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Preliminary genetic characterisation of Southern Smooth Snake *Coronella girondica* (Serpentes, Colubridae) populations in Italy, with some considerations on their alpine distribution

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Abstract. The Southern smooth snake, *Coronella girondica*, is a small-sized colubrid found in Northwest Africa and Southwest Europe. Mitochondrial DNA-based studies showed that the species can be split into five clades: two from Northwest Africa (one Moroccan and one Tunisian-Algerian) and three from Europe (one in the south-west of the Iberian Peninsula, one in the south-east of Spain and one in the rest of the European range). With regards to Italy, to date, only two samples have been analysed both from the Province of Pisa, Tuscany, pointing at that fact that genetic characterisation of Italian populations is still lacking. Accordingly, we have increased the sampling coverage with 19 new samples from northern and central regions of Italy, including two populations, apparently disconnected from the rest of the known range, and analysed their phylogenetic relationships using a portion of the mitochondrial cytochrome b gene. Our results confirm the general phylogenetic arrangement detected in previous studies; specifically for Italian populations, no variability emerged from the Apennine populations, and a slight differentiation could be shown for the Alpine and subalpine ones. This pattern can be explained assuming past spread and recent isolation of *C. girondica* relict populations in the Alpine region, likely during the Last Glacial Maximum. Later, during the Holocene, the Italian Alps and the Po Plain went through various climatic variations and high anthropization which may have influenced *C. girondica* distribution through expansion and contraction processes.

Keywords. *Coronella girondica*, Italy, distribution, relict populations.

INTRODUCTION

The Southern Smooth Snake *Coronella girondica* (Daudin, 1803) is a small-sized Colubridae with a Mediterranean chorotype (West-Mediterranean, Sindaco et al., 2013). The species is present in Northwest Africa (north-central Morocco, northern Algeria and north-western Tunisia) and Southwest Europe (Portugal, Spain, Southern France and Peninsular Italy), where it usually lives in dry and stony Mediterranean scrub habitats (Sindaco et al., 2013; Geniez, 2018; Di Nicola et al., 2021).

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With regards to Italy, the secretive and mainly crepuscular habits of the species (Ferri and Morimando, 2004; Razzetti and Bernini, 2011) hampered the full description of its distribution framework for a long time (Razzetti and Bonini, 2006) and for some areas its presence has even only been recently confirmed (Razzetti et al., 2000; Capula et al., 2010; Rugiero et al., 2018; Di Nicola et al., 2020; Ferri and Soccini, 2020; Iversen et al., 2020).

The species, originally described by Daudin (1803) as *Coluber girondicus*, is currently considered monotypic (Razzetti and Bernini, 2011; Santos and Pleguezuelos, 2015; Sindaco and Razzetti, 2021). Subsequently, *Rhinechis amaliae* (Boettger, 1881) was described from Moroccan specimens, on the basis of a single morphological character relating to the rostral zone (Santos and Pleguezuelos, 2015). However, further morphological investigations did not provide support for the validity of this taxon (Saint Girons, 1956; Domergue, 1962; Santos and Pleguezuelos, 2003). More recently, Santos et al. (2012a) performed a molecular study across the distribution range of the whole species using analysis of mitochondrial genes and highlighted the presence of three major clades: one from Northwest Africa, one from the southeast of Spain and the latter occurring in the rest part of the European range. According to their calibration, the divergence among the clades occurred around 1.4-2.0 Ma, roughly coinciding with the Plio-Pleistocene transition. Within two out of the three major clades, further differentiation was detected into five clades (see Santos et al., 2012b): two from Northwest Africa (one Moroccan and one Tunisian-Algerian) and three from Europe (one in the south-west of the Iberian Peninsula, one in the south-east of Spain and one in the rest of Europe).

