; [20]) representing *Coluber caspius eiselti* Zinner, 1972 from Lindos, Rhodes, Greece. (**A**,**B**) Dorsal and ventral view of the body. (**C**–**F**) Dorsal, ventral, and lateral view of the head. (**G**–**I**) Dorsal and ventral view of the body's scalation, colouration, and pattern. Photos: Daniel Jablonski.

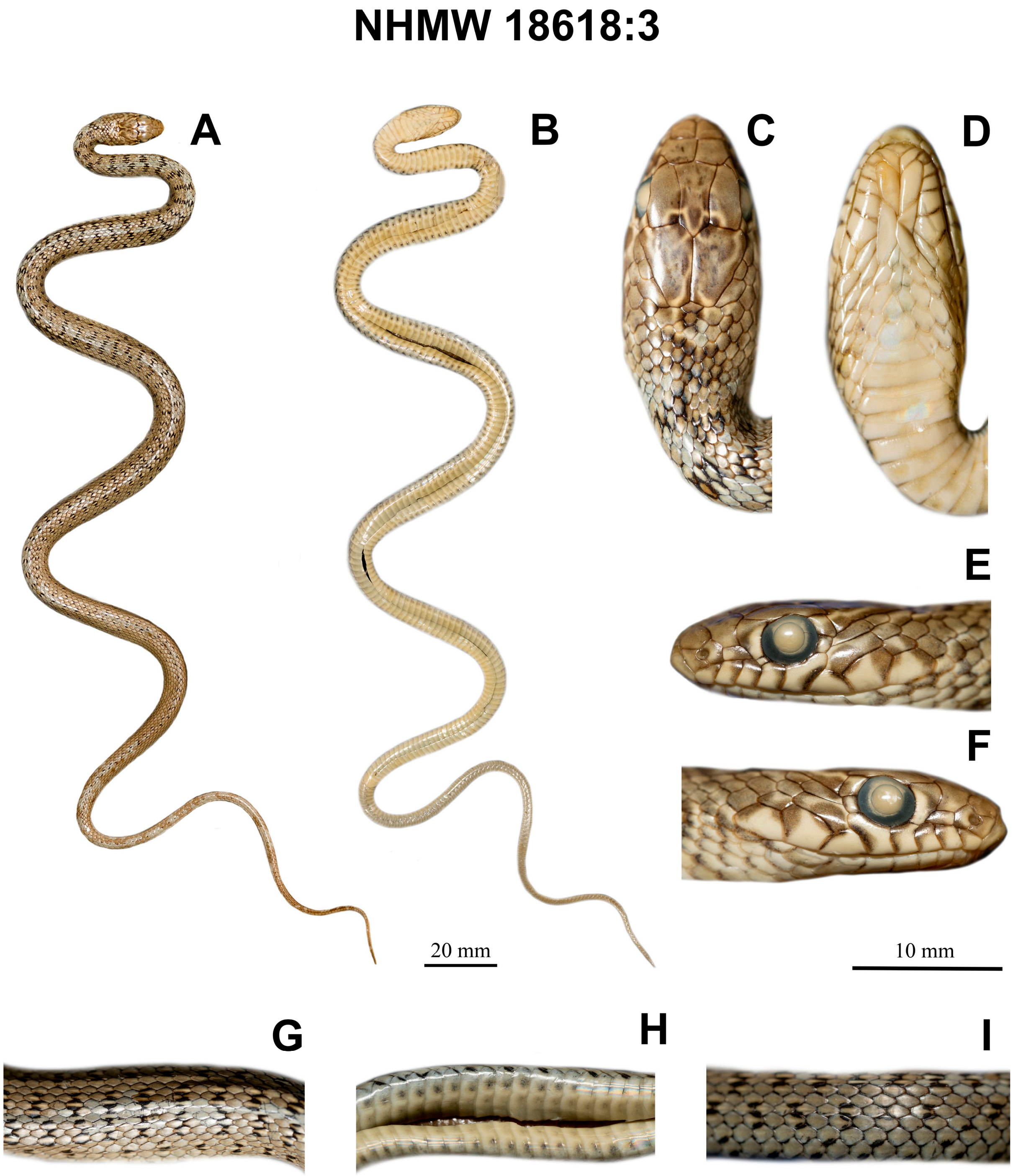

Figure 5. The juvenile specimen NHMW 18618:3 (paratype; [20]) representing *eiselti* Zinner, 1972 from Lindos, Rhodes, Greece. (**A**,**B**) Dorsal and ventral view of the body. (**C**–**F**) Dorsal, ventral, and lateral view of the head. (**G**–**I**) Dorsal and ventral view of the body's scalation, colouration, and pattern. Photos: Daniel Jablonski.

Nevertheless, the question regarding the similarity in colour and pattern of some Rhodes specimens resembling *D. caspius*, which led Zinner to describe the new subspecies under this name, remains open. Although we and existing literature do not anticipate the sympatric presence of both species on the island [14,16,17] as observed in Kos, we cannot exclude the possibility that both species coexisted on Rhodes in the past (Pleistocene) when the island was connected with the mainland due to the sea level being lower than it is today [47]. Hypotheses involving remnants of populations or overseas colonisation cannot be disregarded either. Such interactions could have resulted in historical hybridisations and the fixation of specific alleles leading to phenotypes resembling either species or another (see [14]). Also, the presence of shared BDNF alleles within Kos and the differentiation of this allele may support the presumption of the common evolution of both species.

