

A western representative of an eastern clade: Phylogeographic history of the gypsum-associated plant *Nepeta hispanica*

Ignacio Ramos-Gutiérrez^{a,b,*}, Juan Carlos Moreno-Saiz^{a,b}, Mario Fernández-Mazuecos^{a,b,c}

^a Departamento de Biología, Universidad Autónoma de Madrid, C/ Darwin 2, 28049 Madrid, Spain

^b Centro de Investigación en Biodiversidad y Cambio Global (CIBC-UAM), Universidad Autónoma de Madrid, C/ Darwin 2, 28049 Madrid, Spain

^c Real Jardín Botánico (RJB-CSIC), Plaza de Murillo 2, 28014 Madrid, Spain

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ABSTRACT

The preference of certain plant species for gypsum soils with a patchy distribution leads to disjunct population structures that are thought to generate island-like dynamics potentially influencing biogeographic patterns at multiple evolutionary scales. Here, we study the evolutionary and biogeographic history of *Nepeta hispanica*, a western Mediterranean plant associated with gypsum soils and displaying a patchy distribution with populations very distant from each other. Three approaches were used: (a) interspecific phylogenetic analyses based on nuclear DNA sequences of the ITS region to unveil the relationships and times of divergence between *N. hispanica* and its closest relatives; (b) phylogeographic analyses using plastid DNA regions *trnS-trnG* and *psbJ-petA* to evaluate the degree of genetic isolation between populations of *N. hispanica*, their relationships and their genetic diversity; and (c) ecological niche modelling to evaluate historical distributional changes. Results reveal that *N. hispanica* belongs to an eastern Mediterranean and Asian (Irano-Turanian) clade diversified in arid environments since the Miocene-Pliocene. This species represents the only lineage of this clade that colonised the western Mediterranean, probably through the northern Mediterranean coast (southern Europe). Present Iberian populations display a high plastid genetic diversity and, even if geographically distant from each other, they are highly connected according to the distribution of plastid haplotypes and lineages. This can be explained by a scenario involving a complex history of back-and-forth colonisation events, facilitated by a relative stability of suitable conditions for the species across the western Mediterranean throughout the Quaternary.

1. Introduction

Studying the evolutionary history of plant species in combination with their ecological requirements helps us to understand how they have reached their present distribution and population structure. A key factor to consider is soil composition, which often determines the capacity of species to thrive in a certain area. A paradigmatic example of this effect is provided by gypsum soils, which exert an extraordinary selective pressure on plant species, leading to the evolution of highly adapted floras (Kruckeberg, 1986; Escudero et al., 2015). These gypsic floras frequently display peculiar biogeographic patterns due to the patchy distribution of gypsum soils at different scales (Martínez-Hernández et al., 2009), and this is particularly true for the western Mediterranean region. At a regional scale, several gypsum-associated plant lineages show a wide longitudinal disjunction, in which some representatives are distributed in the western Mediterranean, while their closest relatives are found in the eastern Mediterranean and the Irano-Turanian region

(Braun-Blanquet and Bolòs, 1957; Escudero et al., 2015). This distribution pattern has been historically interpreted as the result of vicariance following the Messinian Salinity Crisis during the late Miocene, when the Mediterranean Sea level drastically dropped, providing an ecological corridor that allowed plants associated with salty and evaporitic environments to spread across the Mediterranean basin (Bocquet et al., 1978). At a local scale, gypsum soils are found in continental areas, and usually form island-like patches surrounded by other substrates (Mota et al., 2009). This entails a remarkable population structure in gypsum-associated plants, in which patterns of gene flow and geographic isolation may approach those of island plants (Moore et al., 2014).

Nepeta L. is a genus of the mint family (Lamiaceae) distributed throughout temperate Eurasia and northern Africa including several gypsum-associated species. With nearly 300 species, most of them perennial herbs, it is one of the most diverse genera of the family (Uberta and Valdés, 1983; Jamzad et al., 2003b). The highest species diversity is

* Corresponding author at: Departamento de Biología, Universidad Autónoma de Madrid, C/ Darwin 2, 28049 Madrid, Spain.

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found in the Irano-Turanian region (Jamzad et al., 2003a). Within *Nepeta*, the section *Oxynepea* is characterised by dioecy and comprises five currently accepted species (Budantsev, 1993; POWO, 2021). The type species of the section is *N. ucranica* L., which occurs in eastern Europe and south-western Asia; three more species are found in south-western Asia (*N. congesta* Fisch. & Mey., *N. heliotropifolia* Lam., and *N. stricta* (Banks & Sol.) Hedge & Lamond); and only one species is found in the western Mediterranean Region, specifically in the Iberian Peninsula and north-western Africa, *N. hispanica* Boiss. & Reut. (see Fig. 1). A detailed phylogenetic study of this section has not yet been carried out, and only two species (*N. heliotropifolia* and *N. congesta*) were included in the most comprehensive study of the genus undertaken so far (see Jamzad et al., 2003a). Given the limited availability of phylogenetic data and the vast geographical distance between *N. hispanica* and the rest of *Oxynepea* species (with Irano-Turanian distributions) there remains the possibility that dioecy (the diagnostic character of sect. *Oxynepea*) evolved more than once during the diversification of the genus, as demonstrated for other plant genera such as *Acer* and *Momordica* (Gleiser and Verdú, 2005; Schaefer and Renner, 2010). This would mean that the sectional classification does not reflect the evolutionary history of the group.

Nepeta hispanica is a western Mediterranean endemic and gypsosag species, i.e., it preferentially occurs on gypsum, although it also tolerates other alkaline substrates such as marls (Mota et al., 2009; Pavón et al., 2015). This species shows the peculiar biogeographic patterns described above for gypsum-associated species at both geographic scales (Escudero et al., 2015): an eastern-western Mediterranean disjunction with its

putative closest relatives, and a patchy distribution of its populations. On the one hand, based on morphological traits, some authors have suggested a very close relationship between *N. hispanica* and *N. ucranica* (Aedo, 2010), or even considered the former a subspecies of the latter (*N. ucranica* subsp. *hispanica* (Boiss. & Reut.) Bellot, Casaseca & Ron). On the other hand, *N. hispanica* occurs in patchy populations that are also distant from one another as a result of its preference for gypsum soils. This scattered distribution, with populations separated by hundreds of kilometres, extends from the inner Iberian Peninsula to northern Morocco. Some taxonomists (Ubera and Valdés, 1983) separated central-northern Iberian populations as a different species (*N. beltranii* Pau, currently considered a synonym of *N. hispanica*; Aedo, 2010), while Moroccan populations have been recognised as *N. hispanica* subsp. *statice* Maire (Fennane et al., 1998). Nevertheless, the phylogenetic relationships of *N. hispanica* within the genus and the genetic variability and relationships between populations remain unstudied. Apart from its biogeographic and evolutionary interest, advancing the knowledge on this species is relevant because it is a threatened plant, categorised as Vulnerable in Spain (Moreno, 2008; de la Cruz et al., 2011) and as Endangered in Morocco (Fennane, 2016).