In Italy, *C. girondica* is distributed in the north-western part of the country and extends south to the Gargano peninsula (Northern Apulia) and southern Lazio, including the region of Molise (Capula et al., 2010; Razzetti and Bernini, 2011; Rugiero et al., 2018; Di Nicola et al., 2021). Recently, two apparently isolated populations have been found in western Lombardy (Di Nicola et al., 2020) and around Lake Garda, in an area that is transitional between the Trentino-Alto Adige, Veneto and Lombardy regions (Ferri and Soccini, 2020; Iversen et al., 2020). These isolated populations are out of the geographical range of the other populations by around 70 km east and 160 km north (Fig. 1, 2).

With regards to genetic characterisation of the species in Italy, only two samples have been analysed, both from the province of Pisa (GenBank accession numbers JQ837570 and JQ837571). The present work therefore describes, for the first time, the intraspecific relationships between Italian populations of *C. girondica* including some recently confirmed observations from Northern Italy (Di Nicola et al., 2020; Ferri and Soccini, 2020; Iversen et al., 2020).

MATERIALS AND METHODS

Given the lack of adequate sample coverage within the Italian range of the species, a total of 19 new samples were collected from seven regions of Northern and Central Italy (Table 1; Fig. 1), including five samples from the isolated populations, respectively western Lombardy (n = 1) and Trentino-Alto Adige (n = 4). Small tissue fragments (tail tips, pieces of ventral scales) were removed and preserved in ethanol 96% from individuals that were found dead (most of the samples) or live specimens. For live specimens, a non-invasive scale clipping of ventral scales was performed. *Coronella girondica* was sampled by nocturnal active search only at two separate localities of Arco (Iversen et al., 2020) and Somma Lombardo (Di Nicola et al., 2020), while in other cases samples were recovered from dead specimen as roadkills, domestic cats or human persecution (Table 1).

In order to infer the phylogenetic relationships between Italian samples and other populations, we selected the mitochondrial DNA cytochrome b (cyt b) gene, a marker often used to infer intra-specific diversity on many vertebrates and invertebrates species, including snakes (Carranza et al., 2006; Mezzasalma et al., 2015; Faraone et al., 2020b). Approximately 20 mg of tissue was used to extract total DNA as described in Tagliavia et al. (2016). Genomic DNA was used as a template for PCR amplification with primers CB1(F) (5'CCATCCAACATCTCAGCATGATGAAA3') and CB2(R) (5'CCCTCAGAATGATATTTGTCCTCA3') (Carranza et al., 1999). DNA bands of the expected size (~300 bps) were obtained and then sequenced with the primer CB1(F) (BMR Genomics, Padua, Italy).

The resulting sequences were each around 255 nucleotides long and were analysed and manually proof-read with the DNA sequencing software CHROMAS v. 2.6.6 (Technelysium Pty. Ltd. 1998, Queensland, Australia). The coding gene fragments of cyt b were translated into amino acids to assess the lack of premature stop codons. Later, using CLUSTAL W (Larkin et al., 2007) with default parameters, the sequences from Italian samples generated in this study were aligned with homologous sequence downloaded from GenBank (Carranza et al., 2004; Santos et al., 2008, 2012a; Carvalho et al., 2017). Four species belonging to Colubridae and Psammophiidae families were used as outgroups (Carranza et al., 2006; Santos et al., 2012a; Faraone et al., 2020a, b).

Table 1. Italian samples and observation details of *Coronella girondica* used in the present study. The numbers and letters reported respectively in the first and second column are referred to localities shown in Fig. 1 and Fig. 2. The haplotype code is shown in brackets after the GenBank accession number. Samples marked with an asterisk were previously published by Santos et al. (2012a).

