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Speciation and periodic restricted environments. The case of genus *Ononis* L. (subsections *Natrix* and *Viscosae*)

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ABSTRACT

The morpho–environmental similarity between subsections *Natrix* and *Viscosae* has been pointed out as the reason for the genetic complexity of these groups of taxa. Based on this characterization a question emerges: could a very recent ongoing evolutionary process explain that morpho–environmental similarity? ISSR and cpSSR amplifications for 45 specimens belonging to taxa of *Natrix* and *Viscosae* subsections were developed, along their biogeographic distribution areas. Twenty-nine haplotypes were detected in the biogeographic area of both subsections, 79% were exclusive haplotypes, but the rest is shared between subsections *Natrix* and *Viscosae* species. Could that haplotype sharing be the result of potential hybridization between these taxa? Do current environmental conditions restrict the gene flow among taxa? The combination of ancestral genetic polymorphism, introgression, coalescence processes and periodic restricted environments (PRE) by glacial–interglacial environmental dynamics were discussed to explain the relevant percentage of exclusive haplotypes detected, as well as the persistence of shared haplotypes. These results are in accordance with the morpho–environmental proximity previously described for both subsections.

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Introduction

The morphological differentiation for *Natrix* and *Viscosae* subsections has been based exclusively on their life cycles. Perennial life forms make up the *Natrix* subsection, while annual cycles characterize the *Viscosae* subsection. No other morphological or environmental strategies were observed in the taxonomic and systematic revisions for these groups of plants in the monophyletic genus of the tribe Trifolieae (Steele and Wojciechowski 2003). Nevertheless, a great deal of morphological variability was found for annual forms in contrast to perennial ones. Two morphological strategies have traditionally been used to distinguish taxa in the *Viscosae* subsection: one characterized by *O. pubescens*, where no infraspecific taxa were detected, and the other by *O. viscosa*, with a relevant diversity of subspecific variability (Ŝirjaev 1932; Pignatti 1982; Devesa 1987, 2000). In the first case (*O. pubescens*), the combination of dense and compact inflorescences with three-foliate middle leaves and small and wide fruits distinguishes individuals of this species. This combination occurring in the same individual is not observed for *O. viscosa*, where neither the inflorescences are not so dense nor the fruit sizes or the composition of middle leaves are very variable. In fact, the morphological variability for *O. viscosa* has usually been cited as the main reason to describe six different subspecies (Pignatti 1982; Devesa 1987, 2000).

The relevant morphological similarity for the *Ononis* subsections analyzed here was already observed by Rocha et al. (2018). Three morphological trends were suggested in this morpho–environmental approach, where taxa of subsection *Natrix* could be distinguished from those of subsection *Viscosae* without enough statistical significance (very low F-remove for the discriminant analysis).

Genetic approaches for taxa of genus *Ononis* are very scarce, and the methodology used was based on simple sequence repeats (SSR) analysis (Kloda et al. 2008). In the case of subsections *Natrix* and *Viscosae*, no molecular analyses has been published. This characterization is essential to describe the correlation between the morpho–environmental analysis, previously elaborated by Rocha et al. (2018), and the genetic information. Life forms, annuals or perennials, have traditionally been indicated as the way to distinguish these subsections. Based on the morpho–environmental description, no significant differences were observed at the morphological level. Environmentally, subsection *Natrix* revealed the widest variability, in contrast to subsection *Viscosae* where just *O. pubescens* and *O. breviflora* showed high variations. In this sense, the molecular approach was necessary to confirm the hypothesis suggested by Rocha et al. (2018). These authors proposed *O. natrix* and *O. ramosissima* as probable ancestors of both subsections, since the annual forms should be the mechanism to colonize

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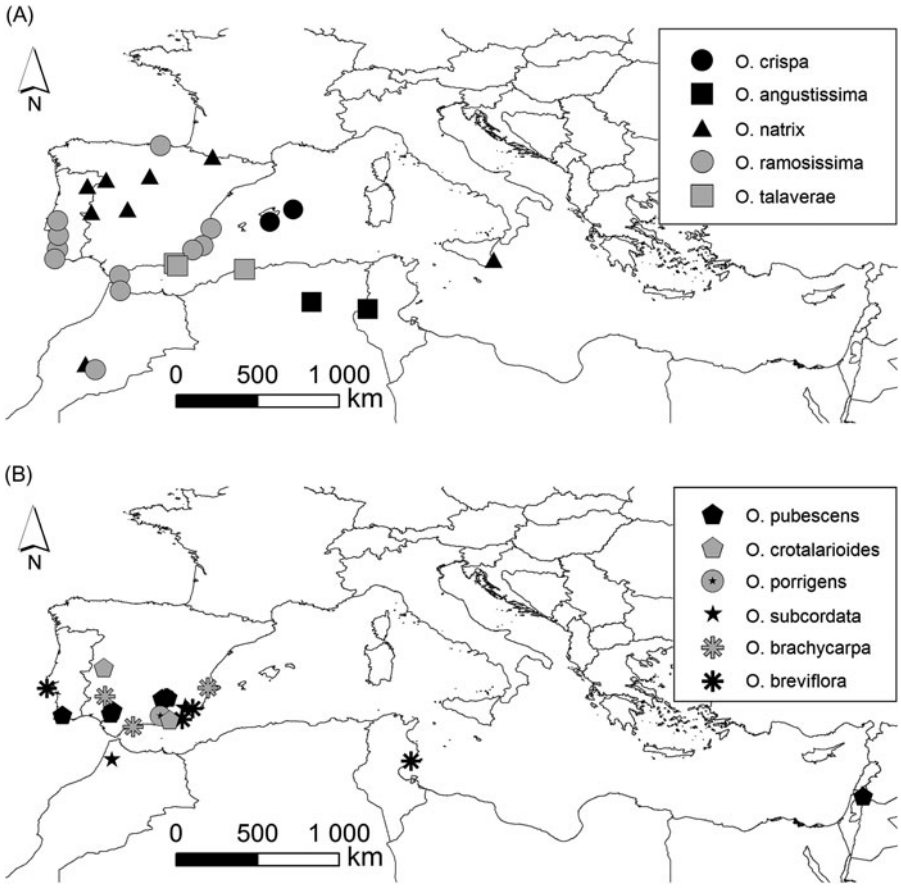