Here we used three approaches to shed light on the evolutionary and biogeographic history of *N. hispanica*. First, we conducted phylogenetic analyses to test the monophyly and estimate divergence times of *Nepeta* sect. *Oxynepea* by combining previously published and newly generated ITS sequences (including those of *N. hispanica*). Second, we performed a phylogeographic analysis of *N. hispanica* based on plastid DNA (ptDNA) sequences to evaluate the degree of genetic isolation between

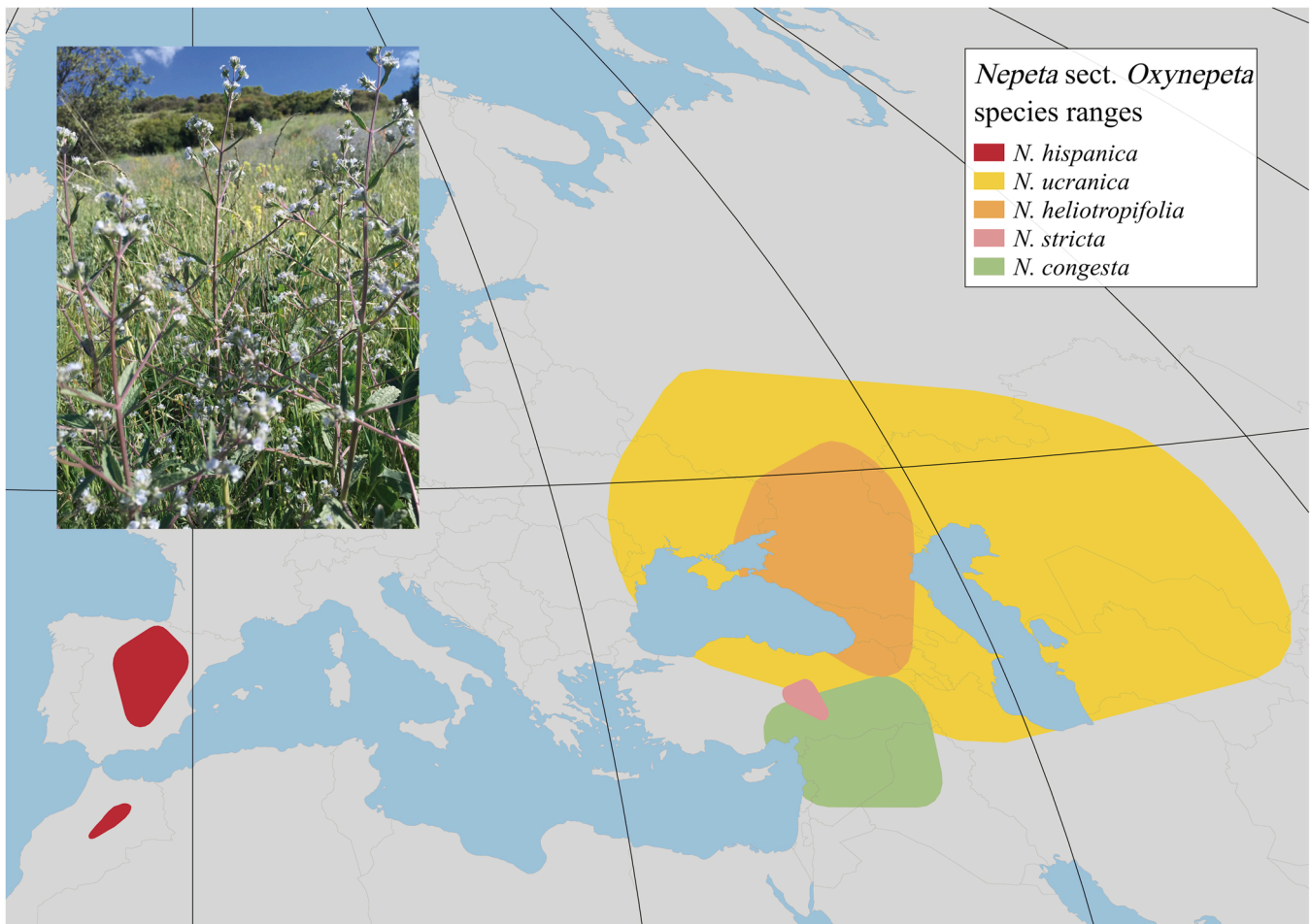


Fig. 1. Distribution ranges of the five accepted species of *Nepeta* sect. *Oxynepea*, based on GBIF and our own distribution data. The inset photo shows the study species *Nepeta hispanica*.

populations, the relationships between them and their genetic diversity. And third, we modelled the present environmental niche of the species and projected it to past periods to help interpret phylogeographic patterns and historical distributional changes. We tested the following hypotheses: (1) sect. *Oxynepete* is a monophyletic group, and therefore *N. hispanica* is closely related to other dioecious species of the genus; and (2) long-term geographic isolation, resulting from a discontinuity in historically suitable areas, has led to a strong ptDNA differentiation between populations of *N. hispanica*.

2. Methods

2.1. Sample collection

For the phylogenetic analysis, we sampled herbarium specimens of the three species of *Nepeta* sect. *Oxynepete* with no DNA sequences available from previous studies, including two geographically distant specimens of *N. hispanica*, one of *N. ucranica* and three of *N. stricta* (Table S1). These were provided by the Royal Botanical Garden of Madrid Herbarium (MA), except for two of the *N. stricta* specimens, which were provided by the University of Salamanca Herbarium (SALA) and the Botanical Garden of Valencia Herbarium (VAL) respectively. Recently collected specimens were used to ensure DNA quality.

For the phylogeographic analysis, we prospected all 14 known Iberian localities of *N. hispanica*. Since the species seems to have disappeared from some of them, we ended up collecting leaf tissue of a total of 111 individuals from 10 populations (12 individuals per population, except population ONT, in which only three individuals were found; cf. Table S2). Whenever possible, similar numbers of male and female individuals were collected. Samples were kept in paper envelopes and dried in silica gel. In populations present in regions where the species was not legally protected, herbarium specimens were prepared and deposited at the Royal Botanical Garden of Madrid (MA) and Autonomous University of Madrid (MAUAM) herbaria. Moroccan populations of *N. hispanica* subsp. *statice* could not be visited, but we obtained one sample provided by the Geneva Herbarium (G) (No. J7635), now deposited at the MA Herbarium.