Fig. 1	Fig. 2	Year	Locality	N	E	Observer/Reference	Accession number
1	C	2019	Arco, Trentino Alto Adige	45.9238°	10.9433°	Iversen et al., 2020	OK573460 (H2)
2	C	2020	Arco, Trentino Alto Adige	45.9238°	10.9433°	Iversen et al., 2020	OK573463 (H2)
3	C	2020	Arco, Trentino Alto Adige	45.9238°	10.9433°	Iversen et al., 2020	OK573462 (H2)
4	C	2021	Arco, Trentino Alto Adige	45.9238°	10.9433°	Iversen et al., 2020	OK573461 (H2)
5	G	2020	Somma Lombardo, Lombardy	45.6713°	8.6833°	Di Nicola et al., 2020	OK573464 (H2)
6		2020	Cassinelle, Piedmont	44.5760°	8.5616°	Cavanna S. pers. obs.	OK573465 (H3)
7		2020	Isola del Cantone, Liguria	44.6432°	8.9664°	De Cresi U. pers. obs.	OK573473 (H1)
8		2018	Albenga, Liguria	44.0970°	8.2129°	Graglia M. pers. obs.	OK573469 (H1)
9		2018	Albenga, Liguria	44.0970°	8.2129°	Graglia M. pers. obs.	OK573468 (H1)
10		2019	Albenga, Liguria	44.0970°	8.2129°	Graglia M. pers. obs.	OK573467 (H1)
11		2020	Peagna, Liguria	44.0989°	8.2013°	Graglia M. pers. obs.	OK573472 (H1)
12		2020	Peagna, Liguria	44.0989°	8.2013°	Graglia M. pers. obs.	OK573471 (H1)
13		2020	Peagna, Liguria	44.0989°	8.2013°	Graglia M. pers. obs.	OK573470 (H1)
14		2020	Aurigo, Liguria	43.9953°	7.9209°	Fecchio L. pers. obs.	OK573466 (H1)
15		2021	Vigolzone, Emilia Romagna	44.9110°	9.6879°	Gereschi V., Mazzotta M. pers. obs.	OK573478 (H4)
16		2020	Foreste casentinesi, Emilia Romagna	43.8874°	11.8939°	Molinari G. pers. obs.	OK573475 (H1)
17		2021	Foreste casentinesi, Emilia Romagna	43.8874°	11.8939°	Molinari G. pers. obs.	OK573476 (H1)
18		N/A	S. Giuliano Terme, Tuscany*	43.7579°	10.4434°	Santos et al., 2012a	JQ837570 (H1)
19		N/A	S. Giuliano Terme, Tuscany*	43.7579°	10.4434°	Santos et al., 2012a	JQ837571 (H1)
20		2020	Piombino, Tuscany	42.9268°	10.5310°	Banchi R. pers. obs.	OK573474 (H1)
21		2020	Capestrano, Abruzzo	42.2867°	13,7942°	D'Amico M. pers. obs.	OK573477 (H1)
	A	2011	Rivoli Veronese, Veneto	45.5872°	10.8215°	Campagnari M. pers. obs.	
	A	2013	Caprino Veronese, Veneto	45.5898°	10.8215°	Campagnari M. pers. obs.	
	B	2020	Avio, Trentino Alto Adige	45.7383°	10.9433°	Secchi M. pers. obs.	
	D	2019	Pietramurata, Trentino Alto Adige	46.0268°	10.9420°	Iversen et al., 2020	
	E	2020	Limone sul Garda, Lombardy	45.8108°	10.7866°	Di Nicola et al., 2020	
	F	2020	Toscolano Maderno, Lombardy	45.6666°	10.6166°	Ferri & Soccini, 2020	
	H	2021	Sostegno, Piedmont	45.6658°	8.2852°	Zonari A. pers. obs.	
	I	2015	Zubiena, Piedmont	45.4833°	8.0333°	Ciracì A. pers. obs.	

The phylogenetic analysis was performed with Maximum Likelihood (ML) under the Akaike Information Criterion using the "Smart Model Selection" (SMS) (Lefort et al., 2017), implemented in PHYML v. 3 (Guindon et al., 2010). Jukes-Cantor (JC) (Jukes and Cantor, 1969) was the most appropriate evolutionary model (-Log likelihood value 1283.58), with a 0.32 gamma estimate of invariable sites and a 1.00 discrete approximation of the gamma distribution. The same model was obtained by using both all available sequences and by previously collapsing the sequences into haplotypes. Node support was estimated by bootstrap (Felsenstein, 1985) with 1,000 replicates and the MEGA X software (Tamura et al., 2021) was used to implement the ML tree. The unrooted minimum spanning network were obtained using the median-joining algorithm (Bandelt et al., 1999) implemented in PopART (http://popart.otago.ac.nz/) (Leigh and Bryant, 2015).