Figure 1. Maps with the location of analyzed taxa, for perennial (subsect. *Natrix*) (A) and for annual (subsect. *Viscosae*) life forms (B). The highest variability of both subsections are observed along the western of Mediterranean basin (Rocha et al. 2018).

inter–glacial environmental restricted areas. That colonization, according to this hypothesis, would be the response of the ancestral haplotype polymorphism of perennial plants to climatic changes, in this case as a result of the recent glacial–interglacial dynamics. Introgression and coalescence processes will be discussed in the light of this biogeographic dynamic hypothesis in order to better describe the evolutionary possibility to distinguish subsection *Natrix* from *Viscosae*. Periodic Restrictive Environments (from now on designated as PRE)—an environmental–spatial driven limitation—will be involved in the glacial–interglacial dynamic, where hybridization mechanisms are not possible and biogeographic isolation is promoted.

Material and methods

Plant material and sampling

The taxa included in subsections *Natrix* are (according to nomenclatural combinations of Devesa 2000 and chromosomal numbers of Devesa 2000 and Baltisberger and Widmer 2006): *O. natrix* L. ($2n = 28$, 30, 32, 64), *O. ramosissima* Desf. ($2n = 32$), *O. talaverae* Devesa & G. López, *O. crispa* L. ($2n = 30$), and *O. angustissima* Lam. ($2n = 30$), and for subsection *Viscosae* are: *O. pubescens* L. ($2n = 32$), *O. viscosa* subsp. *breviflora* (DC.) Nyman ($2n = 32$), *O. viscosa* subsp. *sieberi* (DC.) Širj., *O. viscosa* subsp. *brachycarpa* (DC.) Batt. ($2n = 30$), *O. viscosa* subsp. *crotalarioides* (Coss.) Širj. ($2n = 32$), *O. viscosa* subsp. *subcordata* (Cav.) Širj. ($2n = 32$), and *O. viscosa* subsp. *porrigens* Ball ($2n = 32$). For this work 45 samples were selected from expeditions or voucher specimens in three Iberian herbaria: Herbarium of the University of Trás-os-Montes e Alto Douro (HVR), Herbarium of the University of Salamanca (SALA), and Herbarium of the Royal Botanic Garden of Madrid (MA) (http://sweetgum.nybg.org/ih/herbarium.php? irn¼126327). The relation of these specimens and their original locations are shown in Table 1 and their geographic distribution is represented in Figure 1. According to Rocha et al. (2018), and because of the extreme morpho–environmental similarity between *O. sieberi* and *O. breviflora*, both taxa were here considered as *O. breviflora*.

DNA isolation

Total DNA was extracted from 100 mg of frozen leaf tissue, fresh material from expeditions and dry herbarium material, following the protocol supplied in the DNeasy® Plant Mini Kit (QIAGEN, Düren, Germany). Extracted DNA was quantified by UV spectrometer (Nanodrop® ND-1000, Thermo Fisher Scientific, Waltham, MA, USA) followed by a quality check in 1.0% agarose (w/v) gel electrophoresis using TBE buffer, stained with ethidium bromide. All DNA samples were diluted to a concentration of 10 ng/mL.

ISSR amplification

After pre-screening 14 ISSR primers from the UBC#100/9 set (University of British Columbia, Vancouver, BC, Canada)

Table 1. Relation of analyzed specimens, their locations, herbaria references, cpSSR haplotype detected, and species code used in dendrograms of Figure 2.

