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Research paper

Phylogenetic study of *Elona quimperiana* (Gastropoda, Elonidae): identification of a new haplogroup

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Abstract

Phylogenetic study of Elona quimperiana *(Gastropoda, Elonidae): identification of a new haplogroup.* The land snail *Elona quimperiana* is listed in annexes II and IV of the Europe Habitats Directive. In this study, phylogenetic relationships between populations of this protected species from its whole distribution range were reconstructed based on the sequencing of two mtDNA gene fragments (*COI* and *16S* rRNA) and one nDNA gene fragment (*ITS2*). Haplotype networks were also generated for the *COI* and *16S* rRNA gene fragments. Results yielded three main intraspecific phylogroups, here designated as the lineages Brittany-Spain, Basque, and Navarre-Rioja. This last lineage had not been identified previously. Genetic distances between the three main phylogroups suggest they could have originated by allopatric fragmentation before the Last Glacial Maximum, and then separately evolved in three refugia in the Iberian Peninsula. We here propose that each phylogroup is considered an Evolutionarily Significant Unit. Through extended sampling, we were able to delimit the geographic distribution of all three lineages. Our genetic results support post-glacial colonization of this species from the north-eastern Iberian Peninsula to Brittany in NW-France as proposed previously, and rule out its recent anthropic introduction.

Key words: *Elona quimperiana*, Haplotype network, Multilocus phylogeny, Barriers

Resumen

Estudio filogenético de Elona quimperiana *(Gastropoda, Elonidae): identificación de un nuevo haplogrupo.* El caracol terrestre *Elona quimperiana* está incluido en los anexos II y IV de la Directiva Hábitats de la Unión Europea. En este estudio, se reconstruyeron las relaciones filogenéticas entre las poblaciones de esta especie protegida de todo su rango de distribución a partir de la secuenciación de dos fragmentos de genes de ADNmt (*COI* y *ARNr 16S*) y un fragmento de gen de *ADNn* (*ITS2*). También se generaron redes de haplotipos para los fragmentos de genes *COI* y *ARNr 16S*. Los resultados indican la existencia de tres filogrupos intraespecíficos principales, que hemos designado como los linajes Bretaña-España, Vasco y Navarra-Rioja. Este último linaje no había sido identificado antes. Las distancias genéticas entre los tres filogrupos principales sugieren que podrían haberse originado por fragmentación alopátrica antes del último máximo glacial y que luego evolucionaron por separado en tres refugios en la península ibérica. En este estudio proponemos que cada filogrupo se considere una unidad evolutivamente significativa. Gracias a un muestreo extenso, pudimos delimitar la distribución geográfica de los tres linajes. Por último, nuestros resultados genéticos permiten confirmar la colonización postglacial de esta especie desde el noreste de la península ibérica hasta Bretaña en el noroeste de Francia como se había propuesto anteriormente y descartan su reciente introducción antrópica.

Palabras clave: *Elona quimperiana*, Red de haplotipos, Filogenia multilocus, Barreras

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Introduction

The land snail *Elona quimperiana* (Férussac, 1821) (common name: Quimper snail, spotted snail or caracol de Quimper, caracol moteado in Spanish) is a species of interest in the European Union. It is listed in Annexes II and IV of the Habitats Directive 92/43/EEC and in Appendix II of the Bern Convention. The species also appears in the IUCN red list as of Least Concern (Gómez-Moliner and Seddon 2017). For its proper protection, we need to acquire a good understanding of its natural history. What is known so far is that it shows a preference for an Atlantic climate (or Oceanic) (Köppen and Geiger 1928), inhabiting three separate areas of western Europe: the French departments of Finistère and Côtes du Nord in western Brittany (Daguzan and Gloaguen 1986, Bouchet 1990); the whole Atlantic biogeographical region of the Iberian Peninsula, from northern Portugal and Galicia, through Asturias, Cantabria, and the Basque Country to the North of Navarre and southwestern France (Gómez-Moliner and Madeira 2012, Gómez-Moliner and Seddon 2017); and the southern part of La Rioja in the Iberian System (Altonaga et al 1994, Welter-Schultes 2012). Specimens living in La Rioja are disconnected from the larger haplogroup joining specimens living in Cantabria, and constituted the only haplogroup found in the Mediterranean biogeographical region (Arribas 1992, Altonaga et al 1994, Gómez-Moliner and Madeira 2012). In the Iberian Peninsula, *E. quimperiana* has been detected in 148 UTM 10 x 10 km grid squares (Puente et al 2001), while in France (not including Brittany) it has been reported in five more grid squares in the western region of the western Pyrenees (Gómez-Moliner and Madeira 2012, Bertrand 2020).

Elona quimperiana thrives in damp, shady environments with abundant decaying wood (Gómez-Moliner and Madeira 2012). It is thus mainly found in the undergrowth of humid woods of both oak and beech, often under large logs or stones although it has also been observed in humid, vegetation-rich habitats on walls and scree (Gómez-Moliner and Seddon 2017). This snail also enjoys damp cave entrances (Larraz and Jordana 1984), where it can carry out its complete life cycle maintaining its detritivore and coprophilous diet (Gómez-Moliner and Madeira 2012). In very humid areas, it may occur in the countryside (Altonaga et al 1994).

Using mitochondrial DNA sequencing, Vialatte et al (2008) described two lineages within *E. quimperiana*, which they called Brittany-Spain and Basque, respectively. The Brittany-Spain lineage comprised snails living in Brittany and the western part of its distribution area in the Iberian Peninsula, from Galicia to the village of Ramales de la Victoria (E-Cantabria). The Basque lineage was described to encompass snails from Tolosa (Gipuzkoa) in the East to the eastern limit of its distribution area in the Pyrenees (Vialatte et al 2008). This study did not include snails living between Cantabria and Tolosa or from La Rioja. Vialatte et al (2008) suggested that *E. quimperiana* had survived the Last Glacial Maximum (LGM) in two separate refuges, one probably located in the Picos de Europa area (between Asturias and Cantabria) and the other in the Basque Country. After the LGM, warmer and wetter climate conditions could have allowed for the northwards expansion of this species. The most likely starting point for the colonisation of Brittany could have been a shelter in the Picos de Europa, from where it would have spread across the Atlantic coastal region (Vialatte et al 2008). With the deforestation of a large part of the French Atlantic coast, the habitat of *E. quimperiana* has been largely reduced, which could have led to its disappearance in this area until the current situation of isolation of the snails living in Brittany (Vialatte et al 2008).

The absence of snails in the transition zone (Basque Country and northern Navarre) between the two lineages defined by Vialatte et al (2008) makes it difficult to interpret its intraspecific phylogeny. Moreover, Vialatte et al (2008) did not include specimens from the southernmost area of La Rioja, so their relationships with the two defined lineages are unknown. In the present study, we sequenced an extended sample of *E. quimperiana* populations to: 1) delimit the contact area between the Brittany-Spain and Basque lineages, located between Cantabria and Gipuzkoa; 2) genetically characterise the La Rioja population to establish which lineage they belong to; and 3) infer intraspecific phylogenetic relationships within the haplogroups of *E. quimperiana* and determine the distribution of *E. quimperiana* haplotypes throughout its whole habitat range. These objectives should serve to generate useful information for the management of this snail species, especially in La Rioja, where it seems to occupy a small isolated area.

Material and methods

Taxon sampling

The specimens of *Elona quimperiana* used for this study from Cantabria, the Basque Country, Navarre, La Rioja and SW France were obtained from the Mollusc Collection of the Zoology Laboratory of the University of the Basque Country. In the 1980s, samples were killed by freezing to avoid suffering (Scott 1992) and preserved in 70 % ethanol. More recently, this approach was replaced with 96 % ethanol to preserve DNA for molecular studies (Arrébola and Gómez 1998, Elejalde et al 2005). For the phylogenetic study, we used 35 specimens from 26 localities (table 1). We also incorporated DNA sequences from GenBank for phylogenetic reconstructions (table 1). Maps with all the localities included in this study (35 samples from 26 localities) and previous ones (Manganelli et al 2005: 1 sample from 1 locality; Vialatte et al 2008: 86 samples from 21 localities; Gómez-Moliner et al 2012: 2 samples from 1 locality) are provided in figures 1 and 2A and 2B.

DNA extraction, gene amplification and sequencing

DNA was extracted from a small fragment of the foot of each specimen according to the protocol described in the kit DNAeasy Blood and Tissue (QUIAGEN). The extracted DNA was migrated on 1% agarose gel to check extraction success and verify the presence of high molecular weight DNA suitable for further analysis. Optimal samples were then selected according to the gel results.

Table 1. List of specimens included in this study with voucher codes, GenBank accession numbers (*COI*, *16S* rRNA and *5.8S-ITS2-28S*), tree codes, network groups, locality information (province, country, UTM and height) and the collection to which they belong: ZBCA, Department of Zoology and Animal Cell Biology, University of the Basque Country. Vialatte et al (2008) did not publish UTM data but they communicated them to us personally; ? locality not clear; * Obtained from the bibliography.