With the aim of consolidating current knowledge of *C. girondica* distribution in Northern Italy, bibliographical data from Razzetti and Bonini (2006) were recorded. Furthermore, unpublished observations from the northern Po River area were collected between 2011 and 2021 from authors' field observations and online records. All observations that were not reported by Razzetti and Bonini (2006) were considered new and, subsequently, compared with previous unconfirmed findings. In addition, Citizen Science (see Haklay et al., 2021) was also critical to the generation of distributional datasets through collaborative efforts between herpethologists around Italy and users of a Facebook group managed by two of the authors (MRDN and FPF) "Identificazione

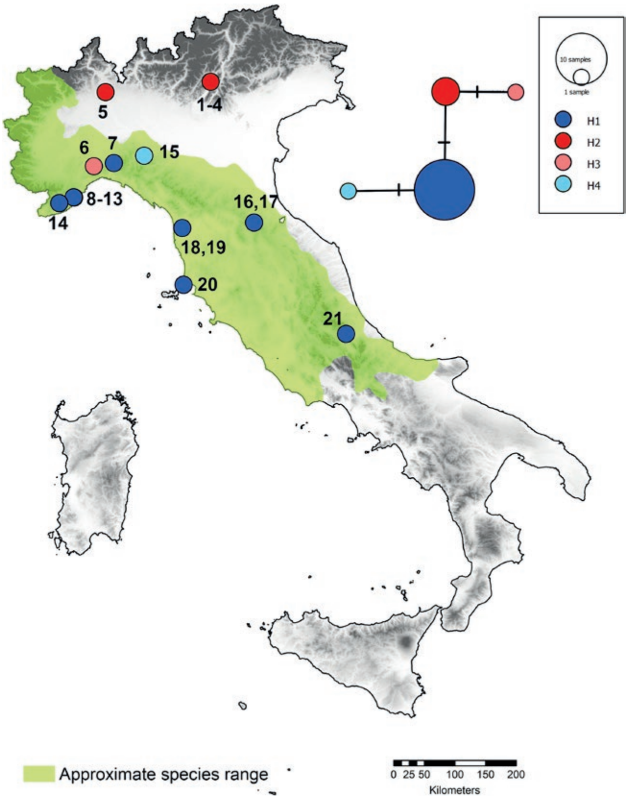

Fig. 1. Minumum spanning network based on the *Coronella girondica* cytochrome b fragment in Italy, and geographic distribution of the haplotypes. Circle sizes are proportional to haplotype frequency, and each black bars represents a mutational step. Samples are numbered as in Table 1. The green area in the map represents the approximate range of *C. girondica* in Italy.

Anfibi e Rettili". The users of the Facebook group provided locations for known European amphibians and reptiles and provided further distributional sites and samples for *C. girondica* found dead in various Italian regions. For each observation recorded through social networks, the authors meticulously checked the original files, the species identification and the location provided by the users through field survey of the coordinates. The new observations were mapped using Adobe Photoshop CC (©1990-2018 Adobe Systems incorporated, Release 19.1.5), together with those available from recent literature (Razzetti and Bonini, 2006; Di Nicola et al., 2020; Iversen et al., 2020; Ferri and Soccini, 2020; Di Nicola et al., 2021).