Herbarium	Taxon	Location	cpSSR Haplotype	**Dendrogram** Figure 2 **code**
MA719361	*O. natrix* L.	Cáceres, Alcántara, regato de Remolinas, vertiente S de las riberas del Tajo, 29SPD7998, 145 m	I	n_1
HVR21564	*O. natrix* L.	Burgos, Fuentenebro	II	
MA580484	*O. natrix* L.	Toledo, Las Herencias, 400 m	III	
MA793886	*O. natrix* L.	Zamora, Moral de Sayago, presa de Villalcampo, 29TQF4397, 580 m	I	
HVR19446	*O. natrix* L.	Huesca, Balneario de Panticosa, N42,75795 W0,23755, 1375 m	IV	n_2
HVR21565	*O. natrix* L.	Vila Nova de Foz Côa, Pocinho	V	
SALA105967	*O. natrix* L.	Marruecos, pista de Telouet, 31°17′18N 7°11′49W, 1890 m	VI	
SALA106548	*O. natrix* L.	Italia, Sicilia, Siracusa, río Ciane	III	
SALA139741	*O. angustissima* Lam.	Túnez, prov. Gabès, Metlaoui, gorges de Seldja, 34°20′19N 08°19′45E, WGS84, 250 m	VII	a_1
SALA22582	*O. angustissima* Lam.	Algérie, wilaya de Biskra, à 7 km à lW de Foughala et à environ 40 Km au SW de Biskra, 200 m	VIII	a_2
MA550868	*O. pubescens* L.	Jaén, Hornos, Garganta de Hornos, Peña Bermeja, 30SWH2730, 1250 m	X	pu_1
MA348055	*O. pubescens* L.	Jaén, Villanueva del Arzobispo, a los Olmillos, 30SVH9926, 620 m	I	
HVR21568	*O. pubescens* L.	Sevilha, Carmona	IX	
HVR19726/ HVR19727	*O. pubescens* L.	Silves, Ribeira de Odelouca	XI	pu_2
SALA129931	*O. pubescens* L.	Israel, Kinnrot Valley (Upper Jordan Valley), 2 km NE of Kibbutz Haon, 150 m	XIII	
HVR21566	*O. pubescens* L.	Sevilha, Alcalá de Guadaira	IX	
HVR10615	*O. ramosissima* Desf.	Cádiz, La Linea, 30STF8805, 10 m	XIII	
SALA99520	*O. ramosissima* Desf.	Valencia, Catamarruch, 30SYH39	I	
HVR21567	*O. ramosissima* Desf.	Bilbao, Punta Lucero	XIV	r_1
HVR20746	*O. ramosissima* Desf.	Murcia, La Manga	XV	
HVR10890	*O. ramosissima* Desf.	Beira Alentejana, Vila Nova de Milfontes, Almograve, 29SNB1767, C.M. 552	I	
HVR16989	*O. ramosissima* Desf.	Algarve, Vila do Bispo, Sagres, C.M. 609, 50 m	III	
HVR20745	*O. ramosissima* Desf.	Alcácer do sal, Comporta	XVI	r_2
HVR20744	*O. ramosissima* Desf.	8 km WSW de Santarém, Santarém	XVII	
HVR17721	*O. ramosissima* Desf.	Marrocos, na direção de Skoura, 5 km antes de Idelsane, 29RPQ141290, N30°58′31″ W06°45′29.4″, 1100 m	VI	
HVR17384	*O. ramosissima* Desf.	Marrocos. UTM 30 288 197E 39 13 89N.	XVIII	
HVR20750	*O. talaverae* Devesa	Almeria, Cabo de Gata, 30S 0575005 4065617, N36°43′99.7″ W02°09′59.7″	XIX	t_2
SALA91783	*O. talaverae* Devesa	Algeria: près Beni-Haoua, sur les collines littorales, à env. 25 km à lE de Ténès (wilaya de Chlef, ex El-Asnam, ex Orléansville), 50 m	V	t_1
HVR20572	*O. viscosa* subsp. *breviflora* (DC.) Nyman	Jaén, barragem do Tranco de Beas	XX	
HVR20747	*O. viscosa* subsp. *breviflora* (DC.) Nyman	Murcia, minas de Mazarrón, N37,59591° W1,32521°	XXI	
SALA47481	*O. viscosa* subsp. *breviflora* (DC.) Nyman	Almería, Carboneras	XXII	bv_1
HVR19790	*O. viscosa* subsp. *breviflora* (DC.) Nyman	Cascais, Alapraia, monte da Cabeça Gorda, a 200 m da margem direita da ribeira da Caparide	XXIII	
HVR19727	*O. viscosa* subsp. *breviflora* (DC.) Nyman	Silves, Ribeira de Odelariça	XXIV	
SALA139851	*O. viscosa* subsp. *breviflora* (DC.) Nyman	Túnez, gobernación de Sfax, cité de Móez, entre Sfax y Thaenae, 34°40′57N 10°42′06E, 10 m	III	bv_2
HVR20748	*O. viscosa* subsp. *brachycarpa* (DC.) Batt.	Málaga, a 5 km de Benalmadena em direcção a Mijas	I	b_2
SALA12177	*O. viscosa* subsp. *brachycarpa* (DC.) Batt.	Alicante, Sierra Mariola, Moncabrer	XXV	
SALA110103	*O. viscosa* subsp. *brachycarpa* (DC.) Batt.	Badajoz, Bienvenida, Sierra de Bienvenida-Capitana, 29SQC4839, 730 m	I	b_1
SALA18472	*O. viscosa* subsp. *porrigens* Ball.	Granada, La Calahorra, 550 m, bordes de la carretera	I	p_1
HVR20656	*O. viscosa* subsp. *porrigens* Ball.	Tavira, Solteiras	I	p_2
SALA152896	*O. viscosa* subsp. *subcordata* (Cav.) Širj.	Marruecos, Chefchaouen (Kénitra), c. 21 km from Ouazzane on road to Souk Elarba du Gharb; 34°47N 5°45W, FLNM468465, 140 m	XXVI	s_2
MA591712	*O. viscosa* subsp. *subcordata* (Cav.) Širj.	Murcia, Lorca, Sierra de la Torrecilla, Rambla del Hortillo	XXVII	s_1
MA346838	*O. viscosa* subsp. *crotalarioides* (Coss.) Širj.	Cáceres, Mirabel, Castillo de Mirabel	XXVIII	c_1
MA415129	*O. viscosa* subsp. *crotalarioides* (Coss.) Širj.	Almería, Alicún	XXVIII	c_2
MA345784	*O. crispa* L.	Cabrera, entre Cabó d'es Forn y el d'es Palangres, rocas marítimas	II	cp_2
MA343550	*O. crispa* L.	Menorca, Cala de Biniancolle, 31SFE0509, rocas del litoral	XXIX	cp_1

Table 2. DNA profile and polymorphism in *Ononis* using six ISSR primers (TL – Total Loci; EL – Exclusive Loci; %PL – Percentage of polymorphic Loci; Ne - Number of effective alleles; I – Shannon's information Index; He – Expected Heterozygosity).