Tabla 1. *Lista de muestras incluidas en este estudio con códigos de Voucher, códigos de acceso en GenBank* (COI, 16S y 5.8S-ITS2-28S)*, códigos en el árbol, grupos de la red, información de localidad (provincia, país, UTM y altitud) y la colección a la que pertenecen: ZBCA, Departamento de Zoología y Biología Celular Animal de la Universidad del País Vasco. Vialatte et al (2008) no publicaron datos UTM, pero nos los comunicaron personalmente; ? la localidad no está clara; * Obtenido de la bibliografía.*

	GenBank accesion number				Network group								
Sample code	***COI***	***16S***	***5.8S-ITS2-28S***	**Tree code**	***COI***	***16S***	**Locality**	**Province**	**Huse**	**X-UTM**	**Y-UTM**	**Height (m)**	**Collection**
EHUMC-2490	OM618575	OM630488	----	ARA01	ARAx	ARA01	Cueva Lazaldai	Araba	30T	516831	4757655	810	ZACB Group
EHUMC-2491	OM618582	OM630507	----	ARA02	ARA02	ARA02	Río Artomaña, Artomaña,	Araba	30T	501824	4759042	347	ZACB Group
EHUMC-2493	OM618578	OM630491	OM688343	ARA03	ARAx	ARA-BIZ-NAVx	Murua, Gorbea Dam	Araba	30T	520992	4761333	701	ZACB Group
EHUMC-2492	OM618576	OM630494	----	ARA04	ARAx	ARA04	Recandi River, Ziorraga, Gorbea	Araba	30T	507127	4764518	317	ZACB Group
EHUMC-2502	OM618572	OM630508	----	ARA05	ARA-BIZ	ARA05	Orduña Port, Tertanga, Arrastaria Valley	Araba	30T	497505	4757335	602	ZACB Group
EHUMC-2504	OM618571	OM630502	OM688338	AST11	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Ruta de las Xanas	Asturias	30T	258499	4794693	470	ZACB Group
EHUMC-2484	OM618590	OM630496	OM688354	BIZ01	BIZ01	BIZ01	Leioa	Bizkaia	30T	502621	4798452	22	ZACB Group
EHUMC-2485	OM618591	OM630497	OM688348	BIZ02	BIZ02	BIZx-CANx-WGIx	Leioa	Bizkaia	30T	502621	4798452	22	ZACB Group
EHUMC-2494	OM618584	OM630515	----	BIZ03	BIZ03	ARA-BIZ-NAVx	Cueva Arko Axpe, Itxina	Bizkaia	30T	515193	4769010	1010	ZACB Group
EHUMC-2495	OM618588	OM630489	----	BIZ04	BIZ04	BIZ04	Arimegorta Erreka, Bajo Atxuri, Gorbea	Bizkaia	30T	520436	4765091	753	ZACB Group
EHUMC-2496	OM618589	OM630506	OM688342	BIZ05	BIZ05	BIZ05	Zugastieta	Bizkaia	30T	524533	4788498	121	ZACB Group
EHUMC-2501	OM618574	OM630499	OM688358	BIZ06	BIZ06	BIZx-CANx-WGIx	Cueva de los cuervos	Bizkaia	30T	492701	4789127	334	ZACB Group
EHUMC-2503	OM618573	OM630501	OM688344	BIZ07	ARA-BIZ	BIZx-CANx-WGIx	Bilbao, Pagasarri	Bizkaia	30T	503760	4785954	626	ZACB Group
EHUMC-2486	OM618585	OM630500	OM688360	BUR01	BUR01	BIZx-CANx-WGIx	Lunada Port	Burgos	30T	447684	4780093	1264	ZACB Group
EHUMC-2470	OM618598	OM630485	----	EGI01	EGI01	EGI01	Errezil	Gipuzkoa	30T	567079	4779503	305	ZACB Group
EHUMC-2475	OM618601	OM630514	OM688355	NAV01	NAV01	NAVx-RIOx	Nacedero del Urederra	Navarre	30T	571140	4739126	660	ZACB Group
EHUMC-2476	OM618604	OM630513	OM688352	NAV02	NAV02	NAVx-RIOx	Nacedero del Urederra	Navarre	30T	571140	4739126	660	ZACB Group
EHUMC-2477	OM618605	----	OM688356	NAV03	NAV03	----	Nacedero del Urederra	Navarre	30T	571140	4739126	660	ZACB Group
EHUMC-2489	OM618577	OM630490	----	NAV04	NAVx	ARA-BIZ-NAVx	Urbasa, Bosque encantado	Navarre	30T	569185	4746396	1015	ZACB Group
EHUMC-2497	OM618579	OM630493	OM688347	NAV05	NAVx	ARA-BIZ-NAVx	Urbasa, Bosque encantado	Navarre	30T	569185	4746396	882	ZACB Group
EHUMC-2498	OM618580	----	OM688346	NAV06	NAV06	----	Urbasa, Salida del Bosque encantado	Navarre	30T	570221	4746366	950	ZACB Group
EHUMC-2499	OM618581	OM630492	OM688339	NAV07	NAV07	ARA-BIZ-NAVx	Puerto de Urbasa, Olazti	Navarre	30T	566860	4745612	883	ZACB Group
EHUMC-2500	OM618587	OM630495	OM688359	NAV08	NAV08	NAV08	Puerto de Urbasa, Olazti	Navarre	30T	566860	4745612	883	ZACB Group
EHUMC-2473	OM618603	OM630512	OM688351	RIO01	RIO01	NAVx-RIOx	San Millán Valley	La Rioja	30T	506108	4677747	1202	ZACB Group
EHUMC-2474	OM618602	OM630511	OM688353	RIO02	RIOx	NAVx-RIOx	San Millán Valley	La Rioja	30T	506108	4677747	1202	ZACB Group
EHUMC-2478	OM618600	OM630510	OM688349	RIO03	RIOx	NAVx-RIOx	Ortigosa de Cameros	La Rioja	30T	524450	4668866	1153	ZACB Group
EHUMC-2479	OM618599	OM630509	----	RIO04	RIOx	NAVx-RIOx	San Millán de la Cogolla	La Rioja	30T	506108	4677747	1202	ZACB Group
EHUMC-2471	OM618597	OM630486	----	SWF03	SWFx I	EGIx-SWFx	Sara	Pyrénées-Atlantiques, Aquitaine	30T	614344	4796625	145	ZACB Group
EHUMC-2472	OM618596	OM630487	----	SWF09	SWFx III	EGIx-SWFx	Bidarraitz	Iparralde	30T	634731	4792021	186	ZACB Group
EHUMC-2480	OM618594	----	OM688357	WGI03	WGIx II	----	Aranzazu	Gipuzkoa	30T	549073	4758657	705	ZACB Group
EHUMC-2481	OM618595	OM630498	OM688350	WGI04	WGI04	WGIx	Txaramela, Elgea	Gipuzkoa	30T	542070	4758192	973	ZACB Group
EHUMC-2482	OM618593	OM630504	----	WGI05	WGIx I	WGIx	Aranzazu	Gipuzkoa	30T	549073	4758657	705	ZACB Group
EHUMC-2483	OM618592	----	OM688345	WGI06	WGI06	----	Escoriaza	Gipuzkoa	30T	537009	4762091	310	ZACB Group
EHUMC-2487	OM618583	OM630503	OM688340	WGI07	WGI07	BIZx-CANx-WGIx	Eibar	Gipuzkoa	30T	542945	4781295	160	ZACB Group
EHUMC-2488	OM618586	OM630504	OM688341	WGI08	WGI08	BIZx-CANx-WGIx	Eibar	Gipuzkoa	30T	542945	4781295	160	ZACB Group
FG13943	AY345051	AY741449	----	AST01*	AST01*	ASTx-BRIx-BUR-CANx	Bendicion y Cueva de San Roman (Peña de Candamo Cave)	Asturias	29T	736837	4815642	180	Manganelli et al 2005
HT01	FJ786457	FJ786408	----	WGI02*	WGIx I	WGIx	Aránzazu	Gipuzkoa	30T	540000	4760000	855	Gómez-Moliner et. al 2012
HT02	FJ786458	FJ786409	----	WGI01*	WGIx II	WGIx	Aránzazu	Gipuzkoa	30T	540000	4760000	855	Gómez-Moliner et. al 2012
EPr5Esp	FJ491886	----	----	AST02*	ASTx-BRIx	----	Praia	Asturias	29T	726391	4821554	80	Vialatte et al 2008
EPr4Esp	FJ491885	FJ491928	----	AST03*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Praia	Asturias	29T	726391	4821554	80	Vialatte et al 2008
EPr3Esp	FJ491874	FJ491927	----	AST04*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Praia	Asturias	29T	726391	4821554	80	Vialatte et al 2008
EPr2Esp	FJ491873	FJ491926	----	AST05*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Praia	Asturias	29T	726391	4821554	80	Vialatte et al 2008
EPr1Esp	FJ491863	----	----	AST06*	ASTx-BRIx	----	Praia	Asturias	29T	726391	4821554	80	Vialatte et al 2008
EPa3Esp	FJ491860	FJ491931	----	AST07*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Panes	Asturias	30T	371533	4797475	82	Vialatte et al 2008
EG1Esp	FJ491826	----	----	AST08*	ASTx-BRIx	----	Gío	Asturias	29T	674703	4802887	235	Vialatte et al 2008
EPr7Esp	FJ491816	FJ491929	----	AST09*	ASTx-BRIx	AST09*	Praia	Asturias	29T	726391	4821554	80	Vialatte et al 2008
EPr6Esp	FJ491815	----	----	AST10*	ASTx-BRIx	----	Praia	Asturias	29T	726391	4821554	80	Vialatte et al 2008
EG4Esp	FJ491854	FJ491925	----	AST12*	AST12*	ASTx-BRIx-BUR-CANx	Gío	Asturias	29T	674703	4802887	235	Vialatte et al 2008
EPa2Esp	FJ491844	FJ491930	----	AST13*	AST13*	ASTx-BRIx-BUR-CANx	Panes	Asturias	30T	371533	4797475	82	Vialatte et al 2008
EG3Esp	FJ491840	FJ491924	----	AST14*	AST14*	ASTx-BRIx-BUR-CANx	Gío	Asturias	29T	674703	4802887	235	Vialatte et al 2008
EG2Esp	FJ491839	FJ491923	----	AST15*	AST15*	AST15*	Gío	Asturias	29T	674703	4802887	235	Vialatte et al 2008
EMS4	FJ491884	----	----	BRI01*	ASTx-BRIx	----	Sud de Morlaix (Dpto. Finisterre)	Brittany	30U	437974	5378731	38	Vialatte et al 2008
EMO4	FJ491883	FJ491907	----	BRI02*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Ouest de Orfaix (Dpto. Finisterre)	Brittany	30U	435872	5380661	65	Vialatte et al 2008
EMH2	FJ491882	FJ491901	----	BRI03*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Site Menez-Hom (Dinéault)	Brittany	30U	408130	5341148	300	Vialatte et al 2008
EMH1	FJ491881	----	----	BRI04*	ASTx-BRIx	----	Site Menez-Hom (Dinéault)	Brittany	30U	408130	5341148	300	Vialatte et al 2008
EMS3	FJ491872	FJ491909	----	BRI05*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Sud de Morlaix (Dpto. Finisterre)	Brittany	30U	437974	5378731	38	Vialatte et al 2008
EMO3	FJ491870	FJ491906	----	BRI06*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Ouest de Orfaix (Dpto. Finisterre)	Brittany	30U	435872	5380661	65	Vialatte et al 2008
EMN5	FJ491869	----	----	BRI07*	ASTx-BRIx	----	Montagnes Noires (Roudouallec)	Brittany	30U	448887	5333180	230	Vialatte et al 2008
EBM1	FJ491866	----	----	BRI08*	ASTx-BRIx	----	??	Brittany	-----	-----	-----	-----	Vialatte et al 2008
EPC5	FJ491862	----	----	BRI09*	ASTx-BRIx	----	Forêt de Pont Calleck (Berné)	Brittany	30T	473637	5312383	150	Vialatte et al 2008
EPC4	FJ491861	----	----	BRI10*	ASTx-BRIx	----	Forêt de Pont Calleck (Berné)	Brittany	30T	473637	5312383	150	Vialatte et al 2008
EMS1	FJ491859	FJ491908	----	BRI11*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Sud de Morlaix (Dpto. Finisterre)	Brittany	30U	437974	5378731	38	Vialatte et al 2008
EMN3	FJ491858	FJ491905	----	BRI12*	ASTx-BRIx	BRI12*	Montagnes Noires (Roudouallec)	Brittany	30U	448887	5333180	230	Vialatte et al 2008