2.2. DNA sequencing

Genomic DNA was extracted from all samples using the *DNeasy Plant Mini Kit* (Qiagen, USA) following the manufacturer's protocol, except that incubation time varied depending on the sample (up to 1.5 h for herbarium samples).

For the phylogenetic analysis, we selected the internal transcribed spacer (ITS) region, which was used in the most extensive phylogenetic analysis of *Nepeta* published to date (Jamzad et al., 2003a). The ITS region was amplified by PCR and sequenced for the *N. hispanica*, *N. ucranica* and *N. stricta* samples using the external primers 17SE-26SE and the internal primers ITS5-ITS4 (Sun et al., 1994; White et al., 1990; Sang et al., 1995).

For the phylogeographic analysis of *N. hispanica*, we first performed a pilot study to select consistently amplified and variable ptDNA regions. Seven regions of the plastid genome, considered potentially informative for angiosperms (Shaw et al., 2007), were sequenced for one individual per population. As a result, we selected the two regions (*trnS-trnG* and *psbJ-petA*) with the highest numbers of nucleotide substitutions. Then these two ptDNA regions were amplified by PCR for ten individuals per population of *N. hispanica*, and for the *N. ucranica* and one *N. stricta* sample. Standard primers were used (Hamilton, 1999; Shaw et al., 2007), in combination with newly designed internal primers for problematic samples (Table S3).

In all cases, PCR was conducted using BIOTAQ DNA polymerase (Bioline, USA). PCR conditions consisted of an initial denaturation step at 94 °C for 1 min and 30 cycles of amplification, each consisting of denaturation at 94 °C for 1 min, annealing at 50–52 °C for 1 min, and

extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. PCR products were checked by electrophoresis in 1 % agarose and submitted to Macrogen Inc. (Madrid, Spain) for Sanger sequencing. Sequences were assembled and edited using Geneious 11.1.13 (Kearse et al., 2012). All sequence variation among individuals was double-checked on electropherograms to prevent mistakes. After multiple attempts, a good-quality ITS sequence of *N. stricta* could not be obtained, possibly due to fungus contamination.

2.3. Phylogenetic analysis and dating

For phylogenetic analyses, the three newly generated ITS sequences (two belonging to *N. hispanica* and one to *N. ucranica*) were combined with 38 other sequences (Jamzad et al., 2003a) obtained from the GenBank database (Benson et al., 2005). These included sequences of two additional species of *Nepeta* sect. *Oxynepete* (*N. heliotropifolia* and *N. congesta*), 31 other species of *Nepeta* and 5 closely related genera to be used as the outgroup. A total of 36 individuals of 35 taxonomically representative species of the genus *Nepeta* were included. All ITS sequences were aligned using the MAFFT algorithm (Katoh et al., 2002), one of the quickest and more precise among those currently available (Thompson et al., 2011). The sequence matrix was analysed using the jModelTest 2.1.10 software (Guindon and Gascuel, 2003; Darriba et al., 2012) to estimate the best-fitting nucleotide substitution model (*GTR+I+G*) according to the Akaike information criterion. A phylogenetic tree based on Bayesian inference was obtained using MrBayes 3.2 (Huelsenbeck and Ronquist, 2001). Two parallel runs of 10 million generations each were performed, and sampled every 1000 generations. A majority-rule consensus tree was calculated with a burn-in phase of 10 %. Mixing and convergence were checked using Tracer 1.7 (Rambaut et al., 2018) by confirming that no parameter had an effective sample size (ESS) lower than the standard threshold of 200.

To obtain a time-calibrated phylogeny, we analysed the ITS matrix (including a single specimen per species) using BEAST2 v2.5.0 (Bouckaert et al., 2019). A secondary calibration was implemented based on a recent exhaustive analysis of the mint family (Rose et al., 2022). Therein, the separation of the genera *Nepeta* and *Agastache* (included in the outgroup of our study) was estimated to have happened between 23.3 and 36.7 Ma. This interval was used as a uniform prior for the root of our tree. A birth-death process and an uncorrelated lognormal relaxed clock were implemented. Four chains of 10 million generations were run and sampled every 1000 generations. Tracer 1.7 was used to confirm mixing and convergence, and determine an appropriate burn-in fraction. After the combination of chains using LogCombiner (with a burn-in phase of 10 %), a maximum clade credibility (MCC) tree was built in TreeAnnotator (both programmes are part of the BEAST2 package) and visualised using FigTree 1.4 (Rambaut, 2014). Additionally, a maximum likelihood (ML) tree was built using RAXML v8.2.12 (Stamatakis, 2014) with the same substitution model and 1000 bootstrap replicates to evaluate the congruence of different phylogenetic approaches.

2.4. Phylogeographic analyses

Sequences of the two ptDNA regions (*trnS-trnG* and *psbJ-petA*) for the 94 individuals of *N. hispanica* and the individuals of *N. ucranica* and *N. stricta* were separately aligned using MAFFT (see Supplementary Material). For phylogeographic analyses, both ptDNA regions were concatenated. To estimate relationships among haplotypes, a haplotype network was inferred using the statistical parsimony method (Templeton, 1992) implemented in the TCS 1.21 software (Clement et al., 2000), with the *gaps = missing* option and a connection limit of 95%. We used DnaSP v6.12.03 (Rozas et al., 2017) to calculate haplotype diversity (*Hd*) per population (Nei, 1987) and to quantify genetic differentiation for all population pairs based on the nearest-neighbour statistic (*S_{nn}*) with 1000 permutation replicates (Hudson, 2000). The distribution of haplotypes across populations was mapped using QGIS 3.4.2 (QGIS

Development Team, 2018).

Additionally, a discrete phylogeographic analysis (DPA; Lemey et al., 2009) was carried out to shed light on migration patterns among biogeographic areas. This analysis combines DNA sequence data with one discrete geographical variable to estimate the ranges of lineage ancestors in a Bayesian framework. The DPA analysis was carried out in BEAST 1.10 (Suchard et al., 2018). For the geographical variable, five discrete areas were defined, including the four Iberian river basins in which *N. hispanica* is found (namely Ebro, Douro, Tagus and Guadalquivir river basins; see Blanco-Sánchez et al., 2021) and northern Africa. *N. ucranica* was used as outgroup with an undetermined area (because we were only interested in migration within *N. hispanica*). An asymmetric substitution model was implemented for the area partition, while substitution models for the DNA partitions followed jModelTest results (GTR+I+G). A strict clock was used for the area partition and an uncorrelated relaxed clock with a lognormal distribution for the DNA partitions, with a constant size coalescent tree prior. The root age was calibrated using a uniform prior distribution between 2.01 and 8.1 Mya based on the divergence time estimated by the dating analysis of ITS sequences (see Results). Two MCMC chains of 10 million generations sampled every 1000 generations were run, and they were combined in LogCombiner with a burn-in phase of 10%. Mixing and convergence of chains were confirmed using Tracer 1.7. A MCC tree was built in TreeAnnotator considering common ancestor heights. To evaluate the statistical support of migration routes, Bayesian stochastic search variable selection (BSSVS) was implemented, and Bayes Factors (BFs) were calculated in SPREAD3 (Bielejec et al., 2016).