RESULTS

Overall, 94 sequences of 255 bp total length were analysed including the outgroups and the results confirm the same overall phylogenetic arrangement previously shown by Santos et al. (2012a) with five major clades (Fig. 3). All the 21 Italian samples (GenBank accession numbers JQ837570, JQ837571 and OK573460-78) fall within 'Clade 5' (sensu Santos et al., 2012a) which also includes sequences from France and most of Iberian Peninsula (Fig. 3). However, the monophily of 'Clade 5' is not adequately supported (bootstrap = 65%) on the basis of the cyt b fragment analysed here and it is considered as an haplogroup.

We found four cyt b haplotypes amongst the Italian *C. girondica* populations, differing by 1-2 mutation steps (Fig. 1). Most of the samples (n: 14) shared the same haplotype (H1), which is also shared with samples from Spain (JQ837574, JQ837587, JQ837589, JQ837607, JQ837610, JQ837635) and Portugal (JQ837569, JQ837582, JQ837595, JQ837634) (Fig. 3). In contrast, the sample from Emilia Romagna (OK573478) share the haplotype H4 with a sample from Ciudad Real, Spain (JQ837591). The following two unpublished haplotypes were detected among the northernmost Italian samples: H2 shared by all the five samples of the recently confirmed separated localities in western Lombardy (OK573464) and Trentino-Alto Adige (OK573460-63) and H3 from southern Piedmont (OK573465). H2 and H3 were shown to belong to the same haplogroup (Figs. 1, 3).

With the exclusion of the observations recently reported in the literature (Iversen et al., 2020; Di Nicola et al., 2020; Ferri and Soccini, 2020), three new localities (B, I, H) that were not recently confirmed (Razzetti and Bonini, 2006) have been registered, see Table 1 and Fig. 2 for details. Observation B resulted from a live adult snake observed on a low wall near Avio (province of Trento, Trentino-Alto Adige) just east of Lake Garda, as part of a cluster which includes observations recently published by Iversen et al. (2020) and Ferri and Soccini (2020) (Table 1, Fig. 2). Observations I and H corresponded to a live and a road-killed snake respectively and were both recorded in the Biella province (Piedmont) falling within the geographic range already known in the north-western regions of the Alps (Razzetti and Bonini, 2006) and the point G, a separated locality recently reported in Lombardy by Di Nicola et al. (2020) (Fig. 2).

DISCUSSION

The results presented here confirm a low genetic diversity for *C. girondica* within the cluster 'Clade 5', as shown by Santos et al. (2012a). For this haplogroup, rapid expansion process from south to north has been hypothesised to be likely derived from climatic warming events and followed by a bottleneck effect (Santos et al., 2012a). This pattern of recent expansion from the Iberian Penin-