EMN2	FJ491857	FJ491904	----	BRI13*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Montagnes Noires (Roudouallec)	Brittany	30U	448887	5333180	230	Vialatte et al 2008
EGSR5	FJ491856	----	----	BRI14*	ASTx-BRIx	----	Guingamp Sud (dpto. Côtes-d'Armor)	Brittany	30U	488677	5378227	77	Vialatte et al 2008
EGSR4	FJ491855	----	----	BRI15*	ASTx-BRIx	----	Guingamp Sud (dpto. Côtes-d'Armor)	Brittany	30U	488677	5378227	77	Vialatte et al 2008
EFLa5	FJ491853	----	----	BRI16*	ASTx-BRIx	----	Forêt de Landerneau (dpto. Finisterre, dstro. Brest y cantón Landerneau)	Brittany	30U	403036	5364998	75	Vialatte et al 2008
EQS5	FJ491847	----	----	BRI17*	ASTx-BRIx	----	Quimper (Dpto. Finisterre)	Brittany	30T	419117	5311699	10	Vialatte et al 2008
EPC2	FJ491845	FJ491910	----	BRI18*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Forêt de Pont Calleck (Berné)	Brittany	30T	473637	5312383	150	Vialatte et al 2008
EMN1	FJ491843	FJ491903	----	BRI19*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Montagnes Noires (Roudouallec)	Brittany	30U	448887	5333180	230	Vialatte et al 2008
EGSR2	FJ491841	FJ491899	----	BRI20*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Guingamp Sud (dpto. Côtes-d'Armor)	Brittany	30U	488677	5378227	77	Vialatte et al 2008
EFLa4	FJ491838	FJ491893	----	BRI21*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Forêt de Landerneau (dpto. Finisterre, dstro. Brest y cantón Landerneau)	Brittany	30U	403036	5364998	75	Vialatte et al 2008
EFLa3	FJ491837	----	----	BRI22*	ASTx-BRIx	----	Forêt de Landerneau (dpto. Finisterre, dstro. Brest y cantón Landerneau)	Brittany	30U	403036	5364998	75	Vialatte et al 2008
EQS4	FJ491830	----	----	BRI23*	ASTx-BRIx	----	Quimper (Dpto. Finisterre)	Brittany	30T	419117	5311699	10	Vialatte et al 2008
EQS3	FJ491829	FJ491914	----	BRI24*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Quimper (Dpto. Finisterre)	Brittany	30T	419117	5311699	10	Vialatte et al 2008
EPC1	FJ491828	----	----	BRI25*	ASTx-BRIx	----	Forêt de Pont Calleck (Berné)	Brittany	30T	473637	5312383	150	Vialatte et al 2008
EGSR1	FJ491827	FJ491898	----	BRI26*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Guingamp Sud (dpto. Côtes-d'Armor)	Brittany	30U	488677	5378227	77	Vialatte et al 2008
EFLa2	FJ491825	FJ491892	----	BRI27*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Forêt de Landerneau (dpto. Finisterre, dstro. Brest y cantón Landerneau)	Brittany	30U	403036	5364998	75	Vialatte et al 2008
EBP5	FJ491824	----	----	BRI28*	ASTx-BRIx	----	Baie de Paimpol (dpto. Côtes-d'Armor, dstro. Saint-Brieuc, cantón Paimpol)	Brittany	30U	494498	5400773	85	Vialatte et al 2008
EBP4	FJ491823	----	----	BRI29*	ASTx-BRIx	----	Baie de Paimpol (dpto. Côtes-d'Armor, dstro. Saint-Brieuc, cantón Paimpol)	Brittany	30U	494498	5400773	85	Vialatte et al 2008
EQS2	FJ491818	FJ491913	----	BRI30*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Quimper (Dpto. Finisterre)	Brittany	30T	419117	5311699	10	Vialatte et al 2008
EQS1	FJ491817	FJ491912	----	BRI31*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Quimper (Dpto. Finisterre)	Brittany	30T	419117	5311699	10	Vialatte et al 2008
EMH4	FJ491814	----	----	BRI32*	ASTx-BRIx	----	Site Menez-Hom (Dinéault)	Brittany	30U	408130	5341148	300	Vialatte et al 2008
EMH3	FJ491813	FJ491902	----	BRI33*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Site Menez-Hom (Dinéault)	Brittany	30U	408130	5341148	300	Vialatte et al 2008
EBP2	FJ491810	FJ491890	----	BRI34*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Baie de Paimpol (dpto. Côtes-d'Armor, dstro. Saint-Brieuc, cantón Paimpol)	Brittany	30U	494498	5400773	85	Vialatte et al 2008
EBP1	FJ491809	----	----	BRI35*	ASTx-BRIx	----	Baie de Paimpol (dpto. Côtes-d'Armor, dstro. Saint-Brieuc, cantón Paimpol)	Brittany	30U	494498	5400773	85	Vialatte et al 2008
EFQ2	FJ491880	----	----	BRI36*	ASTx-BRIx	----	Forêt de Quénécan (Sainte-Brigitte)	Brittany	30U	493332	5336143	195	Vialatte et al 2008
EFQ1	FJ491868	----	----	BRI37*	ASTx-BRIx	----	Forêt de Quénécan (Sainte-Brigitte)	Brittany	30U	493332	5336143	195	Vialatte et al 2008
EFQ4	FJ491812	FJ491897	----	BRI38*	ASTx-BRIx	ASTx-BRIx-BUR-CANx	Forêt de Quénécan (Sainte-Brigitte)	Brittany	30U	493332	5336143	195	Vialatte et al 2008
EMS2	FJ491871	----	----	BRI39*	BRI39*	----	Sud de Morlaix (Dpto. Finisterre)	Brittany	30U	437974	5378731	38	Vialatte et al 2008
EGSR3	FJ491842	FJ491900	----	BRI40*	BRI40*	ASTx-BRIx-BUR-CANx	Guingamp Sud (dpto. Côtes-d'Armor)	Brittany	30U	488677	5378227	77	Vialatte et al 2008
EPC3	FJ491846	FJ491911	----	BRI41*	BRI41*	ASTx-BRIx-BUR-CANx	Forêt de Pont Calleck (Berné)	Brittany	30T	473637	5312383	150	Vialatte et al 2008
EFLo1	FJ491879	FJ491894	----	BRI42*	BRIx I	ASTx-BRIx-BUR-CANx	Forêt de Lorge (dpto. Costas de Armor, comuna nueva Plœuc-l'Hermitage)	Brittany	30U	512330	5355901	245	Vialatte et al 2008
EFLo2	FJ491811	FJ491896	----	BRI43*	BRIx I	ASTx-BRIx-BUR-CANx	Forêt de Lorge (dpto. Costas de Armor, comuna nueva Plœuc-l'Hermitage)	Brittany	30U	512330	5355901	245	Vialatte et al 2008
EBP3	----	FJ491891	----	BRI44*	----	ASTx-BRIx-BUR-CANx	Baie de Paimpol (dpto. Côtes-d'Armor, dstro. Saint-Brieuc, cantón Paimpol)	Brittany	30U	494498	5400773	85	Vialatte et al 2008
ERV2Esp	FJ491849	FJ491935	----	CAN01*	CAN01*	BIZx-CANx-WGIx	Ramales de la Victoria/Bustablado	Cantabria	30T	461853	4789468	75	Vialatte et al 2008
ERV1Esp	FJ491832	FJ491936	----	CAN02*	CAN02*	BIZx-CANx-WGIx	Ramales de la Victoria/Bustablado	Cantabria	30T	461853	4789468	75	Vialatte et al 2008
EC4Esp	FJ491878	----	----	CAN03*	CANx	----	Cobreces	Cantabria	30T	401045	4804250	41	Vialatte et al 2008
EC3Esp	FJ491867	FJ491934	----	CAN04*	CANx	CANx	Cobreces	Cantabria	30T	401045	4804250	41	Vialatte et al 2008
EC1Esp	FJ491852	FJ491932	----	CAN05*	CANx	CANx	Cobreces	Cantabria	30T	401045	4804250	41	Vialatte et al 2008
EC2Esp	----	FJ491933	----	CAN06*	----	ASTx-BRIx-BUR-CANx	Cobreces	Cantabria	30T	401045	4804250	41	Vialatte et al 2008
ET1Bq	FJ491889	FJ491942	----	EGI02*	EGIx	EGIx-SWFx	Tolosa-Betelu	Gipuzkoa-Navarre	30T	579036	4770086	460	Vialatte et al 2008
ET4Bq	FJ491835	FJ491943	----	EGI03*	EGIx	EGIx-SWFx	Tolosa-Betelu	Gipuzkoa-Navarre	30T	579036	4770086	460	Vialatte et al 2008
ET2Bq	FJ491821	----	----	EGI04*	EGIx	----	Tolosa-Betelu	Gipuzkoa-Navarre	30T	579036	4770086	460	Vialatte et al 2008
ET3Bq	FJ491822	----	----	EGI05*	EGI05*	----	Tolosa-Betelu	Gipuzkoa-Navarre	30T	579036	4770086	460	Vialatte et al 2008
ERe7Esp	FJ491888	FJ491919	----	LUG01*	LUGx II	LUGx II	Reme	Lugo	29T	656882	4817584	20	Vialatte et al 2008
ERe6Esp	FJ491887	----	----	LUG02*	LUGx II	----	Reme	Lugo	29T	656882	4817584	20	Vialatte et al 2008
ERe3Esp	FJ491864	FJ491917	----	LUG03*	LUGx II	LUGx II	Reme	Lugo	29T	656882	4817584	20	Vialatte et al 2008
ERe1Esp	FJ491848	FJ491915	----	LUG04*	LUGx II	LUGx II	Reme	Lugo	29T	656882	4817584	20	Vialatte et al 2008
ERe8Esp	FJ491819	FJ491920	----	LUG05*	LUGx II	LUGx II	Reme	Lugo	29T	656882	4817584	20	Vialatte et al 2008
ERe4Esp	FJ491875	FJ491918	----	LUG06*	LUGx II	LUGx II	Reme	Lugo	29T	656882	4817584	20	Vialatte et al 2008
ERe2Esp	----	FJ491916	----	LUG07*	----	LUGx II	Reme	Lugo	29T	656882	4817584	20	Vialatte et al 2008
ERe5Esp	FJ491876	----	----	LUG08*	LUGx I	----	Reme	Lugo	29T	656882	4817584	20	Vialatte et al 2008
ERe10Esp	FJ491831	FJ491922	----	LUG09*	LUGx I	LUGx I	Reme	Lugo	29T	656882	4817584	20	Vialatte et al 2008
ERe9Esp	FJ491820	FJ491921	----	LUG10*	LUGx I	LUGx I	Reme	Lugo	29T	656882	4817584	20	Vialatte et al 2008
ES9Bq	----	FJ491940	----	SWF01*	----	EGIx-SWFx	Sare	Pyrénées-Atlantiques, Aquitaine	30T	614346	4796587	150	Vialatte et al 2008
ES10Bq	----	FJ491941	----	SWF02*	----	EGIx-SWFx	Sare	Pyrénées-Atlantiques, Aquitaine	30T	614346	4796587	150	Vialatte et al 2008
ES4Bq	FJ491851	----	----	SWF04*	SWFx I	----	Sare	Pyrénées-Atlantiques, Aquitaine	30T	614346	4796587	150	Vialatte et al 2008
ES3Bq	FJ491850	----	----	SWF05*	SWFx I	----	Sare	Pyrénées-Atlantiques, Aquitaine	30T	614346	4796587	150	Vialatte et al 2008
ES1Bq	FJ491833	FJ491937	----	SWF06*	SWFx I	EGIx-SWFx	Sare	Pyrénées-Atlantiques, Aquitaine	30T	614346	4796587	150	Vialatte et al 2008
ES2Bq	FJ491834	FJ491938	----	SWF07*	SWF07*	EGIx-SWFx	Sare	Pyrénées-Atlantiques, Aquitaine	30T	614346	4796587	150	Vialatte et al 2008
PB1_1Bq	FJ491836	FJ491836	----	SWF08*	SWFx III	EGIx-SWFx	Pays basque français??	-----	-----	-----	-----	-----	Vialatte et al 2008
ES7Bq	FJ491877	FJ491939	----	SWF10*	SWFx II	EGIx-SWFx	Sare	Pyrénées-Atlantiques, Aquitaine	30T	614346	4796587	150	Vialatte et al 2008
ES6Bq	FJ491865	----	----	SWF11*	SWFx II	----	Sare	Pyrénées-Atlantiques, Aquitaine	30T	614346	4796587	150	Vialatte et al 2008