Primer	Primer sequence (5′-3′)	TL	EL	%PL	Ne	I	He
UBC811	$(GA)_8C$	23	1	100	1.316	0.373	0.225
UBC835	$(AG)_8YC$	14	0	100	1.429	0.417	0.266
UBC857	$(AC)_8YG$	22	1	100	1.502	0.472	0.302
UBC881	$(GGGTG)_3$	14	0	100	1.603	0.527	0.352
UBC886	$VDV(CT)_7$	23	3	100	1.258	0.315	0.185
UBC888	$BDB(CA)_7$	10	0	100	1.504	0.493	0.319
Natrix (average)		16.67	0	100	1.440	0.434	0.277
Viscosae (average)		17.17	0.83	99.03	1.414	0.418	0.265
Total (average)		17.67	0.83	100	1.413	0.418	0.265

Higher average exclusive loci are observed for annual forms (subsection *Viscosae*) than for perennial ones (subsection *Natrix*).

tested in DNA bulks from each subspecies, six primers were selected (UBC811, UBC835, UBC857, UBC881, UBC886, UBC888) for further analysis (Table 2), which combined high polymorphism with clear and reproducible fragments (two replications). Each amplification for ISSR primers was performed in a reaction volume of 20.0 μL containing 10 ng of genomic DNA, 0.5 mM of primer, 10 mM of dNTPs, 25 mM of $MgCl_2$ and 1.0 U of Taq DNA polimerase in the manufacture's buffer (Thermo Fisher Scientific, Waltham, MA, USA). The ISSR-PCR reactions were carried out using the T-Professional Basic thermocycler (Biometra, Germany). After an initial denaturation of 5 min at 94 °C, 45 cycles for 30 s at 94 °C, 45 s at 52 °C, 2 min at 72 °C were performed, followed by a final 10 min extension at 72 °C. PCR products were separated in 1.8% (w/v) agarose gels using TBE buffer, stained with ethidium bromide; the GeneRulerTM 100 bp DNA Ladder Plus (Thermo Fisher Scientific, Waltham, MA, USA) was included as size standard. The electrophoretic patterns of the PCR products were digitally recorded using a Molecular Image Gel-Doc™ XRþ with Image Lab™ Software (BIO RAD, Hercules, CA, USA). The images were analyzed using the same software, which assigns a fragment size to each band using an algorithm based on the 100 bp ladder.

cpSSR amplification

Five chloroplast SSR loci (ccmp3, ccmp4, ccmp6, ccmp7 and ccmp10), were amplified in all samples using the consensus primer pairs designed by Weising and Gardner (1999). The forward primer of each pair was fluorescently labelled with 6-FAM (ccmp3 and ccmp4), HEX (ccmp6) or NED (ccmp7 and ccmp10). PCR reactions were performed in a 20 μL final volume containing 10 ng of template DNA, 0.4 mM of forward and reverse primers, 0.15 mM of each dNTP, 2 mM of $MgCl_2$ and 1.0 U of FIREpol® DNA polymerase in the manufacturer's buffer (Solis BioDyne, Estonia). Amplifications were carried out in a T-Professional Basic thermocycler (Biometra, Germany) initially set for 3 min at 94 °C, followed by 30 cycles of 94 °C for 45 s, 50 °C (ccmp4, ccmp6, ccmp7 and ccmp10) or 55 °C (ccmp3) for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. PCR products were visualized by electrophoresis on 2.5% agarose gels (w/v), in TBE buffer and ethidium bromide staining. Amplified products were run on the ABI Prism® 3730 Genetic Analyzer using the GeneScan™ 500 LIZ® size standard (PE Applied Biosystems, Foster City, CA, USA).

Data analysis

For the genetic similarity analysis, ISSR-PCR products were scored as present (1) or absent (0) on the basis of size comparison, using the GeneRulerTM 100 bp DNA Ladder Plus (Thermo Fisher Scientific, Waltham, MA, USA) to produce a set of binary data. Based on the dominancy of these markers it was assumed that each DNA fragment position corresponds to a locus with two alleles revealed by band absence or presence (Powell et al. 1996).

The resulting binary data matrix of ISSR amplifications was analyzed by means of GeneAlEx software to estimate genetic diversity parameters assuming Hardy-Weinberg equilibrium: the total number (TL) and percentage of polymorphic loci (%PL), the exclusive loci (EL), number of effective alleles (Ne), Shannon's information index (I) and expected heterozygosity (He) (Peakall and Smouse 2006), measured at subsection level.

Genetic similarity matrices among specimens from ISSR data were calculated using the simple matching (SM) similarity index (Sneath and Sokal 1973) and employed to construct UPGMA dendrograms with the NTSYS-pc version 2.20 software package (Rohlf 2005).

Labelled products of cpSSRs were analyzed and sized by means of Peak Scanner v1.0 free software (PE Applied Biosystems, Foster City, CA, USA). Median-joining network analysis (Bandelt et al. 1999) of haplotypes obtained by cpSSR was performed by means of NETWORK 5.0.0.0 software (Fluxus Technology Ltd., Suffolk, England).

Results

ISSR amplification

A total of 106 markers was obtained, with an average of 17.67 per ISSR primer, with 100% of polymorphism per primer (Table 2). Higher genetic diversity parameters were obtained for *Natrix* subsection. The highest numbers of loci were amplified with the UBC 811 and UBC 886 primers. However, the greatest genetic variability was obtained with the UBC811 primers: highest number of effective alleles (Ne = 1.603), information index ($I = 0.527$) and expected heterozygosity (0.352) (Table 2).