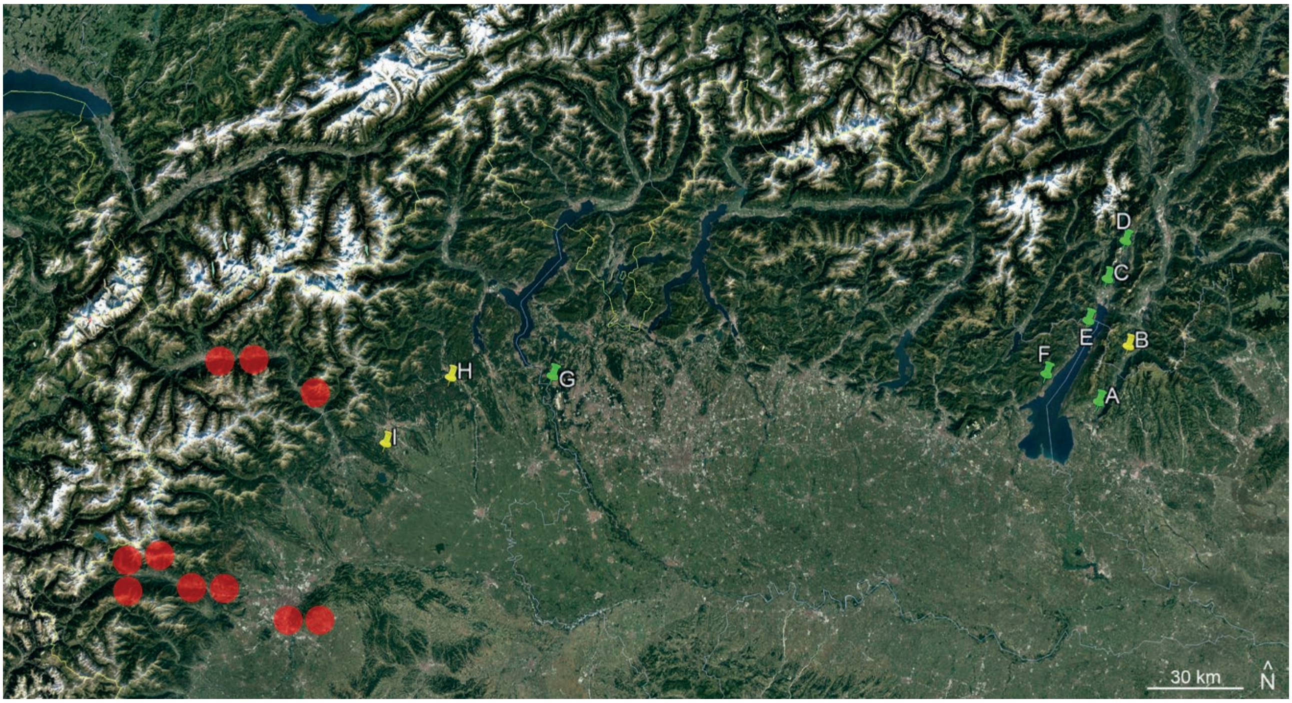

Fig. 2. Distribution of *Coronella girondica* in the Italian Alps north of the Po river. Red circle are the areas reported up to Razzetti and Bonini (2006), green landmarks represent the observations published after 2006 (Di Nicola et al., 2020; Iversen et al., 2020; Ferri and Soccini, 2020), yellow landmarks are the unpublished findings. The details of each point are shown in Table 1.

sula towards the north-east has also been hypothesised for other snake species such as *Natrix maura* (Guicking et al., 2002).

The genetic structure that emerges from the Italian *C. girondica* samples shows a lack of mitochondrial variability in the Apennine cyt b samples and a slight differentiation in Alpine and subalpine samples (Fig. 1, 3), which highlight that the endemic haplotypes H2 and H3 belong to the same cluster. This mtDNA pattern partially matches with that from other species of the Italian herpetofauna (Canestrelli et al., 2007; Canestrelli and Nascetti, 2008; Salvi et al., 2013; Chiocchio et al., 2021), including the Barred grass snake *Natrix helvetica* (Schultze et al., 2019) and confirms the margin of the Northern Apennines as a suture area of the Italian Peninsula (Hewitt, 2011; Chiocchio et al., 2021). This is compatible with recent spreading and isolation of *C. girondica* in Northern Italy, likely during the last glacial maximums, which allowed a slight haplotype differentiation. Subsequently, the Italian Alps and the Po Plain went through various climatic fluctuations and extensive human impact during the Holocene (Colombaroli et al., 2010; Nussbaumer et al., 2011; Joannin et al., 2013). These factors may have modulated the distribution of *C. girondica* with expansion and contraction processes.

The H4 haplotype, found in a single Italian sample (OK573478) collected in Emilia Romagna (Table 1, Figs. 1, 3), is identical to JQ837591 from Ciudad Real, central Spain (Fig. 3). This observation is similar to other cases reported for *Natrix natrix* and *N. helvetica* (Kindler et al., 2017; Schultze et al., 2019, 2020) and could be indicative of human translocation events. However, this hypothesis still needs further investigation through testing additional markers.