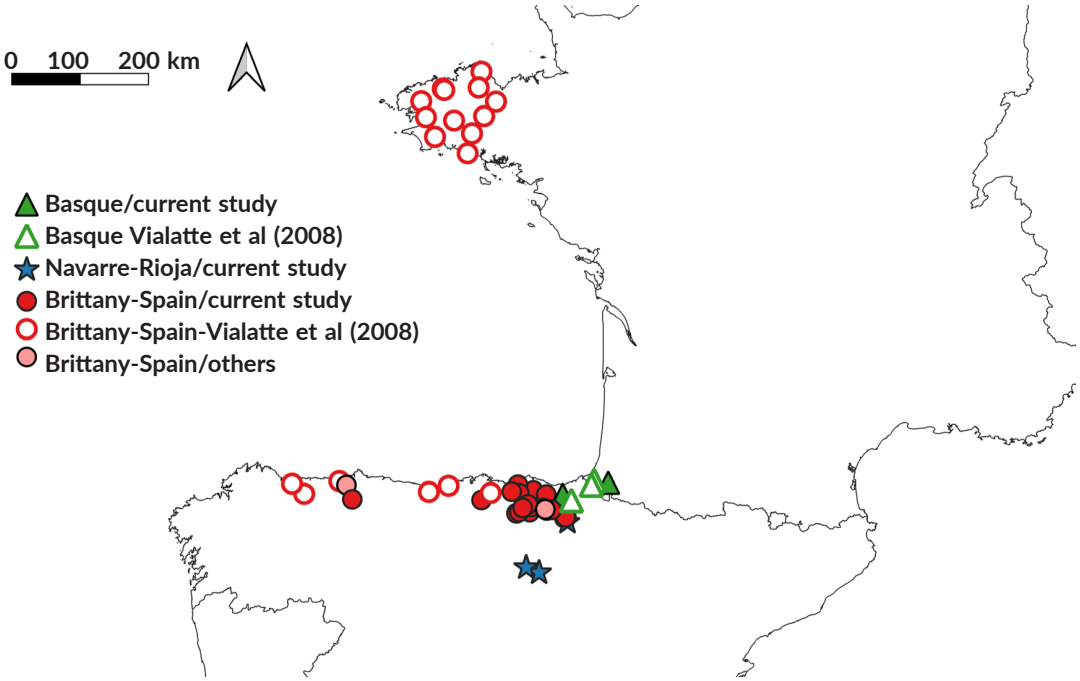

Fig. 1. Distribution map of the study samples coloured according to the main haplogroups. (For the UTM coordinates of the study area, please, see table 1).

Fig. 1. *Mapa de distribución de las muestras estudiadas coloreado según los principales haplogrupos. (Para las coordenadas UTM del área de estudio, véase la tabla 1).*

Two fragments of the mitochondrial genes cytochrome c oxidase I (*COI*) and *16S* rRNA (*16S*) were amplified by polymerase chain reaction (PCR) using the primers LCO 1490 and HCO 2198 (Folmer et al 1994), and 16Scs1 and 16Scs2 (Chiba, 1999), respectively. In addition, the 3' end of the *5.8S* rRNA gene, the complete *ITS2* region, and the 5' end of the large subunit *28S* rRNA gene (*5.8S-ITS2-28S*) were also amplified using the primers LSU-1 and LSU-3 (Wade et al 2006). The PCR conditions were: an initial denaturation step at 96 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, and ending with a final extension step of 10 min at 72 °C. PCR products were migrated by electrophoresis on a 1.5 % agarose gel to confirm correct amplification. Amplicons were sequenced at Macrogen Inc. using an ABI3730XL sequencer. The resulting forward and reverse sequences were assembled using Geneious 5.1.7 (Kearse et al 2012). Following automatic assembly, each contig was checked for errors/ambiguities. Double peaks with equally high intensities in the chromatograms were assigned as heterozygous sites. These polymorphic sites were coded as ambiguous nucleotides following IUPAC-IUB code.

Phylogenetic analyses

Two phylogenetic analyses were performed: one using a matrix combining the mitochondrial *COI* and *16S* gene fragment data (mitochondrial dataset), and the other using a combined matrix of data from the *COI*, *16S* and *5.8S-ITS2-28S* genes (multilocus dataset). For the first analysis, the new sequences obtained here were aligned with the sequences published in GenBank for the species (Manganelli et al 2005, Vialatte et al 2008, Gómez-Moliner et al 2012). The second analysis was performed with only the new sequences obtained here (table 1: Zoology and Animal Cell Biology ZACB Group of the University of the Basque Country). Alignments were carried out with MAFFT 7.313 online version (Katoh et al 2017) using the L-INS-I strategy for the *COI* gene fragment and the Q-INS-i algorithm for *16S rRNA* and *5.8S-ITS2-28S*. For each codon position in *COI*, substitution saturation was assessed through the entropy-based information method (Xia et al 2003) as implemented in DAMBE v.6.1.19 (Xia 2013). The best gene partition scheme for the two datasets was obtained with Partition Finder V1.1.1 (Lanfear et al 2012). The best evolutionary model for each gene partition was estimated with jModelTest 3.7 (Darriba et al 2012) according to the Akaike Information Criterion (AIC) and employing CIPRES Science Gateway (Miller et al 2010). Molecular character statistics including parsimony informative sites and base frequency were calculated with MEGA X (Kumar et al 2018) for each gene partition (table 2). Genetic distances between the main clades (table 3) were calculated by MEGA X (Kumar et al 2018) using the 'Between Group Mean Distance' method and *p*-distance model with the default values.