2.5. Ecological niche modelling

For ecological niche modelling, a maximum entropy algorithm was used (Phillips et al., 2006) because it is considered to work well with presence-only data (Elith et al., 2011). To prevent geographic bias, the analysis was performed only for the Iberian Peninsula, where the distribution range of *N. hispanica* is well known. Northern Africa was not included because of the limited available information on the species distribution in this region. Occurrence points included those obtained during our own fieldwork and those obtained from the biodiversity database GBIF (GBIF.org, 2018). However, we only used high-accuracy occurrence data postdating 1901 (at least 4 decimal places in their coordinates, an error corresponding to less than 10 m at that latitude) with associated herbarium specimens. Therefore, the taxonomic accuracy of all occurrences was confirmed. Oversampling issues (redundant data in more accessible areas) were corrected by the random elimination of points that were less than 0.05° apart from each other using a custom R script.

Bioclimatic, edaphic and lithological variables at 30 arc-second resolution were included in the model (see Table S4). Bioclimatic data were obtained from the CHELSA database (<https://chelsa-climate.org>; Karger et al., 2017), whereas edaphic data were taken from the SoilGrids database (<https://soilgrids.org/>; Hengl et al., 2017). To avoid collinearity issues in these sets of variables, only non-redundant ones were selected. To that end, a two-step procedure was implemented using the R packages “raster” (Hijmans, 2018) and “HH” (Heiberger, 2020) for bioclimatic and edaphic variables separately. First, we selected variables with a correlation below 70 % in the study area (Dormann et al., 2013). Then, we excluded additional variables with a high variance inflation factor (VIF) until all remaining variables had $VIF < 10$. Selected variables are marked in Table S4. Additionally, a lithological categorical variable, mapping nine lithological classes potentially influencing plant distributions (Fernández-Mazuecos & Glover, in prep.) was included because *N. hispanica* is known to be associated with gypsum soils. This lithological variable is based on the Lithostratigraphic Map of Spain (del Pozo Gómez, 2009). Maximum entropy modelling was implemented in MaxEnt 3.4.1 (Phillips et al., 2006). The model was generated by averaging 100 subsample replicates, each one being assigned 90% of

point data for modelling and 10 % for validation.

To estimate the areas with environmentally favourable conditions for *N. hispanica* in the past, the present model was projected to nine late Quaternary time slices. Layers of bioclimatic variables for these periods at 2.5 arc-minute resolution were taken from the PaleoClim database (<http://www.paleoclim.org/>), which compiles information from Brown et al. (2018), Fordham et al. (2017), Karger et al. (2017) and Otto-Bliesner et al. (2006) (see Table S5). For these projections, only climatic conditions were considered to vary, while edaphic and lithological conditions were assumed to have stayed constant throughout these geologically recent periods.

3. Results

3.1. Phylogenetic analysis and dating

The phylogeny of 35 species of *Nepeta* obtained using the ITS region is shown in Fig. 2a. The four species of section *Oxynepea* included in the analysis were recovered as a monophyletic group with a high posterior probability (PP = 0.96). Within this clade, *N. ucranica* was recovered as the earliest-diverging species, with the remaining three species (*N. hispanica*, *N. congesta* and *N. heliotropifolia*) forming a moderately supported clade (PP = 0.86). The tree resulting from the ML analysis showed a similar topology (identical for section *Oxynepea* species), although support values were generally weaker (see Fig. S1).

The dated ITS phylogeny of *Nepeta* sect. *Oxynepea* and close relatives is shown in Fig. 2b. The sect. *Oxynepea* clade was estimated to have split from its sister group at some point between the late Miocene and the Pliocene (highest posterior density, HPD: 3.38–10.22 Ma; PP = 0.83). The most recent common ancestor (MRCA) of the extant *Oxynepea* species had an estimated age between the late Miocene and the early Pleistocene (HPD: 2.01–8.10 Ma; PP = 0.95), while the common ancestor of *N. hispanica*, *N. congesta* and *N. heliotropifolia* had an estimated age between the Pliocene and the Pleistocene (HPD: 0.55–4.07 Ma; PP = 0.96).

3.2. Phylogeographic analyses

The geographic distribution and relationships of ptDNA haplotypes are shown in Fig. 3. A high intraspecific haplotype diversity (16 extant haplotypes; 22 missing haplotypes) and a high number of nucleotide substitutions were detected. In the haplotype network (Fig. 3b), haplotype A was interpreted as ancestral given that it is linked to the outgroup species (*N. ucranica*), has the highest frequency (widely distributed across CE Spain in five populations) and shows the highest number of connections. Haplotype A was also found in *N. stricta*, although the *psbJ-psbA* sequence of this species differed from that of haplotype A individuals of *N. hispanica* in the presence of a 5-bp insertion (indels were not considered when building the haplotype network). From the ancestral haplotype A, eight derived lineages radiate, each of them including between one and four haplotypes separated from the ancestral A by one to six substitutions. The B1-B3 lineage formed a loop with the ancestral haplotype.

The geographic distribution of haplotypes (Fig. 3a) shows that the same haplotype, or phylogenetically close ones, can be found in very distant populations. For example, the derived haplotype B2 is found in two populations, one of them in central Spain (RPI) and the other in northeastern Spain (CAN). More strikingly, haplotype B1 is found in northeastern Spain (CAN), southeastern Spain (PFA) and the single Moroccan sample (MOR). Furthermore, very distant haplotypes can be found in the same or geographically close populations. For example, the population in southeastern Spain (PFA) contains the distant haplotypes C4 and B1/B3, and a population in northeastern Spain (CAN) contains the ancestral haplotype A together with the distant derived haplotypes G2 and B1/B2. Regarding diversity patterns, the highest numbers of haplotypes and haplotype diversity were found in central, northeastern

