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When island-like populations at high elevation show genetic divergence despite no morphological variability: the case of *Lupinus montanus* in Central Mexico

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Abstract: *Lupinus montanus* subsp. *montanus* var. *montanus* Kunth is a widespread taxon occurring throughout the highlands of Central Mexico and Guatemala. Populations of this variety show little variation in plant morphology, but their highly disjunct island-like distribution suggests that genetic differentiation between populations should be expected. To test this idea, we assessed genetic diversity among 13 populations of *Lupinus montanus* var. *montanus* growing on the 6 main volcanoes of the Trans-Mexican Volcanic Belt (TMVB) using 4 intersimple sequence repeat markers to quantify genetic divergence. The 93 loci identified showed significant differentiation between populations ($F_{ST} \approx 0.5$) and high levels of genetic diversity among populations (P = 97.9%, He = 0.29, H = 0.44). A clear segregation between eastern and western populations was revealed. Among the eastern populations, we did not find a significant structure, but there was a trend towards a site-dependent effect, indicating very recent divergence. The results reveal in situ diversification events, an East-West split suggestive of older divergence, and more recent incomplete divergence among the eastern populations, which can be attributed to a combination of the impacts of Pleistocene glaciations and the geological history of formation of the TMVB volcanoes. Lack of morphological differentiation may be the product of recent isolation and stabilising selection.

Key words: Intersimple sequence repeat, *Lupinus*, population genetics, species complex, Mexican Volcanic Belt

1. Introduction

Lupinus L. is a diverse genus within Fabaceae, comprising 200–500 species and subspecies (Smith, 1944; Dunn and Gillet, 1966; Dunn, 1984), although recent work has estimated there to be around just 275 species (Eastwood et al., 2008). Most of the taxa are distributed throughout the New World with major centres of species diversity in western North America and the Andes. The genus is thought to have originated in Europe during the Miocene (16–21 Myr BP) (Drummond et al., 2012) and then colonised North America >10 Myr BP. Drummond et al. (2012) showed that *Lupinus* diversified in western North America 5–13 Myr BP and in Mexico 1.2–3.5 Myr BP.

Three geographic regions of the Americas are at the centre of evolutionary radiations: the Rocky Mountains, the Andes, and Mexico. In South America, Hughes and Eastwood (2006) showed that Andean *Lupinus* represent "the most spectacular example of plant species diversification documented to date", which can be explained by the fast orogeny of the Andes, which provided multiple new ecological opportunities for *Lupinus* diversification (Janzen, 1967; Simpson, 1975). The phylogenetic relations within the genus are still under discussion because of lack of resolution (Käss and Wink, 1997; Hughes and Eastwood, 2006). Moreover, some species are highly variable, forming widespread polymorphic species alliances with complex infraspecific taxonomies, as exemplified by the *Lupinus albifrons* (Huang and Friar, 2011) and *Lupinus microcarpus* (Drummond and Hamilton, 2007) complexes in California and the *Lupinus montanus* complex (Dunn and Harmon, 1977) in Mexico, which is the focus of this study.

These alliances are potential examples of incomplete speciation, in which the subspecies/varieties correspond to incipient or ephemeral species, as described by Rosenblum et al. (2012) and Levin (2005). They concluded that 3 main processes are involved in speciation, as suggested by Givnish (2010): 1) the initial origin of genetic differentiation among populations, 2) the evolution of reproductive isolation, and 3) the evolution of ecological divergence in the form of the speciation process. However,

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they do not point systematically to complete speciation (Kocher, 2004). Incipient or ephemeral species often fail to persist due to extinction by hybridisation (after secondary contact) with the parent species (Seehausen, 2006; Behm et al., 2010) and/or through changes in environmental conditions (Thuiller et al., 2005, 2008). In both cases, the slow process of isolation/speciation is cancelled by a much faster one: hybridisation/environmental change. Rundell and Price (2009) also hypothesised that speciation (and, by extension, radiation) can nonetheless be successful, especially when species occupy a large geographic distribution and ecological niche divergence, as is the case for the genus *Lupinus*.