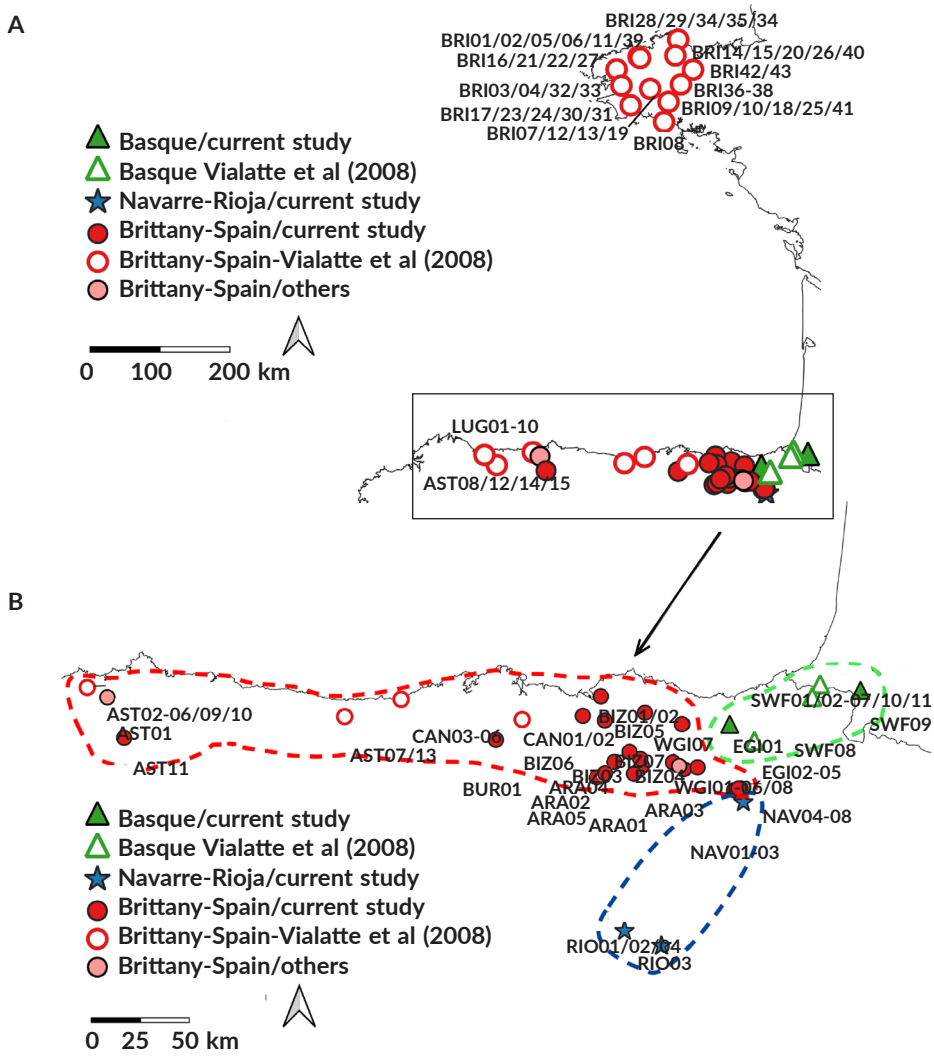

Fig. 2. Magnification of the distribution map of samples, with the tree codes, coloured according to the main haplogroups focusing on the northern Iberian Peninsula. Dashed lines delimit the main haplogroups. (For the UTM coordinates of the study area, please, see table 1).

Fig. 2. *Ampliación del mapa de distribución de las muestras, con los códigos de los árboles, coloreado según los principales haplogrupos del norte de la península ibérica. Las líneas discontinuas delimitan los principales haplogrupos. (Para las coordenadas UTM del área de estudio, véase la tabla 1).*

For phylogenetic analyses of the various datasets we used the Bayesian Inference (BI) method and applied the partition schemes obtained with Partition Finder (table 2). A Bayesian search of tree space was performed with MrBayes v3.2.2 (Ronquist et al 2012) at CIPRES Science Gateway, specifying for each partition the best evolutionary model obtained with jModelTest (table 2). *Sphincterochila candidissima* (*COI*: FJ786500, *16S*: KJ458556 and *ITS2*: KJ458636) and *Norelona pyrenaica* (*COI*: ON211918, *16S*: KJ458544 and *ITS2*: KJ458627) were used as outgroups. Analyses were run for 95 million generations in two parallel runs, sampling every 1,000 generations with the first 25% of trees discarded as burn-in. Convergence between runs was assessed by comparing traces using Tracer v1.7.1 (Rambaut et al 2018). In the BI analysis, posterior probability (PP) values above 0.95 were interpreted as significant statistical support.

Haplotype network and genetic barriers

Haplotype networks for the mitochondrial markers were constructed using the computer program PopART (Leigh and Bryant 2015) using TCS Network model (Clement et al 2000). Including the new sequences obtained here and those previously published, the sequence dataset of *COI* employed for this analysis consisted of 119 sequences, while the *16S* matrix contained 88 sequences. Sequences were aligned as described above. It is important to note that when constructing networks, PopART does not take into account insertion/deletion polymorphisms or indeterminate positions. Finally, barriers to gene flow between locations were calculated within the *COI* gene fragment as it has the largest number of samples. They were analysed with Alleles In Space v.2.2, AIS, (Miller

Table 2. Gene partition determined for each dataset including alignment length, number of taxa, selected evolutionary model (with MrBayes equivalences), base frequencies and parsimony informative sites for each gene fragment: GP, gen partition; EM, volutionary model; EE, evolutionary MrB equivalences; PS, parsimony informative sites

Tabla 2. *Partición de los genes determinada para cada conjunto de datos incluyendo la longitud de alineamiento, el número de taxones, el modelo evolutivo seleccionado (con equivalencias de MrBayes), las frecuencias de bases y los sitios informativos de parsimonia para cada fragmento de gen: GP, partición del gen; EM, modelo evolutivo; EE, equivalencias evolutivas de MrB; PS, sitios informativos de parsimonia.*

					Base frequencies (%)				
GP	Length	Taxa	EM	EE	T	C	A	G	PS
Mitochondrial									
COI codon 1	1-651\3	119	TrN	6	29	16	28	27	60
COI codon 2	2-651\3		F81	1	46	22	12	20	
COI codon 3	3-651\3		TIM2+G	6+G	45	9	32	12	
16S	820	88	TPM1uf+I	2+I	31	13	38	18	44
Multilocus									
COI codon 1	1-651\3	35	TPM1uf	6	30	16	27	27	60
COI codon 2	2-651\3		F81	1	46	22	12	20	
COI codon 3	3-651\3		TIM2+G	6+G	45	9	32	12	
16S	741	31	HKY+I	2+I	32	13	38	17	44
5.8S-5'/28S	88/354	24	JC	1	19	27	24	30	1
ITS2	521		HKY	2	28	25	19	28	1

2005) using Monmonier Maximum Difference Algorithm (MMDA). The number of detected barriers was configured with the default parameters and for the parameter of use, the 'Residual genetic distances'option. These conditions were used in case there were correlations between genetic and geographic distances in our data set. AIS perform analyses that can detect or characterize patterns of spatial genetic structure. For example, it can assess correlations between genetic and geographic distances of sampled individuals and it can also perform a generalized form of spatial autocorrelation analysis that allows detection of genetic structure (Miller 2005).

Results

Phylogenetic analyses

The final alignment of the mitochondrial dataset included 124 *E. quimperiana* individuals (35 new) and comprised 1,481 bp (see table 1 and section 2.3 for GenBank accession numbers). The alignment of the multilocus dataset contained 35 specimens and comprised 2,355 characters. For datasets, alignment length, number of taxa, parsimony informative sites, average base frequencies and the best evolutionary model for each marker are provided in table 2.

The phylogenetic reconstruction based on the two mitochondrial gene fragments (*COI* and *16S* rRNA) including both the new and GenBank sequences revealed three main clades, although not supported, and support for their phylogenetic relationships was also weak (fig. 3). The phylogenetic reconstruction of the three concatenated loci with added information for the nuclear gene fragment (*5.8S-ITS2-28S*) recovered these three main clades with statistical support, and also supported their phylogenetic relationships (fig. 4). One clade joined together the specimens from the eastern part of the Iberian Peninsula and SW-France, inhabiting areas from Bidarraitz (Iparralde) to Errezil (Gipuzkoa) (fig. 1, 2). In consequence, we named this the Basque lineage. Snails from Port of Olasti, in the northern Urbasa Mountains comprised the westernmost specimens belonging to this lineage. All specimens described by Vialatte et al (2008) from this geographical region clustered within this Basque lineage. A second clade brought together the sequences of 101 specimens, all from localities spanning from the western Cantabrian region to western Gipuzkoa and NW-Navarre (fig. 1, 2). All published GenBank sequences for specimens of *E. quimperiana* from Brittany clustered within this lineage, so we designated it the Brittany-Spain lineage. Finally, the last clade, referred to here as the Navarre-Rioja lineage, joined together all samples from La Rioja and the specimens from the southern Sierra de Urbasa (Nacedero del Urederra). The Navarre-Rioja lineage was recovered as sister to the Brittany-Spain phylogroup, while the Basque lineage was recovered as the most divergent. Genetic distances between the main lineages were: 2.4% between Brittany-Spain and Basque, and 3.0% and 3.4% for Navarre-Rioja and the other two lineages, respectively (table 3). The contact area between the Basque and Brittany-Spain lineages is located in Gipuzkoa (fig. 1, 2) between

Table 3. Genetic distance estimates between the main lineages. The number of base differences per site from averaged sequence pairs between groups are shown (p-distance with uniform rates).

Tabla 3. *Estimaciones de la distancia genética entre los principales linajes. Se muestra el número de diferencias de bases por sitio de pares de secuencias promediados entre grupos (distancia p con tasas uniformes).*

	Brittany-Spain	Basque
Basque	0,024	
Navarre-Rioja	0,030	0,034

Table 4. Results of Monmonier's Maximum Difference Algorithm Analysis applied to Data Type 'DNA sequence data' and used in computation the 'Residual Genetic Distances': SSV, subsegment value: SCP, segment connection point.

Tabla 4. *Los resultados del análisis del algoritmo de diferencia máxima de Monmonier se aplicaron al tipo de datos "datos de secuencia de ADN" y se utilizaron en el cálculo de las distancias genéticas residuales: SSV, valor de subsegmento: SCP, punto de conexión del segmento.*

Barrier	
Number of segments	2
Number of subsegments	10
Sum of subsegment values	4.093E-01
Average subsegment value	4.093E-02

Segment 1: 9 subsegments				
X-coord1	Y-coord1	X-coord2	Y-coord2	SSV
42.97522	-2.48406	42.80138	-2.13001	5.25E+12
42.86017	-2.18157	42.80138	-2.13001	4.70E+12
42.86702	-2.15302	42.80138	-2.13001	4.49E+12
42.86666	-2.14034	42.80138	-2.13001	4.65E+12
43.07937	-2.02908	42.80138	-2.13001	4.86E-02
43.27696	-1.60815	42.80138	-2.13001	4.78E+12
43.26896	-1.33978	42.80138	-2.13001	4.83E+12
42.17155	-2.70398	42.80138	-2.13001	2.12E+12
42.97522	-2.48406	42.80138	-2.13001	5.25E+12
Segment 2: 1 subsegments				
42.80138	-2.13001	42.97522	-2.48406	SCP
42.80138	-2.13001	42.17155	-2.70398	2.12E+12

Eibar and Errezil-Tolosa, localities separated by the valleys of the Deba and Urola rivers. The Navarre-Rioja lineage is present in two areas separated by the Ebro River: Nacedero del Urederra in the southern Sierra de Urbasa, and the Iberian System''s foothills in La Rioja (fig. 1, 2; table 1).