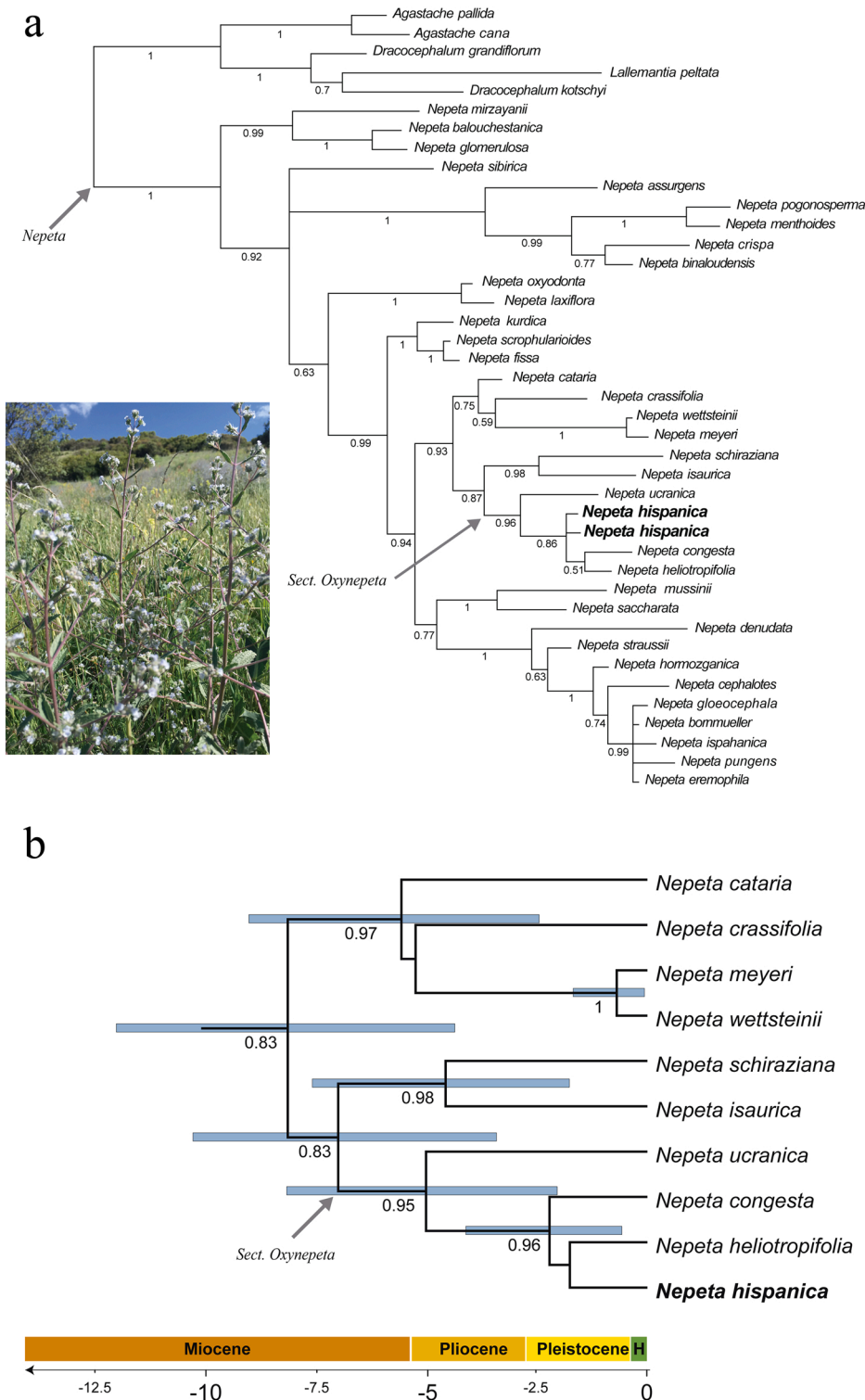


Fig. 2. Phylogenetic position of *Nepeta hispanica* within the genus, and divergence times of *Nepeta* sect. *Oxynepea* and its closest relatives based on ITS sequences. (a) Majority-rule consensus tree obtained in the Bayesian phylogenetic analysis of 35 species of the genus *Nepeta* and five outgroup species. (b) Dated phylogeny of *Nepeta* sect. *Oxynepea* and close relatives; for clarity, other lineages included in the analysis are not shown in this figure (the dated phylogeny for the complete set of species is shown in Fig. S2). In both trees, numbers below branches are Bayesian posterior probabilities. In b, bars indicate 95% highest posterior density intervals for divergence times. The clades representing the genus *Nepeta* and section *Oxynepea* are indicated with arrows.

and southeastern Spanish populations (five populations with 3–4 haplotypes per population, frequently from distant lineages, and H_d ranging from 0.511 to 0.733; cf. Table S6). Five Spanish populations had no haplotype diversity (a single haplotype per population, $H_d = 0$). Various degrees of differentiation were found according to pairwise S_{nm} values. Two distant populations (RPI-CAN) displayed the only non-significant value ($S_{nm} = 0.54$), while significant values ranged from 0.66 (RPI-RCO) and 1.00 (27 comparisons).

The results of the DPA are shown on Fig. 4. The most probable

location of the most recent common ancestor of all plastid lineages of *N. hispanica* was the Ebro river basin in north-eastern Iberia (PP = 0.54), followed by the Tagus basin in central Iberia (PP = 0.41). According to Bayes factors, the best-supported migration route was that from the Ebro basin to the Tagus basin (BF = 18.8), followed by the route from the Ebro basin to the Guadalquivir basin (BF = 9.41). Six other migration routes connecting adjacent and distant areas received positive support (BF > 3; Kass and Raftery, 1995).