The dendrograms elaborated by these amplifications are represented in Figure 2(A) and (B). Wider variability was obtained for subsection *Natrix*, when compared with subsection *Viscosae*. Three groups are observed in these clustering representations (A, B and C) with *O. natrix* present in all of them, while *O. ramosissima* and *O. pubescens* in groups A and B, and the rest (*O. angustissima*, *O. talaverae* and *O. crispa*, for subsection Natrix -Figure 2(A)-, and all the other subspecies of subsection *Viscosae* -Figure 2(B)-) in only one (group B). According to this distribution of genetic distances, *O. natrix* is the most diverse. *O. pubescens* and

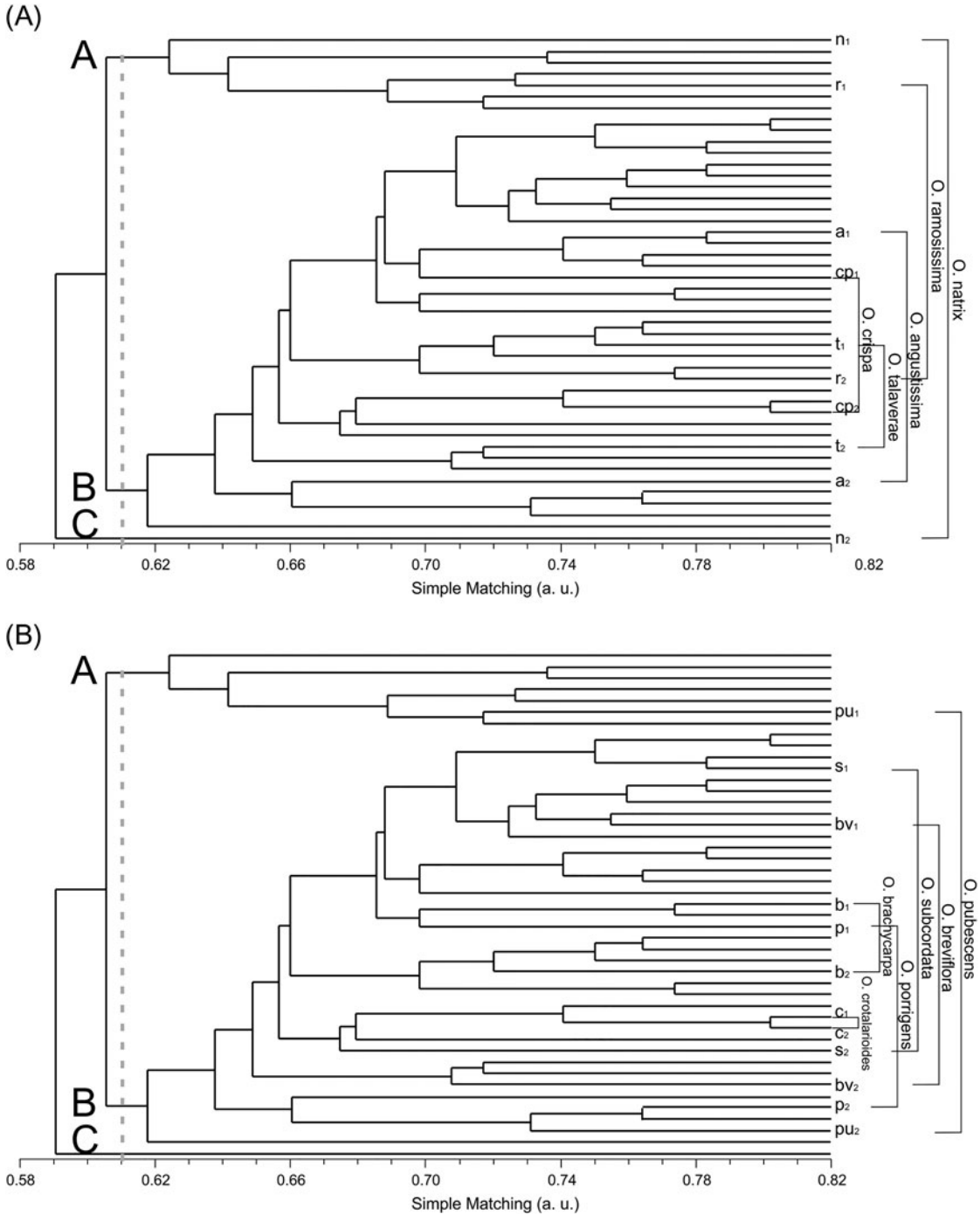

Figure 2. Dendrograms of 45 *Ononis* studied accessions, obtained using UPGMA cluster analysis for ISSR marker data. The genetic variability obtained per taxon is represented on the right of these graphs (and the first three groups obtained: A, B and C) for, perennial (A) and annual life forms (B). Locations for each species range are indicated by code references exposed in Table 1.

O. ramosissima showed a remarkable variability too, and the other taxa presented a more restricted behavior. In this last case, *O. angustissima* and *O. subcordata* were the most variable ones (Figure 2(A)) in contrast with *O. brachycarpa* and *O. crotalarioides*, or the intermediate range of variability of *O. porrigens* (Figure 2(B)).

cpSSR amplification

A high polymorphism was detected for both subsections, based on the 29 haplotypes obtained for the five cpSSR loci amplified in the 45 samples analyzed and their frequencies (Table 1 and Figure 3): 20% in haplotype I; 9% in haplotype III; 4% for haplotypes II, V, VI, and IX; and 2% for the rest. Haplotypes I and III were represented in six taxa: *O. natrix*, *O. ramosissima*, *O. pubescens*, *O. breviflora*, *O. brachycarpa*, and *O. porrigens*. The best represented taxa per haplotype were *O. ramosissima* (in 32% of haplotypes), *O. natrix* (25% of haplotypes), *O. breviflora* (21%) and *O. pubescens* (in 18%). Haplotypes II, V and VI, all of them detected in *O. natrix*, were shared with other species of subsection *Natrix* (*O. ramosissima*, for haplotype VI; *O. talaverae*, for haplotype V; and *O. crispa*, for haplotype II). Haplotype IX was just shared