Haplotype network

The haplotype networks recovered are presented in figures 5 (*COI*) and 6 (*16S rRNA*). Haplotypes are labelled according to the codes in table 1. Grey circles without a code represent a hypothetical intermediate haplotype linking the observed haplotypes. Dashes on the lines connecting circles represent single base changes. In both networks, three main haplogroups were identified, here designated as Brittany-Spain, Basque and Navarre-Rioja (fig. 3, 4), and corresponding to the three main clades recovered in the phylogenetic trees. The Brittany-Spain haplogroup contained 35 haplotypes for *COI* and 18 for *16S*, joining the specimens from Lugo, Asturias, Cantabria, Bizkaia, W-Gipuzkoa and Navarre (except samples from Nacedero del Urederra) to those from Brittany. The most frequent haplotype identified in our *COI* analysis was ASTx-BRIx, which joined together 48 specimens distributed across Asturias and Brittany. The haplogroup ASTx-BRIx-BUR-CANx was the most frequent in the *16S* analysis, joining 33 specimens from Asturias, Burgos, Cantabria and Brittany. All haplotypes identified from Asturias and Brittany showed a star-like pattern in the *COI* network, with no large genetic gaps between them. Samples from Lugo and Cantabria were included in the same star-like haplogroup together with the Asturias and Brittany samples in the *16S* analysis, with only one or two mutational steps between them. As many as 13 haplotypes were recovered for *COI* within this star-like haplogroup. The remaining 22 haplotypes identified for *COI* from Bizkaia, Araba, Navarre and W-Gipuzkoa belonging to the Brittany-Spain haplogroup, showed greater genetic distances, and no more than three samples shared the same haplotype for *COI*. The Basque haplogroup brought together the specimens from E-Gipuzkoa and SW-France, showing 7 haplotypes for *COI* separated by several genetic gaps, and as many as 13 mutational steps between samples EGI01 and SWF07. This Basque haplogroup showed only two haplotypes for *16S*, separated by four mutational steps. Finally, the Navarre-Rioja haplogroup joined with the samples from Nacedero del Urederra (Navarre) and all those from La Rioja. The three samples from Nacedero del Urederra shared no haplotype for *COI*. Sample NAV01 was five and six mutational steps away from NAV02 and NAV 03, respectively (fig. 5, 6). Samples from La Rioja showed from six to 13 mutational steps with respect to the Navarre haplotypes for *COI*. On the contrary, all samples from Nacedero del Urederra and La Rioja shared the same haplotype for *16S*. The Brittany-Spain and Basque haplogroups were separated by 14 single mutations in the *COI* network, and six single mutations in the *16S* network. The Navarre-Rioja haplogroup featured 14 single mutations in the *COI* network and seven single mutations in the *16S* network with respect to the Brittany-Spain haplogroup. The barrier obtained in table 4 with Alleles In Space software are presented in the figure 7. Table 4 showed that 2 segments were obtained within barrier 1. The first one consists of 9 UTM coordinates and the second one 3 UTM coordinates. SubSegment Values has the desirable properties

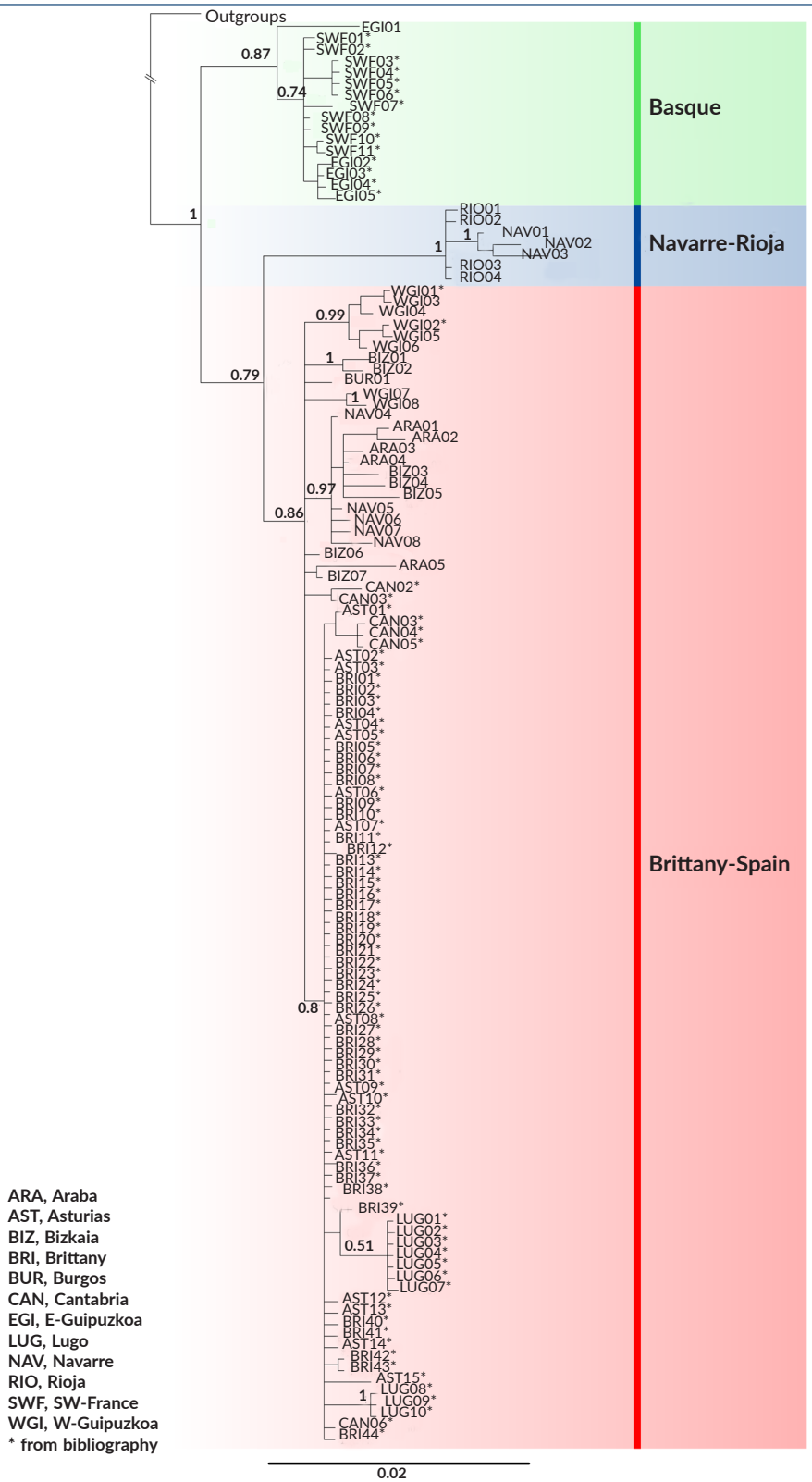

Fig. 3. Phylogenetic reconstruction based on the mitochondrial dataset by Bayesian Inference (*COI* and *16S* rRNA). Numbers on nodes correspond to BI posterior probabilities; if no number is displayed the node is not supported. The tree is coloured to distinguish the main clades.

Fig. 3. *Reconstrucción filogenética basada en el conjunto de datos mitocondriales obtenidos por inferencia bayesiana (IB)* (COI *y ARNr* 16S). *Los números en los nodos corresponden a las probabilidades posteriores de IB; si no hay ningún número significa que el nodo no está soportado. El árbol está coloreado para distinguir los principales clados.*

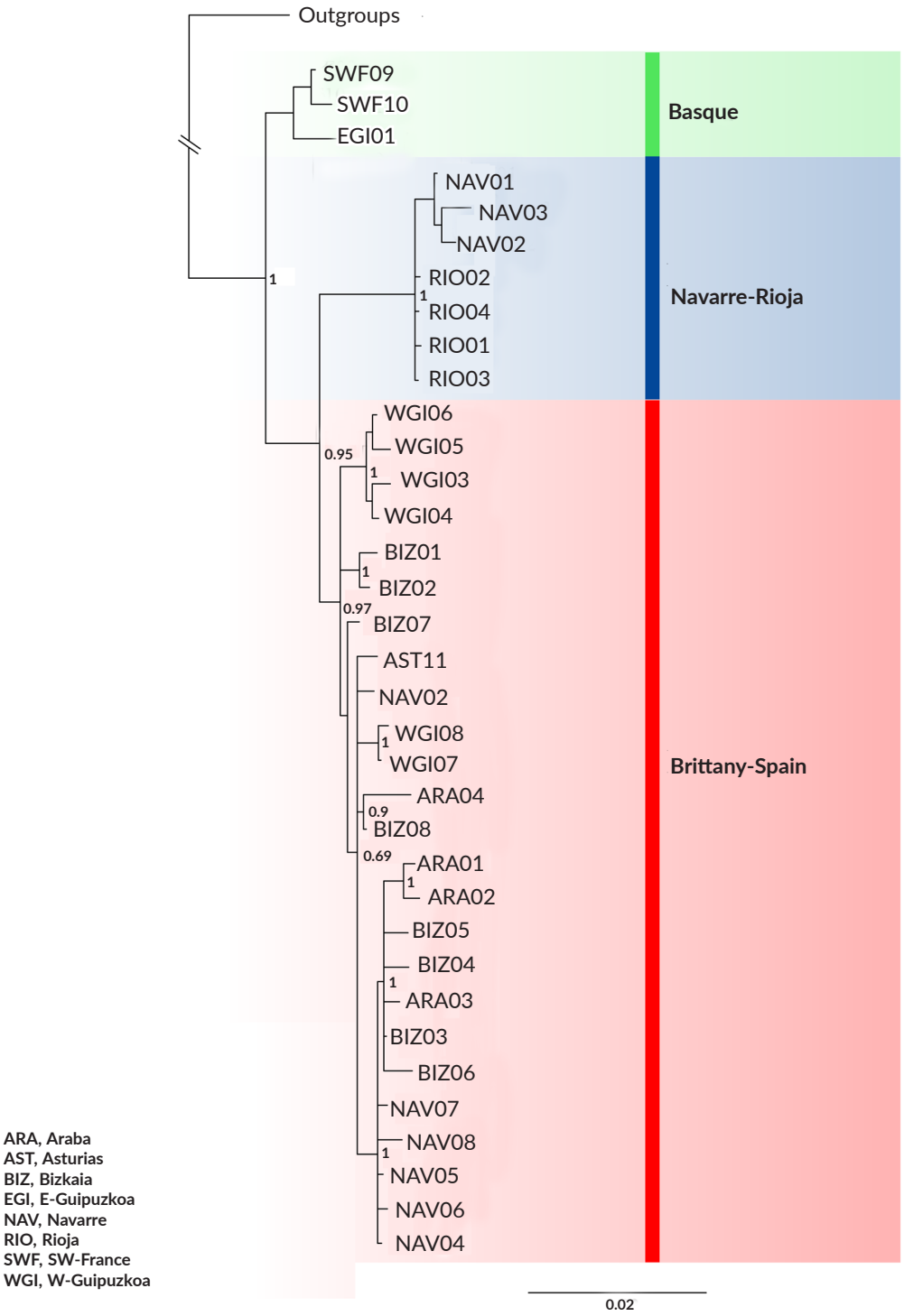

Fig. 4. Phylogenetic reconstruction based on the multilocus dataset by Bayesian Inference (*COI*, *16S* rRNA and *5.8S-ITS2-28S*). Numbers on nodes correspond to BI posterior probabilities; if no number is displayed the node is not supported. The tree is coloured to distinguish the main clades.