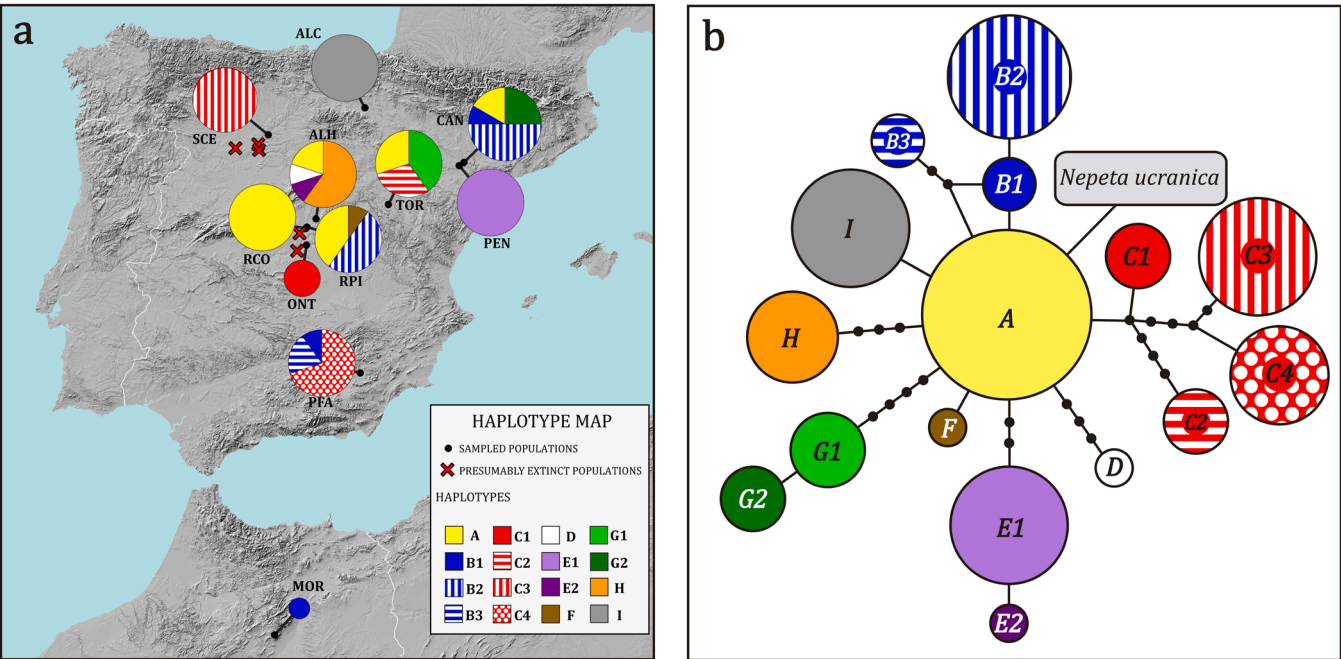


Fig. 3. Phylogeographic analysis of *Nepeta hispanica* based on plastid DNA regions *trnS-trnG* and *psbJ-petA*. Each colour represents a haplotype. (a) Geographic distribution of haplotypes across the Iberian Peninsula and northern Africa. Pie charts indicate the frequencies of haplotypes in populations. Pie chart sizes are proportional to the number of sampled individuals per population. (b) Haplotype network. Circle sizes are proportional to haplotype frequencies. Each line represents a nucleotide substitution, and black dots represent missing haplotypes (extinct or not sampled).

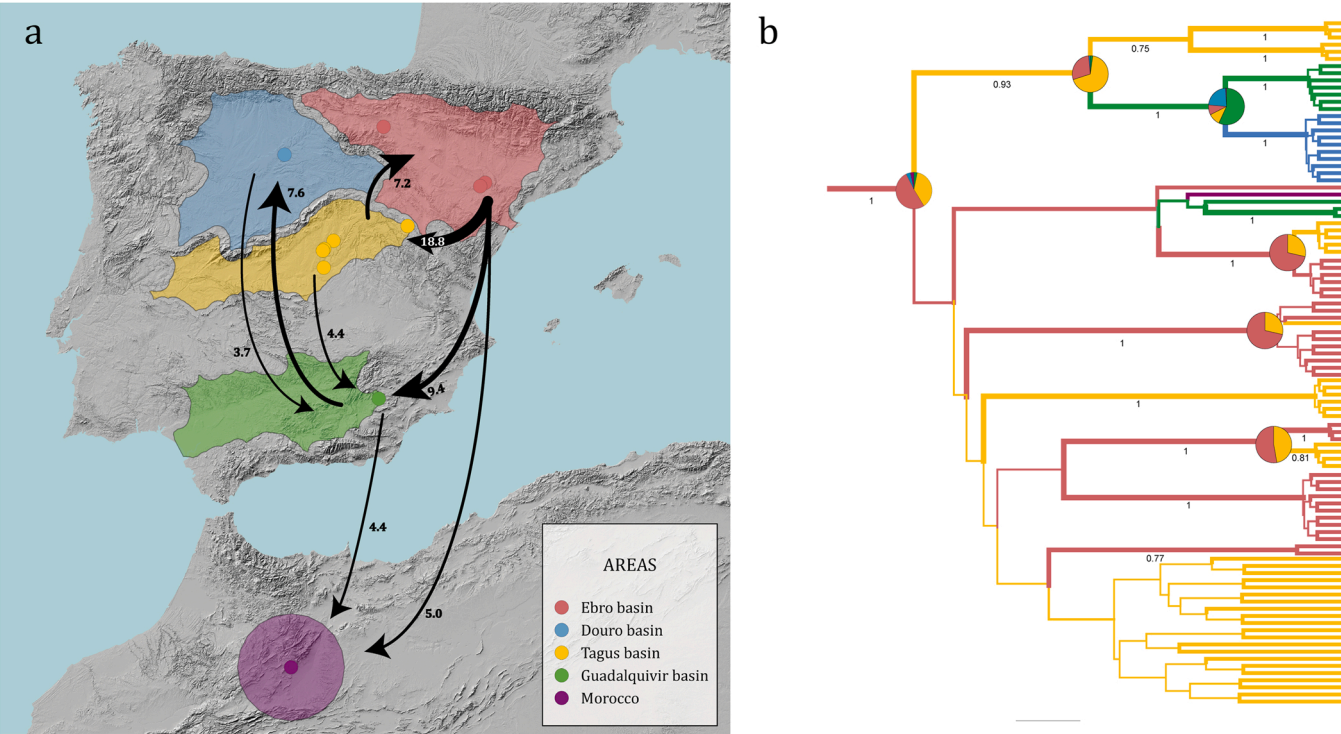


Fig. 4. Discrete phylogeographic analysis (DPA) of *Nepeta hispanica* in the Iberian Peninsula and northern Africa based on plastid DNA sequences. (a) Map of sampled populations in the five geographic areas (four river basins in the Iberian Peninsula, and northern Africa). Arrows represent routes of migration between areas with Bayes factor (BF) values above 3 (numbers and width indicate BF values). (b) DPA maximum clade credibility tree, in which branch widths represent posterior probabilities, and colours indicate areas as in (a). Numbers below branches show posterior probabilities above 0.75. Pie charts show probabilities of ancestral areas for nodes with posterior probability above 0.9. The outgroup is excluded for clarity.