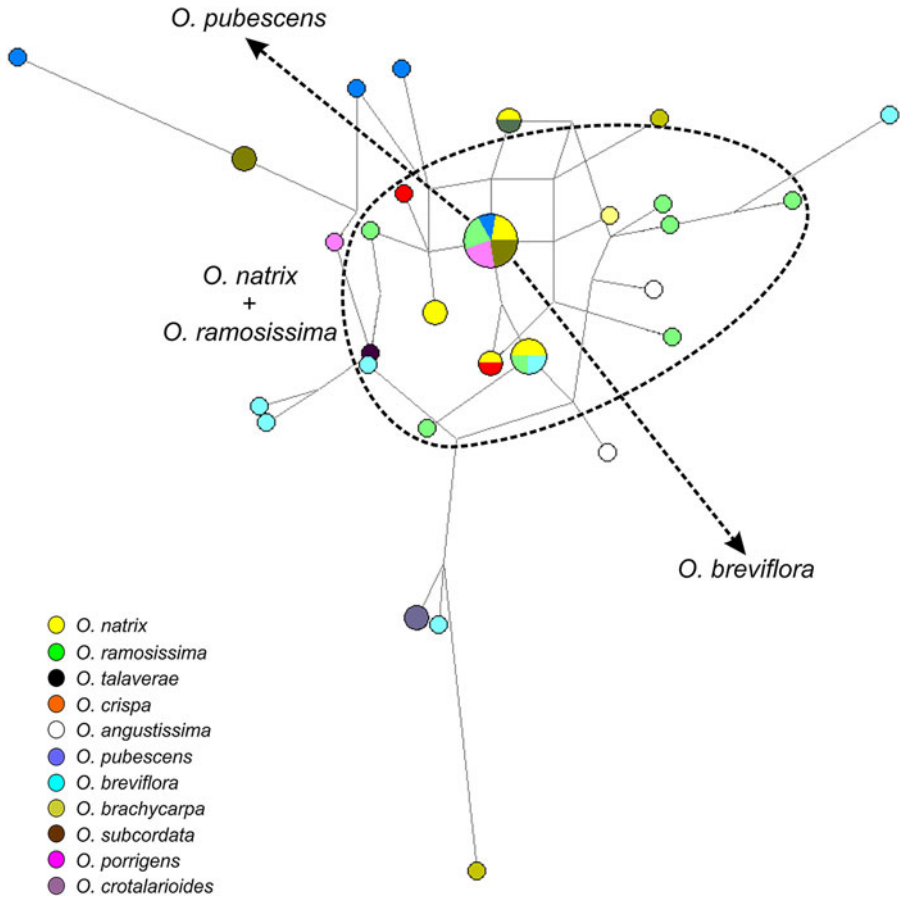

Figure 3. Evolutionary network obtained from the cpSRR amplification for the taxa analyzed. Perennial forms, *O. natrix* and *O. ramosissima*, are concentrated in the center of the haplotype presentation, while annual forms, *O. pubescens* and *O. breviflora*, show opposite and divergent trends.

between taxa of subsection *Viscosae* (*O. pubescens* and *O. porrigens*). Only *O. angustissima* had no haplotypic combination with any other taxon, but two different cpSSR profiles were obtained for this taxon (haplotype VII and VIII). A similar result was observed for *O. crotalarioides*, where only one exclusive haplotype was obtained (haplotype XXVIII). Two opposite trends are observed in the network of Figure 3, both characterized by taxa of subsection *Viscosae*: *O. pubescens* and *O. breviflora*. The highest variability for subsection *Natrix* is supported by *O. ramosissima*, with more haplotypic variability than *O. natrix*. The geographic distribution of these haplotypes is represented in Figure 4(A), for taxa of subsection *Natrix*, and Figure 4(B), for taxa of subsection *Viscosae*.

Discussion

A remarkable similarity for both *Natrix* and *Viscosae* subsections, morpho–environmentally revealed by Rocha et al. (2018), was confirmed again in this work (now at the genetic level). At the same time, two of the species of subsection *Natrix*, *O. natrix* and *O. ramosissima*, and two of the subsection *Viscosae*, *O. pubescens* and *O. breviflora*, were shown to have an important genetic role in explaining the short genetic distance between both subsections. High polyploid variability has been observed for *O. natrix* (Devesa 2000), which could suggest this taxon as a potential ancestor of both subsections, confirmed by its wider environmental profile as a response to higher ecological variability (Han et al. 2015). In this sense, a huge molecular diversity has been observed through both subsections, more remarkable for *O. natrix*, *O. ramosissima* and *O. pubescens*. Not just the ISSR but the cpSSR amplifications confirmed the results obtained by Rocha et al. (2018), where the highest morphological and environmental variability was observed in these three taxa. Twenty-one of the twenty-nine haplotypes observed by cpSSR amplification were concentrated in four taxa (*O. natrix*, *O. ramosissima*, *O. pubescens* and *O. breviflora*), while 74% were exclusive haplotypes for subsection *Natrix* and 77% for *Viscosae* (more frequent for *O. subcordata*, *O. brachycarpa*, *O. porrigens* and *O. crotalarioides*). On the other hand, three haplotypes were shared between subsection *Natrix* and *Viscosae*. In this sense, the diversification of subspecies of *O. viscosa* showed an important disconnection from taxa of subsection *Natrix* (with very low haplotype sharing percentages, 10%). That genetic connection between subsection *Natrix* and *Viscosae* was observed along four taxa: *O. pubescens*, *O. breviflora*, *O. brachycarpa* and *O. porrigens*. Just two subspecies of *O. viscosa* had no haplotype sharing (*O. crotalarioides* and *O. subcordata*). The morpho–environmental proximity between both subsections could be explained by this genetic share, where migrations episodes should be necessary to join these taxa biogeographically. Introgression processes (Mallet 2005) and the haplotype coalescent emergence involved (Velasco 2010) could be the main consequence of a