Fig. 4. *Reconstrucción filogenética basada en el conjunto de datos multilocus obtenido por inferencia bayesiana (IB)* (COI, *ARNr* 16S y 5.8S-ITS2-28S). *Los números en los nodos corresponden a las probabilidades posteriores de IB; si no hay ningún número significa que el nodo no está soportado. El árbol está coloreado para distinguir los principales clados.*

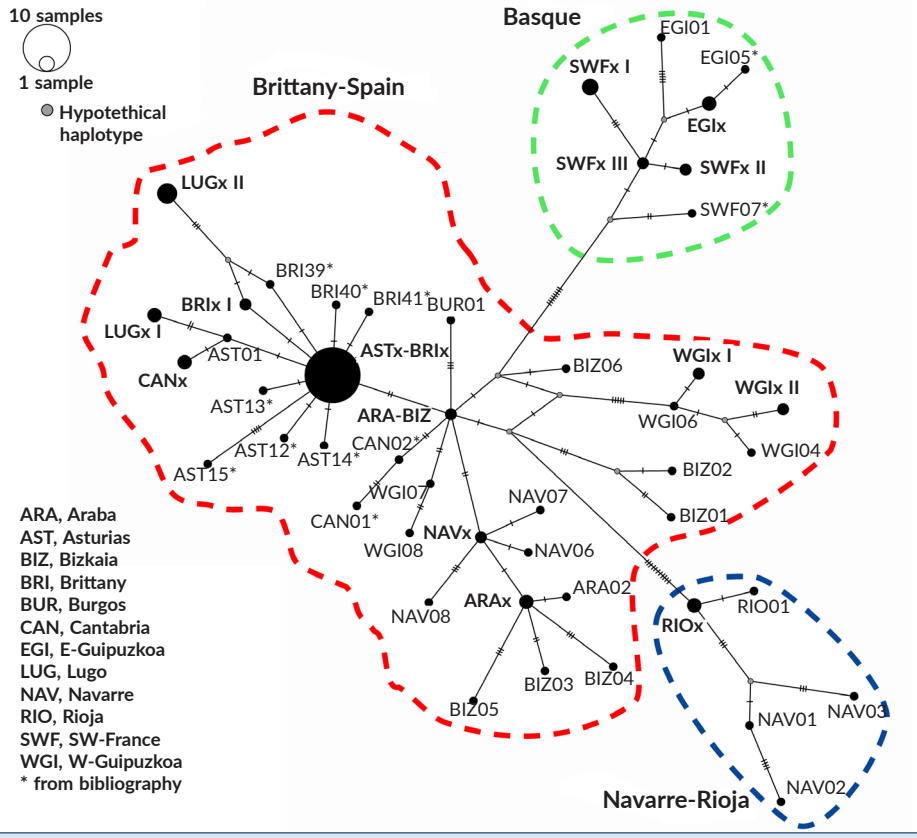

Fig. 5. Haplotype network for the *COI* gene. Dashed lines delimit the three main haplogroups identified as Basque, Navarre-Rioja and Brittany-Spain. Black circles represent haplotypes named according to the codes in table 1 and their size indicates haplotype frequency (scaled according to number of specimens). Small grey circles are hypothetical missing intermediate haplotypes necessary to link the observed haplotypes. Dashes on the lines connecting circles represent single base changes.

Fig. 5. *Red de haplotipos para el gen* COI. *Las líneas discontinuas delimitan los tres principales haplogrupos identificados como Vasco, Navarro-Riojano y Bretaña-España. Los círculos negros representan haplotipos nombrados de acuerdo con los códigos de la tabla 1 y su tamaño indica la frecuencia de los haplotipos (ajustada al número de muestras). Los pequeños círculos grises son hipotéticos haplotipos intermedios faltantes necesarios para vincular los haplotipos observados. Los guiones que cruzan las líneas que conectan los círculos representan cambios en una sola base.*

of taking on values of 0 when genotypes are identical and 1 when genotypes are completely dissimilar. This geographical regionalization procedure was used to detect the locations of putative barriers to gene flow by iteratively (MMDA) identifying sets of contiguous, large genetic distances along connectivity networks (Miller, 2005). The graphical representation obtained of 'barriers' was superimposed over the map (fig. 7) of all the samples where each of the 2 segments separate the previously reported haplotypes. Both lines allowed us to check the separation of the samples from the three haplotypes.

Discussion

Elona quimperiana is a protected species and so requires strategies for its conservation and management based on solid knowledge of its preferred habitats and distribution. In this phylogenetic study based on multilocus DNA sequences from additional samples and genes to those examined previously, we provide new helpful information for the design of such strategies.

Both our phylogenetic reconstruction and network analyses identified three main lineages within *E. quimperiana*. The Brittany-Spain and Basque lineages are clearly related to those described by Vialatte et al (2008) based on the same mitochondrial loci. Haplogroups Hg1 (*16S*) and HgA (*COI*) reported by Vialatte et al (2008) assigned the populations from Brittany, Asturias, Cantabria and Lugo to our Brittany-Spain haplogroup. In contrast, haplogroups Hg11 (*16S*) and HgW (*COI*) of Vialatte et al (2008) joined together samples from E-Gipuzkoa and SW-France and were incorporated in our Basque haplogroup. The additional specimens from the Basque Country included in our study allowed us to extend eastwards the distribution range of the Brittany-Spain lineage. In the study of Vialatte et al (2008), the easternmost population of this lineage was Ramales de la Victoria (Cantabria). Our study expands that limit by about 120 km eastwards to the Eibar region (Gipuzkoa) and the Sierra de Urbasa's northern slope (Navarre). In contrast, the Basque lineage was restricted to the easternmost

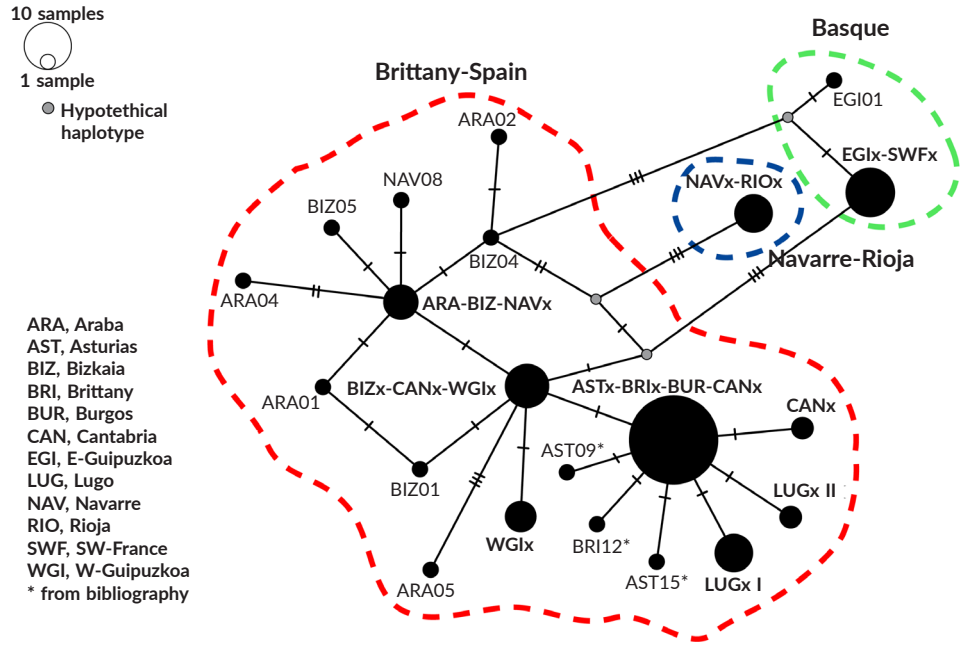

Fig. 6. Haplotype network for the *16S* rRNA gene. Dashed lines delimit the three main haplogroups identified as Basque, Navarre-Rioja and Brittany-Spain. Black circles represent haplotypes named according to the codes in table 1, and their size indicates haplotype frequency (scaled according to number of specimens). Small grey circles are hypothetically missing intermediate haplotypes necessary to link the observed haplotypes. Dashes on the lines connecting circles represent single base changes.

Fig. 6. *Red de haplotipos para el gen ARNr 16S. Las líneas discontinuas delimitan los tres principales haplogrupos identificados como Vasco, Navarro-Riojano y Bretaña-España. Los círculos negros representan haplotipos nombrados de acuerdo con los códigos de la tabla 1 y su tamaño indica la frecuencia de haplotipos (ajustada al número de especímenes). Los pequeños círculos grises son hipotéticos haplotipos intermedios faltantes necesarios para vincular los haplotipos observados. Los guiones que cruzan las líneas que conectan los círculos representan cambios de una sola base.*

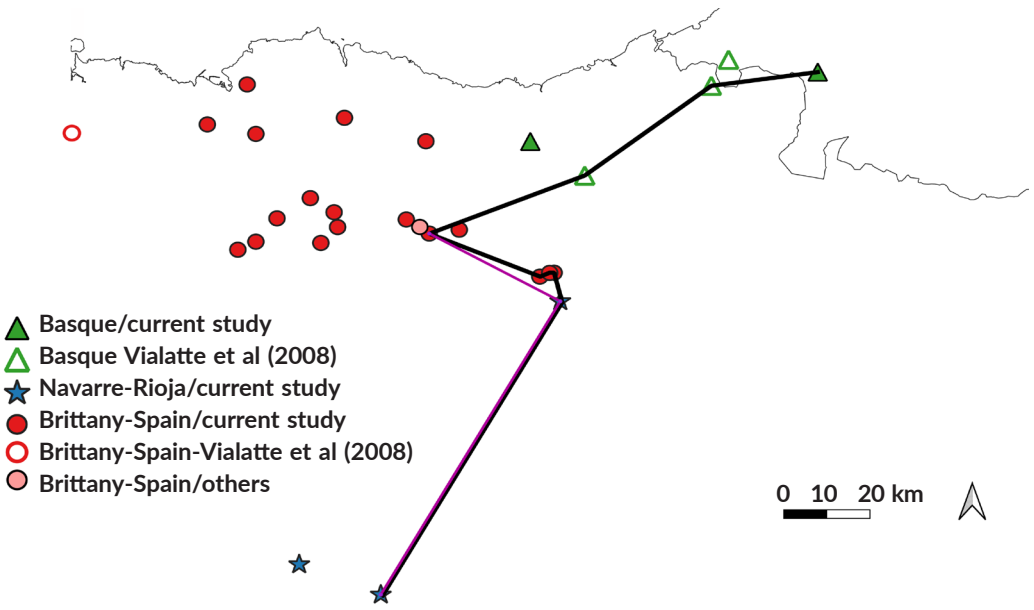

Fig. 7. Barriers obtained by Alleles in Space v.2.2 within *COI* gene fragment dataset. The black line represents the Segment 1 in Barrier 1 and the purple line represents the Segment 2 in Barrier 1. (For the UTM coordinates of the study area, please, see table 1).