3.3. Ecological niche modelling

Fig. 5 shows the result of the maximum entropy distribution

modelling, both for the present and for past time slices. Percent contributions and permutation importance of variables are shown in Table 1. According to these, the most relevant variables included

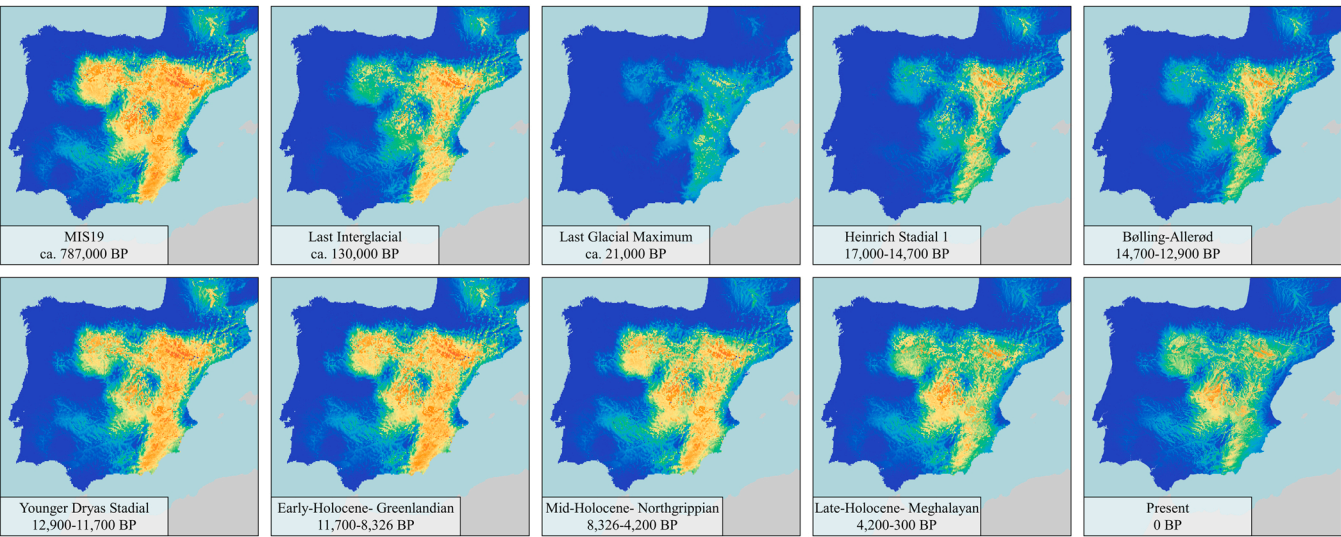


Fig. 5. Ecological niche modelling of *Nepeta hispanica* in the Iberian Peninsula based on climatic, edaphic and lithological variables. Environmental suitability values for the present and nine past time slices according to maximum entropy modelling are represented, with colder colours indicating low suitability, and warmer colours indicating higher suitability.

Table 1
Bioclimatic, edaphic and lithological variables included in the ecological niche model of *Nepeta hispanica*, sorted by their percent contributions to the model. Permutation importance and effect direction are also shown for every variable.

Variable	Variable description	Percent contribution	Permutation importance	Effect direction
bio16	Precipitation of wettest quarter	35.1	82.2	Sigmoid-negative
eda09	pH index measured in water solution	22.4	0.0	Neutral
bio04	Temperature seasonality	19.0	5.9	Linear-positive
iberlit	Lithology	19.0	7.4	Preference for categories “gypsic” and “fine-grained clastic”.
bio11	Mean temperature of coldest quarter	2.7	1.6	Hump-shape
eda06	Cation exchange capacity of soil	0.6	1.3	Sigmoid-positive
eda03	Weight percentage of clay particles (<2.10–4 mm)	0.4	0.5	Linear-negative
bio17	Precipitation of driest quarter	0.3	0.1	Almost neutral-negative
eda02	Volumetric percentage of coarse fragments (>2 mm)	0.3	0.7	Almost neutral-positive
bio08	Mean temperature of wettest quarter	0.1	0.3	Neutral
eda07	Soil organic carbon content	0.1	0.1	Sigmoid-negative
eda05	Weight percentage of sand particles (0.05–2 mm)	0.0	0.0	Neutral
bio09	Mean temperature of driest quarter	0.0	0.0	Neutral

climatic (precipitation of wettest quarter, temperature seasonality), edaphic (soil pH) and lithological variables. Suitable areas for *N. hispanica* in the present were estimated throughout the northeastern half of the Iberian Peninsula, with the highest suitability values in areas with continental climate and gypsic or fine-grained clastic lithologies (gypsum, gypsiferous marl, lutite, silt, clay). Projections to past time slices showed a relatively constant distribution range, with some waxing and waning. In particular, the greatest range contraction was obtained for the Last Glacial Maximum (LGM), when estimated suitable areas were confined to the eastern continental areas of the Iberian Peninsula.

4. Discussion

This study provides a two-scale picture of the evolutionary and biogeographic history of the gypsum-associated Mediterranean species *Nepeta hispanica*. On the one hand, phylogenetic results confirm a close relationship between the western Mediterranean *N. hispanica* and the other geographically distant species of *Nepeta* sect. *Oxynepete*, distributed in the eastern Mediterranean and Irano-Turanian regions. On the other hand, phylogeographic results based on plastid DNA revealed a remarkable lack of genetic isolation between the distant island-like patches making up the distribution range of *N. hispanica*.

4.1. A western Mediterranean representative of a predominantly Irano-Turanian clade

Phylogenetic analyses are congruent with the classical morphological criterion used to define *Nepeta* sect. *Oxynepete* (Budantsev, 1993). Indeed, results support that dioecy, the diagnostic character of the section, has evolved only once in the genus *Nepeta*. As a result, the four sampled species of sect. *Oxynepete* (out of a total of five currently accepted species) form a monophyletic group in our ITS phylogeny. Additionally, the only species of sect. *Oxynepete* not included in the ITS phylogeny (*N. stricta*) displayed plastid DNA sequences nearly identical to those of *N. hispanica* and *N. ucranica*. This makes us believe the group would probably still be still monophyletic if *N. stricta* was included in the ITS phylogeny. The common ancestor of this group is estimated to have split from its sister lineage around the late Miocene to early Pliocene, possibly in the eastern Mediterranean or Irano-Turanian region, based on the distribution ranges of species in the sister lineage (*N. isaurica*, *N. schiraziana*) and of the earliest-diverging species of sect. *Oxynepete* (*N. ucranica*). It is also estimated that, at some point in the last 4 million

years, a lineage of sect. *Oxynepetea* migrated to the western Mediterranean, eventually became isolated and gave rise to a distinct species, *N. hispanica*. Therefore, *N. hispanica* stands as the only western-Mediterranean representative of this otherwise eastern clade. This longitudinal Mediterranean disjunction has been widely discussed for different species (Feliner, 2014; Manafzadeh et al., 2014; Thompson, 2020). It has long been hypothesised that migrations from the eastern Mediterranean and the Irano-Turanian region to the western Mediterranean occurred mainly in the Messinian Age (7.2–5.3 Ma), coinciding with the Messinian Salinity Crisis (Bocquet et al., 1978). However, this hypothesis has received limited phylogenetic support so far, since the disjunctions involving taxa from the eastern vs western Mediterranean Basin have turned out to have a Pliocene or even more recent origin (Kadereit and Yaprak, 2008; Xiang et al., 2017; Seidl et al., 2020). The divergence of *N. hispanica* appears to have happened also more recently (Pliocene or later), and therefore may be the result of long-distance dispersal between environmentally similar eastern and western habitats. It should be noted that our phylogeny (based on that of Jamzad et al., 2003a) has been obtained using a single DNA region and a limited sample of *Nepeta* species, and divergence time estimates have wide confidence intervals. While divergence times should be taken with caution, we consider that the close relationship between *N. hispanica* and the eastern species of sect. *Oxynepetea* is well supported based on the congruence of ITS sequences and morphology. A more deeply sampled time-calibrated phylogeny of *Nepeta* based on multi-locus data combined with explicit biogeographic analyses will be needed to get deeper insights into the diversification history of Mediterranean and Irano-Turanian *Nepeta* lineages (e.g. Manafzadeh et al., 2014; Gorospe et al., 2020).