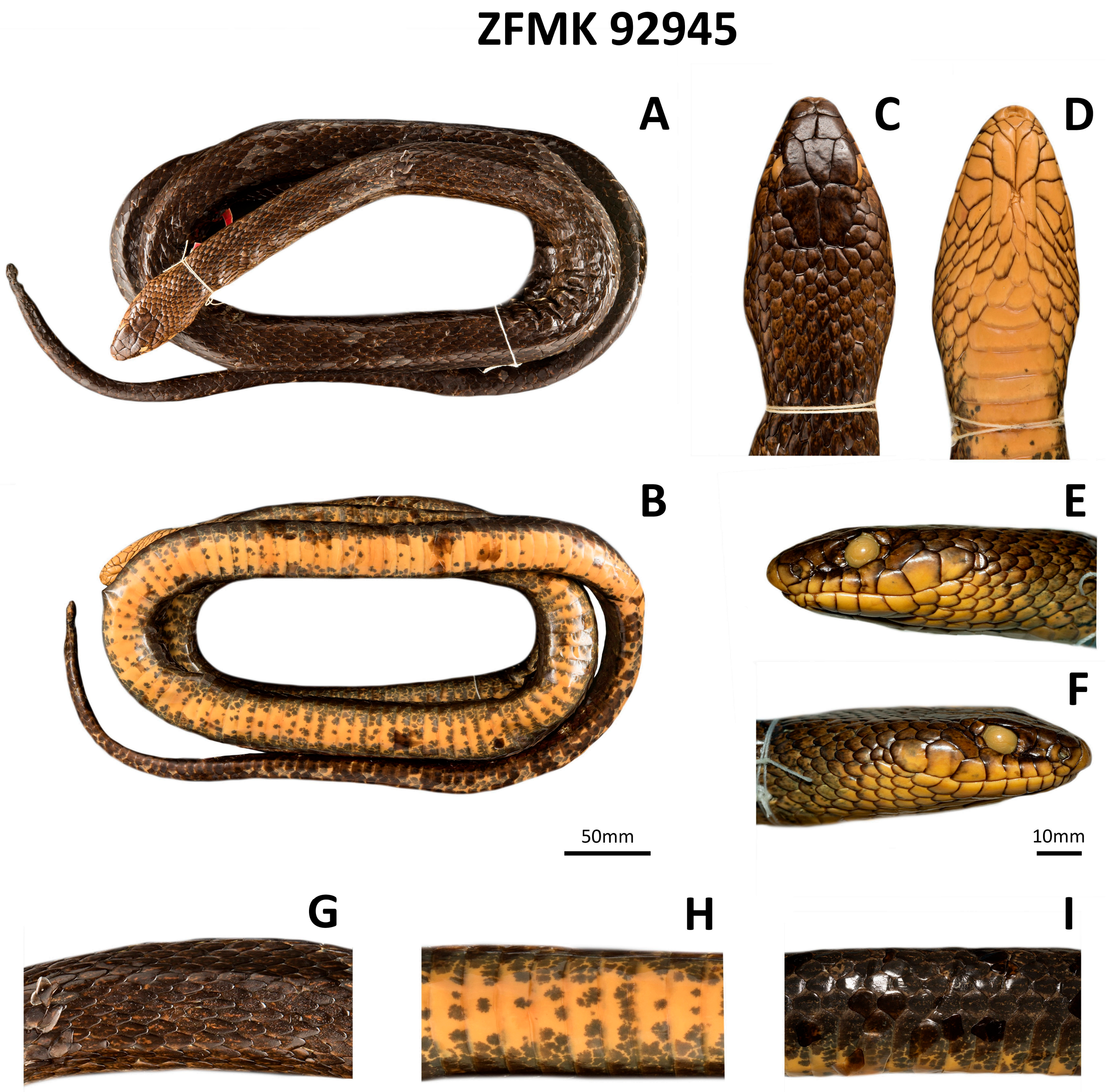

Figure 6. The adult specimen ZFMK 92945 (holotype; [15]) from the type collection of *Dolichophis jugularis zinneri* Cattaneo, 2012. (**A**,**B**) Dorsal and ventral view of the body. (**C**–**F**) Dorsal, ventral, and lateral view of the head. (**G**–**I**) Dorsal and ventral view of the body's scalation, colouration, and pattern. Photos: Morris Flecks (**A**–**D**) and Daniel Jablonski (**E**–**I**).

However, the distinct phenotype differentiation of *D. jugularis* from Rhodes may also signify an environmentally related phenomenon in the Aegean region. Jablonski et al. [4] discuss that specific environmental and ecological conditions, combined with genetic components and selection, might underlie the high phenotype variability (mostly in colour and pattern) observed in island populations. The Aegean region presents several instances where morphologically based taxonomy does not align with genetic data, such as the Aegean *Natrix natrix schweizeri* Müller, 1932, or *Macrovipera schweizeri* (Werner,

1935) [4,49,50]. Additionally, Eastern Mediterranean reptile populations often exhibit exceptions to their common colouration and pattern (see [51–53]), a trend also observed in *Dolichophis* populations [22] (genotyped specimens ZMHRU 2012/50-51; Table 1 and Figure 3) from western Anatolia and the western Aegean [54].

Conclusively, while the Rhodes population appears genetically distinct, its morphological differentiation may also result from the specific ecological conditions on the island. However, without genomic data, we also cannot exclude the possibility of historical hybridisations. In this context, Kos Island and, generally, the Dodecanese represent a great, naturally isolated outdoor laboratory for exploring historical and recent hybridisations between both present *Dolichophis* species in the Aegean region and their ecological divergence. Extensive field studies, along with DNA barcoding and genomics, are imperative to unravel the evolutionary processes in *Dolichophis*, not solely in the Aegean region but throughout its range.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d16030184/s1: Table S1. Primers and PCR conditions used in this study; primer orientation (OR): F = forward, R = reverse [55–59].

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