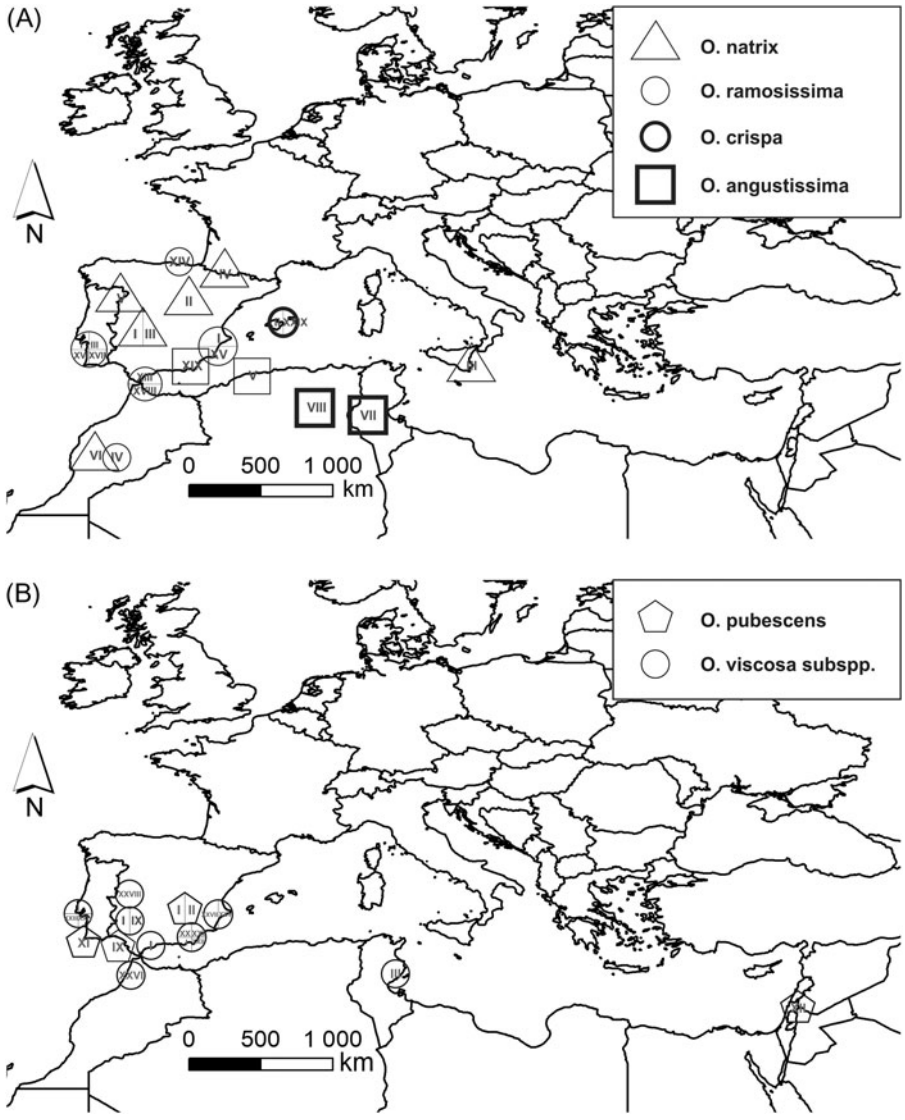

Figure 4. Geographic distribution of cpSRR haplotypes of analyzed taxa, for perennial (subsect. *Natrix*) (A), and for annual (subsect. *Viscosae*) life forms (B). High haplotypic variability are concentrated in southern of Iberian Peninsula and northern Morocco for both subsections.

periodic gene flow, as suggested for other taxa of the Mediterranean flora (Blanco-Pastor et al. 2012; Eaton and Ree 2013; Jordan et al. 2017).

This molecular proximity between all the taxa of both subsections has been morpho–environmentally described previously (Rocha et al. 2018). In this work, much more morpho–environmental variability was obtained for *O. natrix*, *O. ramosissima*, *O. pubescens*, and *O. breviflora*, once again the most genetically similar four taxa. The percentage of common haplotypes shared between them could be described by monophyletic relationships (Steele and Wojciechowski 2003; Turini et al. 2010). Those monophyletic behaviors have been described in Leguminosae, supported by their significant loci polymorphism as a consequence of high frequencies of genome rearrangement (Doyle and Luckow 2003; Tangphatsornruang et al. 2009; Yoder et al. 2013; Martin et al. 2014).

In the case of the rest of the perennial and annual forms, distributions and environmental profiles were clearly more restrictive, especially when compared with *O. natrix* and *O. ramosissima*. This biogeographic trend could be described as an adaptive process to diverse environmental pressures (Davis et al. 2005; Turner et al. 2010; Anderson et al. 2011). For the taxa analyzed here, and according to the strong recent glacial and interglacial horizontal dynamics (Perrin and Bosellini 2012) and the consequent glacial–induced altitudinal and geographical migrations, periodical processes of connection and disconnection between them might be involved. A process such as this promotes PRE, since restricted environmental profiles emerge under these periodic changes. Based on the coalescent theory, introgression processes among taxa should be forced (Schaal et al. 1998; Cruzan and Templeton 2000). At the same time, environmental conditions also restrict the hybridization processes (Jordan et al. 2017). Thus, ecological reasons may restrict genetic recombinations and, consequently, the capacity to isolate haplotypes, as an explanation for obtaining a relevant number of exclusive haplotypes. In this case, those periodic connections between wider environmental profile taxa (*O. natrix*, *O. ramosissima*, *O. pubescens* and *O. breviflora*) with

those isolated under more restricted environmental conditions (*O. angustissima*, *O. talaverae*, *O. crispa*, *O. subcordata*, *O. brachycarpa*, *O. porrigens* and *O. crotalarioides*) explain the occurrence of shared and exclusive haplotypes as a result of successive transitory hybridizations processes. The combination of 29 haplotypes and five loci obtained in this work describe a remarkable molecular plasticity, as well as the genetic continuity along the taxa of both subsections.