4.2. Lack of phylogeographic structure despite an archipelago-like range

We hypothesise that the migration and arrival of the ancestor of *N. hispanica* in the western Mediterranean from the east (eastern Mediterranean and Irano-Turanian regions) happened along the northern Mediterranean coast (southern Europe), a route also followed by the genus *Haplophyllum* (Manafzadeh et al., 2014). Indeed, the most likely ancestral areas for *N. hispanica* populations are found in central and northern Iberia, and the haplotype of the only sample from northern Africa (Morocco) is derived from an ancestral Iberian haplotype and is shared with several Iberian populations. Colonisation of northern Africa was likely the result of at least one relatively recent event of long-distance dispersal from the Iberian Peninsula (Blanco-Sánchez et al., 2021; Terrab et al., 2008). Consequently, allopatric differentiation across the Strait of Gibraltar between subsp. *statice* (Morocco) and subsp. *hispanica* (Spain) appears to be limited, at least based on ptDNA sequences (cf. Fernández-Mazuecos and Vargas, 2010; Martín-Rodríguez et al., 2020). Nevertheless, additional northern African samples would be necessary to confirm these patterns and evaluate the evolutionary history of the species in this region. Because of this limited availability of information about northern African populations, here we have focused on disentangling the phylogeographic structure of the better-known Iberian populations.

Since the establishment of *N. hispanica* in the Iberian Peninsula, estimated to have happened during the late Pliocene or Pleistocene (not before 4 Ma), a history of sequential colonisation of areas started, with substantial migration between areas in multiple directions, as revealed by ptDNA lineages. Throughout Iberian populations, high variability and low phylogeographic structure of ptDNA sequences is observed at the global and population scales, which is congruent with a history of genetic connection rather than isolation. Species distribution modelling revealed a relatively wide extension and temporal continuity of available habitats with continental climates and suitable soils throughout the late Quaternary. Several patches with highly suitable habitats appear to have remained essentially unchanged, with varying degrees of habitat isolation between them through time. All this appears to have allowed

the preservation of high genetic variation and facilitated long-term genetic exchange between populations. The only period in which this temporal continuity was partially interrupted is the LGM (approximately 21,000 years ago, cf. Fig. 5). However, suitable areas remained in the inner-eastern areas of the Iberian Peninsula, where populations thrive nowadays. These areas therefore may have acted as genetic sources from where the remaining populations were recolonised. This hypothesis is in line with DPA results, in which Ebro basin populations are estimated to be the ancestral ones, from where other populations were colonised in a series of back-and-forth events. A north-to-south pattern has been described for some Iberian gypsophytes and xerophytes (Santos-Gally et al., 2012; Salmerón-Sánchez et al., 2017), although the opposite pattern has also been found in other taxa (Pérez-Collazos et al., 2009; Martín-Hernanz et al., 2016; Perfectti et al., 2017). Quaternary climatic oscillations may have favoured expansion of these taxa until reaching the gypsum outcrops available in both directions (Salmerón-Sánchez et al., 2017). These north-to-south and south-to-north long-distance dispersal (LDD) events seem to be related to the migratory routes followed by steppe ancestors from their ancestral Iranian-Turanian area to the western Mediterranean, namely the European route towards Iberia (as suggested for *N. hispanica*) or the African route towards the Maghreb. Given the maternal inheritance of plastids in *Nepeta* (Corriveau and Coleman, 1988), phylogeographic patterns based on ptDNA reflect seed dispersal rather than pollen flow. Although most of the seeds of this species fall in the vicinity of the mother plant (de la Cruz et al., 2011), two hypotheses may explain an active seed dispersal through long distances: epizoochory, as the calyx of this species becomes coriaceous when mature, with hook-like sepals that may get stuck on the fur or feathers of vertebrates, including livestock (Pueyo et al., 2008); and tumbleweed dispersal, in which the whole or a part of a plant (including seeds) is blown by the wind (Mehlman, 1993; de la Cruz et al., 2011).

In addition to long-distance dispersal, we need to consider a potential role of incomplete lineage sorting (Edwards and Beerli, 2000) in the lack of phylogeographic structure found for plastid DNA sequences of *N. hispanica*. This would imply that divergence of haplotypes occurred in a widely distributed ancestral population, followed by fragmentation of populations and incomplete sorting of haplotypes among them. The wide distribution of suitable habitats in MIS19 (c. 787 ka) partially supports this hypothesis, but the wide HPD intervals in DPA prevent any robust conclusions about the timing of haplotype divergences. Future studies based on additional genetic markers will be required to address this question.

In summary, *N. hispanica* does not conform to the assumption of a strong genetic isolation between populations of gypsum-associated plant species resulting from a supposed archipelago-like range (Salmerón-Sánchez et al., 2017; Blanco-Sánchez et al., 2021). Indeed, long-term availability of suitable habitats loosely associated with gypsum (*N. hispanica* is a gypsogastrophyte rather than a gypsophyte) appear to have permitted the preservation of high genetic variation and an active genetic interchange between populations of *N. hispanica*, at least based on plastid DNA markers. Future studies will determine the degree to which this pattern is also supported for genome-wide markers of this species, and for other gypsum-associated species from the western Mediterranean. Understanding the genetic structure of populations may be key for the preservation of this species, whose conservation status may deteriorate in the near future. *Nepeta hispanica* stands as the most threatened non-endemic species in eastern Andalusia, where only a few individuals thrive in a single population (PFA) (Gutiérrez et al., 2019). Several other Spanish populations widely cited in the literature seem to have disappeared or significantly shrunk in recent decades, and the species is rare in Morocco (Fennane, 2018). The high lineage diversity displayed by this species must be taken into account in case in situ or ex situ preservation programmes have to be carried out.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ppees.2022.125699](https://doi.org/10.1016/j.ppees.2022.125699).

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