Fig. 7. *Barreras obtenidas por Alleles in Space v.2.2 dentro del conjunto de datos de fragmentos de gen* COI. *La línea negra representa el Segmento 1 de la Barrera 1 y la línea Púrpura, el Segmento 2 de la Barrera 1. (Para las coordenadas UTM del área de estudio, véase la tabla 1).*

part of the Cantabrian Mountains region and western end of the Pyrenees. The westernmost localities to which this lineage is assigned are Tolosa (Vialatte et al 2008) and Errezil (present work). This means that the Brittany-Spain and Basque lineages are geographically separated by the Deba and Urola river valleys. More samples from these two valleys and adjacent regions are needed to identify possible contact points between the Spain-Brittany and Basque lineages.

In addition to the two lineages identified by Vialatte et al (2008), we detected a third, previously unidentified lineage, the Navarre-Rioja clade. This lineage comprises the specimens of *E. quimperiana* living in the Iberian System, but also the specimens from Nacedero del Urederra, on the southern slope of the Sierra de Urbasa. These localities occur in the Mediterranean biogeographic region. Our phylogenetic reconstruction recovered with support from a sister group relationship between the Navarre-Rioja and Brittany-Spain lineages (PP = 0.95) (fig. 4), indicating a closer relationship between the two. The specimens of *E. quimperiana* living in the Iberian System (La Rioja) constitute an isolated haplogroup with a restricted distribution range, which seems to have maintained a reduced population size over a long period of time (Puente et al 2001, Gómez-Moliner and Madeira 2012). The Navarre-Rioja lineage in the mountains to the North and South of the Ebro Valley could be the remains of a past wider distribution range (Puente et al 2001). *E. quimperiana* may have shown a continuous distribution between Navarre and La Rioja throughout the Ebro Valley during the last glaciation period, and Mediterraneanization of this valley after the LGM would have promoted its restriction to forested areas of the northern slopes of the Iberian System mountains (Puente et al 2001, Gómez-Moliner and Madeira 2012, Caro et al 2019).

The relatively long mutational distances observed between the three lineages suggest an old genetic isolation event separating these three intraspecific genetic entities. Indeed, the overall haplogroup pattern obtained for the three haplogroups corresponds to the phylogeographic category I of Avise et al (1987) and Avise (2000): 'Deep gene tree, major lineages allopatric'. These genetic differences seem to be the result of an accumulation of mutations postdating the allopatric separation of the three lineages or of lineage sorting of an ancestral gene pool (Avise 2000, Johnson et al 2015, Uit De Weerd and Gittenberger 2019, Català et al 2021). This would appear to indicate that the three lineages of *E. quimperiana* evolved separately in three allopatric regions. Genetic differences among the three main lineages were 2.4%-3.4%. Several authors have estimated DNA sequence divergence ranges at 1% to 10% per million years for land snails (Chiba 1999, Razkin et al 2015, Chueca et al 2017). However, the small size of populations restricted to reduced geographic areas could promote more rapid evolutionary mutation rates (Frankham et al 2012, Korábek et al 2022).

According to Vialatte et al (2008), *E. quimperiana* could have survived the Quaternary glaciations in two separate refuges, probably in the Basque Country and in Asturias. This isolation would have given rise to the Basque and Brittany-Spain lineages, respectively. The results of our study point to a third refuge between La Rioja and Navarre, when lower temperatures prompted the extension of humid forests in areas today dominated by the dry Mediterranean climate of the Ebro Valley. In effect, multiple refugia within the Iberian Peninsula have been identified for other species (Gómez and Lunt 2007). Sampling efforts by various specialists in Navarre and La Rioja have indicated no continuity between the *E. quimperiana* haplogroup of La Rioja and that of the Cantabrian mountain range (Larraz and Jordana 1984, Prieto 1986, Arribas 1992, Altonaga et al 1994). Thus, there is currently no genetic flow between populations living in these two regions. The specimens of Nacedero del Urederra in the southern slope of the Urbasa Mountains (Navarre) are closed off in geographic terms to the populations living on the northern slope of these mountains. Hence, the former belongs to the Navarre-Rioja lineage and the second to the Brittany-Spain lineage. No hybrids or gene flow have been identified between snails living on every slope of the Urbasa Mountains. Accordingly, non-forested regions in the upper part of the Urbasa Mountains may have prevented secondary contact between them. The genetic distances between the Navarre-Rioja haplogroup and the other two groups are 3.0 %–3.4 %. The divergences in mitochondria may well pre-date the present separation of populations, and lack of gene flow could be inferred from lack of geographic overlap between mitochondrial clades. We therefore propose that the Navarre-Rioja lineage, just like the Brittany-Spain and Basque lineages, represents ESU (Evolutionarily Significant Unit) in the sense of Crandall et al (2000) and deserves special conservation measures.

The specimens examined here from NE-Spain (Asturias, Lugo and Cantabria) and Brittany (i.e., Brittany-Spain lineage) showed a star-like haplogroup network pattern for both mitochondrial DNA gene fragments. This fits well with network category V of Avise et al (1987): 'shallow gene tree, lineage distribution varied'. This classification describes common lineages that are widespread, and closely related lineages that are confined to one or a few nearby localities. The specimens from Asturias and Brittany share one common haplotype for each gene fragment, the most frequent in both areas. The most common haplotype is considered to be ancestral for this haplogroup, while other genotypes are derived (Avise et al 1987). In this context, the ASTx-BRIx haplotype should be considered the ancestral *COI* haplotype for NE-Spain and Brittany, and the haplotype ASTx-BRIx-BUR-CANx is ancestral for the *16S* gene fragment. There are several private haplotypes for the Brittany specimens (BRIx, BRI39, BRI40 and BRI41 for *COI*; BRI12 for *16S*) that probably arose after the separation of the Brittany and Spain populations. In addition, the barrier segments from AIS divide the haplogroups of the three regions. These divisions collected by the AIS program are the same as those obtained in the haplotype networks and therefore the results complement each other. Within the samples of the Basque haplogroup, the EHUMC-2470 is left out within Segment 1, showing that it is quite different from the remaining samples. This differentiation

is also found in the haplotype networks where this sample has 5 private base changes in the *COI* gene fragment and another private mutation in the *16S* gene fragment. Due to the information provided from the networks, this sample really belongs to the Basque haplogroup. Based on these findings, we support the theory of Vialatte et al (2008) ruling out the recent anthropic introduction into Brittany of *E. quimperiana*. The recent colonization of Brittany would have meant low DNA polymorphism, as the recurrent human introduction of this species is unlikely as it is of no economic interest. The star-like network of the Brittany haplogroup indicates its origin is not that ancient either (Gómez and Lunt 2007, Vialatte et al 2008, Hutchinson et al 2020) and it probably did not predate the last glaciation period. This, along with the presence of some private haplotypes in Brittany, supports the suggestion of its expansion after the last glaciation, following forest expansion from the south (Ferris et al 1995) as was proposed by Vialatte et al (2008). Nevertheless, its origin as a result of passive transport from the Iberian Peninsula to Brittany in ancient times cannot be ruled out. Indeed, long distance passive transport has been documented in land snails (Chueca et al 2015, Korábek et al 2018, Somoza-Valdeolmillos et al 2021) and the absence of the species in intermediate coastal areas of western France makes this option equally plausible.

Conclusions

Elona quimperiana is a protected species listed in annexes II and IV of the Europe Habitats Directive, determining a need for adequate conservation measures for this taxon. The large number of specimens included in our phylogenetic analyses enabled us to identify a new haplogroup of *E. quimperiana*, designated Navarre-Rioja. Through extended multilocus analysis, statistical support was obtained for the phylogenetic relationships between the three main clades identified within *E. quimperiana*: Basque, Brittany-Spain and Navarre-Rioja. The various haplogroups were generated by allopatry in the absence of gene-flow. The haplotype networks observed here support this conclusion as a high number of mutational steps exist among haplogroups. The results obtained by both analyses, haplotype networks, and barriers to gene flow highlight the presence of three haplogroups within *E. quimperiana*, each of them being well delimited geographically and with several private mutations separating haplogroups. We were therefore able to delineate the distribution ranges of the three groups and the contact areas between them. Hence, the Basque and Brittany-Spain lineages are separated by the Deva and Urola river valleys, and the Basque and Navarre-Rioja lineages by the Sierra de Urbasa. The main lineages identified here represent three ESU, and thus special conservation actions are warranted. We propose that these measures should focus on preserving all three lineages, taking into account their genetic singularities and polymorphisms, their phylogenetic relationships, and their geographic ranges.

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Competing interest information
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Author contributions
BJ Gómez-Moliner and **MJ Madeira** conceived the original idea of the presented study, developed the theory and supervised the findings of this work. **E Somoza-Valdeolmillos**, **A Caro** and **LJ Chueca** obtained the samples, performed the computations, verified the analytical methods and carried out the experiments. **BJ Gómez-Moliner** and **E Somoza-Valdeolmillos** wrote the first draft of this publication. All these authors provided critical feedback and helped to shape the research, analysis and manuscript. **AI Puente** supervised the project and provided institutional support for the development of this study.

Conflicts of interest
No conflicts declared

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