Recent Italian findings of *C. girondica* north of the Po River can be grouped into two main clusters. A Northwestern Alpine cluster includes some Alpine valleys of Northern Piedmont and Aosta Valley, and it is apparently separated from both the neighbouring French and Italian populations (Razzetti and Bonini, 2006; Sillero et al., 2014). An Eastern Alpine cluster is located around Lake Garda, and falls within the territories of Lombardy, Trentino-Alto Adige and Veneto (Iversen et al., 2020; Di Nicola et al., 2020; Ferri and Soccini, 2020). The populations around Lake Garda have been reported from the literature with many detailed records (De Betta, 1857; Dalla Torre, 1912) and later attested only by a few museum specimens dated up to 1977 (see Iversen et al., 2020). Other more recent information has been reported by Lorenzi and Bruno (2006) which, however, do not

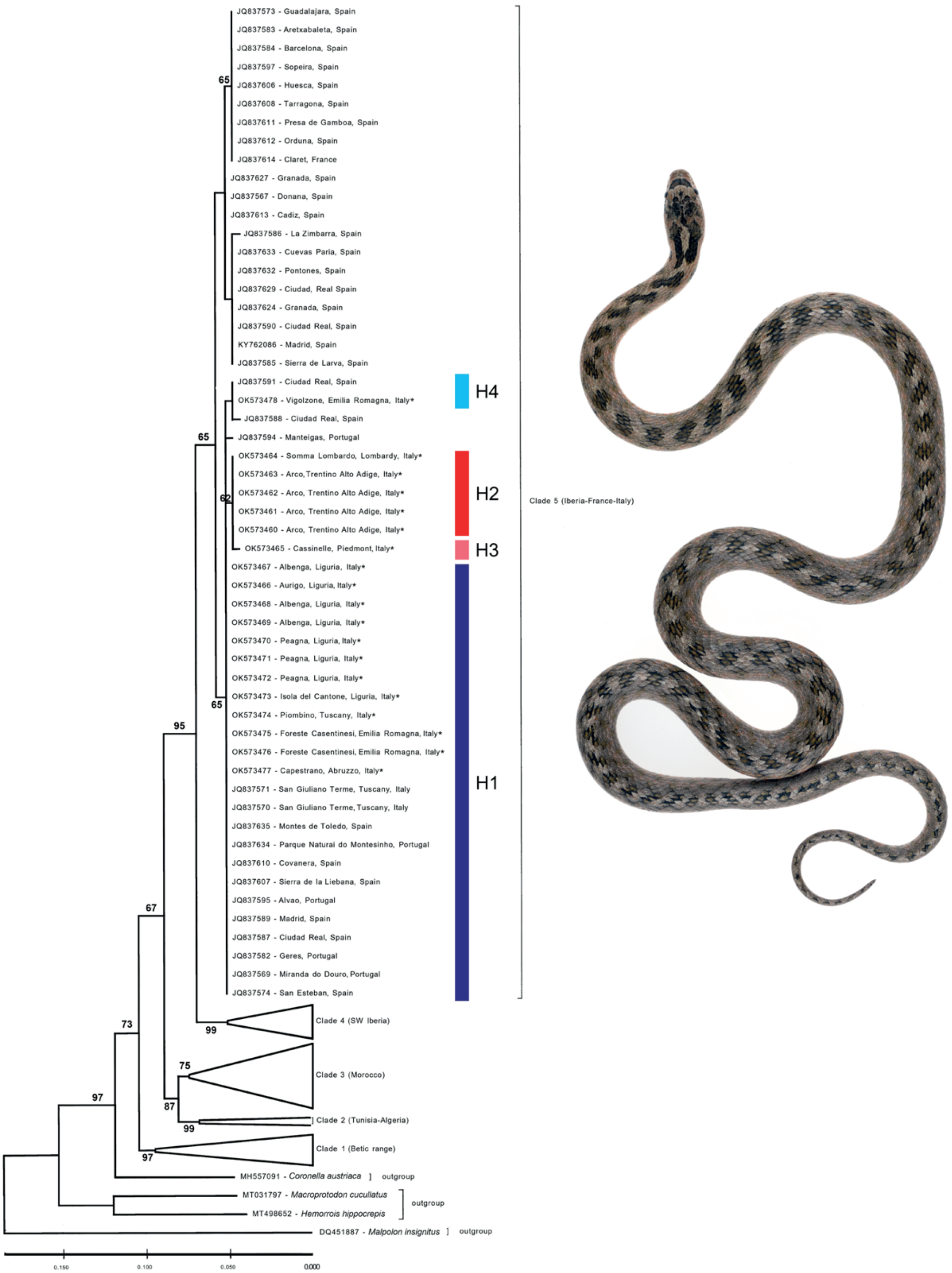

Fig. 3. Maximum Likelihood (ML) tree of *Coronella girondica* inferred from the mitochondrial cytochrome b gene, with a detail on the haplotypes of the 'Clade 5' (sensu Santos et al., 2012a) cluster. The haplotypes that occur in Italy are marked with the same colors used in Fig. 1. The numbers at nodes are ML bootstrap percentages. With the exception of the unpublished samples (see Table 1) all the others are taken from literature (Carranza et al., 2004, 2006; Santos et al., 2008, 2012a, b; Carvalho et al., 2017; Faraone et al., 2020a, b).

clearly contextualise their personal observations. The scarcity of findings during the twentieth century has led zoologists to consider this population close to extinction (Razzetti and Bonini, 2006) or probably extinct (Razzetti and Bernini, 2011). Recently, new observations led to the confirmation of the presence of *C. girondica* in Veneto (Novarini et al., 2017; Iversen et al., 2020), Trentino-Alto Adige (Iversen et al., 2020) and Lombardy (Di Nicola et al., 2020; Ferri and Soccini, 2020). New observations around Lake Garda largely confirmed previous records provided by Dalla Torre (1912), including the more recent point "B" (Fig. 2), which falls within a locality highlighted by the author between 1896 and 1900.