Based on this hypothesis, the combination or rearrangement of genome frequencies by glacial–interglacial geographic isolation processes (Feliner 2014) demostrates the relevance of extreme environmental variability in recent periods. The gene flow associated with those environmental variations explains coalescence and introgression phenomena between the complex *O. natrix*, *O. ramosissima*, *O. pubescens*, and *O. breviflora* and the other taxa. A similar possibility has been suggested by several authors for other Leguminosae genera (Byrne et al. 2002; Ramos et al. 2009; Yoder et al. 2013; Liu et al. 2017) and other botanic families (Gutiérrez-Larena et al. 2002, Palme et al. 2004; Molins et al. 2011; Blanco-Pastor et al. 2012; Tamaki and Okada, 2014; Valcárcel et al. 2017) as a gradual speciation (Pfeil et al. 2017). In the case analyzed here, the low haplotype sharing between *O. natrix* or *O. ramosissima* with the species of subsection *Viscosae* (only three, 23% of haplotypes detected for this subsection), as well as the huge percentage of exclusive haplotypes for the other subspecies of *O. viscosa* (more specially *O. subcordata*, *O. crotalarioides*, *O. brachycarpa* and *O. porrigens*, 77% of haplotypes), better explains the restrictive environmental conditions for most of the subspecies of *O. viscosa*, and the strong current PRE that they are subject to. On the other hand, the results obtained for the other perennial taxa (*O. crispa* and *O. angustissima*), also exposed to narrow current PRE, confirm this environmental relevance in the evolutionary process for these subsections. The expansion of subsection *Natrix* to the Arabic-Saharian region or to the Balearic Islands, and their latter biogeographic isolation from *O. natrix* and *O. ramosissima*, are reflected in the low percentage of haplotype sharing (for *O. crispa*), as well as in the occurrence of exclusive haplotypes (for *O. angustissima*).

The low percentages of shared haplotypes is interpreted here as the ancestral polymorphism subject to periodic glacial–interglacial environmental restrictions, as detected for several other taxonomic groups (Gutiérrez-Larena et al. 2002; Bowie et al. 2006; Vitelli et al. 2017). In this sense, the shared haplotypes could be considered as the rest of that ancestral genetic polymorphism, and the exclusive haplotypes as the results of biogeographic and genetic restrictions by cyclical PRE. That ancestral polymorphism is suggested based on the polyploidy detected in *O. natrix*, and its relevance to share haplotypes with the other taxa, as a response to successive PRE along the Pliocene–Holocene period (Han et al. 2015).

Results like these are explained by the intensive climatic changes, topographic and geologic barriers observed along the western Mediterranean basin in the Pliocene–Holocene period (Blanco-Pastor et al. 2012; Jiménez-Moreno et al. 2013; Cheddadi et al. 2016; Tosal and Martín-Closas 2016; Valcárcel et al. 2017), with summer drought intensification (Rundel et al. 2016). The current climatic complexity of these areas (Rodríguez-Puebla et al. 1998; Knippertz et al. 2003) could be the consequence of an equally diverse recent climatic evolution (Goñi et al. 1999; Sánchez Goñi et al. 2002). Climatic dynamics like this have been used to explain the concentration of phylogeographic refugia in this region (Brito 2005; Gómez and Lunt 2007; Médail and Diadema 2009), and the subsequent genetic isolation and the relevant percentages of exclusive haplotypes for taxa with restricted distributions (*O. crispa*, *O. talaverae*, *O. brachycharpa*, *O. porrigens*, *O. subcordata* and *O. crotalarioides*. Environmental profiles associated with climatic dynamics for taxa with large potential distributions (wider environmental profiles) involve the possibility of colonizing areas, where no occurrence was available under current climatic conditions (Rocha et al. 2017). Based on this hypothesis, the Iberian Peninsula and the northwestern Africa could have hosted most of the taxa of subsections *Natrix* and *Viscosae*. So, the genetic diversity detected here may be the consequence of the admixture of lineages colonizing this region (Petit et al. 2003): ancestral genetic polymorphism (shared haplotypes) exposed to isolation processes (exclusive haplotypes), and periodic introgressions and coalescence along glacial periods, when the contact probability between individuals was higher (Jesus et al. 2006). This explanation is also supported in the biogeographic ancestry hypothesis (Shriver and Kittles 2004), where ice ages could be discussed as the environmental factors to explain current biogeographic distributions.

Conclusions

According to this set of results and the possible interpretations suggested here, the strategy of perennial and annual life forms were the result of colonizing and isolation processes under diverse climatic dynamics or PRE. *O. natrix*, *O. ramosissima*, *O. pubescens* and *O. breviflora* showed higher morpho–environmental and genetic variability, in contrast to many other annual and perennial taxa (*O. talaverae*, *O. crispa*, *O. brachycharpa*, *O. porrigens*, *O. subcordata* and *O. crotalarioides*). Introgression and coalescence processes reflect hybridization among taxa, in this case represented by shared haplotypes. Contrary to the expected results based on the intensive climatic variations in the Mediterranean basin along the Pliocene–Holocene period, the shared haplotype percentages was not very relevant (and this was much more concentrated in the more widely distributed taxa). Thus, PRE was also pointed out as the main reason to explain those restricted genetic connection among taxa, and shared haplotypes as the rest of the ancestral common genetic polymorphism. Based on these results, morpho–environmental similarity confirms the genetic dynamic observed for both subsections.

Disclosure statement

The authors declare that they have no competing interests.

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