The observations "I" and "H" are located between the Northwestern cluster and point "G", recently reported in Lombardy by Di Nicola et al. (2020) (Table 1, Fig. 2). Point "I" is an unpublished locality, while "H" surprisingly confirms a site with a single specimen (MSNM RE 1439) preserved in the Civic Natural History Museum of Milan (see also Andreone and Sindaco, 2002), dated back to 1926. The geographic position of points "H" and "I" (Fig. 2) suggests that the North-western cluster and point "G" could be connected by small, scattered and low-density populations that may be detected by increasing the environmental monitoring of the species.

As indicated by Bombi et al. (2009), with the exclusion of the Maritime Alps and the Northern Apennines, Northern Italy has very low suitability values for *C. girondica* since only a few scattered sub-optimal patches in the area north of the Po River have been found. The results obtained here confirm this scenario, since *C. girondica* has a clearly fragmented distribution in the Alpine and sub-alpine areas. Furthermore, this species is mostly found in habitats within sub-Mediterranean climates, often characterised by xerophilous faunistic and vegetational communities which are very specific compared to other habitats and surrounding territories (La Greca, 1956; Gratani and Varone, 2003; Agabiti et al., 2005).

In conclusion, the results generated in this study suggest that the fragmented distribution of *C. girondica* north of the Po river can be mainly attributed to relict populations, based on the following points: (a) the finding of endemic haplotypes, compatible with a recent separation occurred during the last glacial events; (b) the few and scattered observations, highly localised in small patches with xeric Mediterranean features, suitable for the species; (c) current records largely match with historical records which, on the other hand, indicate few Alpine areas without recent confirmation; (d) the post-glacial history of Northern Italy is characterised by changes in ecosystems caused by climatic fluctuations and a strong human impact, and this could have caused the expansion and contraction of *C. girondica* as a thermophilic snake. This hypothesis will be further investigated through more extensive sampling in the field and the analysis of a greater number of loci in order to further detail the genetic structure of the Italian *C. girondica* populations. A greater fieldwork effort will be necessary, especially on the Western Alps, where our results could indicate a probably incomplete *C. girondica* distribution, which may be slightly less fragmented than previously thought.

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