Lupinus montanus Kunth presents a wide but highly disjunct montane distribution from northern Mexico (Chihuahua) to Guatemala (Dunn, 2001). Most of the populations are found in the Trans-Mexican Volcanic Belt (TMVB) at altitudes between 2500 and 4100 m a.s.l., where they grow in forests of pine (*Pinus hartwegii* Lindl.), oak (*Quercus* spp.), and sacred fir [*Abies religiosa* (Kunt.) Schltdl. & Cham.] and in alpine and subalpine prairies (Benitez, 1986; Acosta-Percastegui and Rodríguez-Trejo, 2005). Several allied species (*Lupinus muelleri* Standl., *Lupinus kellermanianus* C.P.Sm., and *Lupinus cacuminis* Standl.), subspecies (*Lupinus montanus* subsp. *montesii*, *Lupinus montanus* subsp. *glabrior*, and *Lupinus montanus* subsp. *montanus*), and varieties of the nominal subspecies (*Lupinus montanus* subsp. *montanus* var. *montanus*, *Lupinus montanus* subsp. *montanus* var. *nelsonii*, and *Lupinus montanus* subsp. *montanus* var. *austrovolcanicus*) have been recognised (Dunn and Harmon, 1977). The 3 allied species differ vegetatively, but their flowers are morphologically very similar (large flowers with the banners reflexing near the midpoint). A key character for identification of the *Lupinus montanus* complex is the presence of unusually long (3–10 cm) adnate stipules (Dunn, 2001).

From the work of Dunn and Harmon (1977), a specific characteristic of most of the taxa of this complex is known to be their extremely restricted distributions; the allied *Lupinus kellermanianus* is reported from 2 volcanoes in Guatemala, while *Lupinus muellerii* and *Lupinus cacuminus* have their main distributions in Cerro Potosí and Nuevo León, Mexico. The subspecies *Lupinus montanus* subsp. *montesii* is known from Sinaloa and Durango, and *Lupinus montanus* subsp. *glabrior* is present in Chihuahua and northern Durango. Concerning varieties, they seem to occur in mixed populations with the nominal form, while *Lupinus montanus* var. *nelsonii* is restricted to the highlands of Oaxaca and *Lupinus montanus* var. *austrovolcanicus* to Volcán Santa María in Guatemala.

In this study, despite the presence of these various morphological taxa, we restricted our sampling to *Lupinus montanus* var. *montanus*, which presents the wider distribution (from Guatemala to Central Mexico) and is morphologically uniform. Most populations of *Lupinus montanus* var. *montanus* are found in the TMVB in Central Mexico, with an island-like distribution across a series of more or less isolated volcanoes of different origins, from the Quaternary (e.g., Pico de Orizaba and Nevado de Toluca) or the Tertiary (e.g., Iztaccihuatl and La Malinche) (Marshall and Liebherr, 2000). This pattern of distribution may represent a factor of diversification and differentiation, which is impossible to detect using purely morphological characters (Dunn and Harmon, 1977).

The aim of this study is to investigate patterns of genetic diversification in the absence of obvious morphological differentiation across these volcanoes in order to address the following questions: Is *Lupinus montanus* var. *montanus* undergoing a diversification/differentiation process despite this morphological uniformity? How do patterns of genetic diversity and the structure of natural populations relate to past geological and climate events? In order to answer these questions we employed intersimple sequence repeat (ISSR) markers, which have been widely used to detect genetic diversity in plants (Ge et al., 2005a; Sica et al., 2005; Meloni et al., 2006; Alam et al., 2009; Escaravage et al., 2011; Gürkök et al., 2013). The ISSR technique presents several advantages: high reproducibility due to long primers that permit the use of stringent annealing temperatures (45–60 °C) and high polymorphism, including within a species (Pradeep Reddy et al., 2002). ISSR markers are dispersed and abundant throughout the genome, and no information about the sequences is necessary before amplification (Wink, 2006). Finally, ISSR analysis only requires a small amount of plant material.

2. Materials and methods

2.1. Biological material

Between August and November 2010, a total of 304 individuals of *Lupinus montanus* var. *montanus* representing 13 populations were sampled from 6 volcanoes of the TMVB: Nevado de Colima (NC: 2 populations), Nevado de Toluca (NT: 2 populations), Ajusco (AJ: 2 populations), Iztaccihuatl (IZ: 3 populations), La Malinche (LM: 2 populations), and Pico de Orizaba (PO: 2 populations) (Figure 1). Collection sites were determined based on previous consultation of several herbarium collections (MEXU, UAGC, IBUG, CIMI, HUMO, TLXM, HUAP, ENCB, MO, K, NY, P, and PC) (Thiers, continuously updated). Geographical coordinates and sample sizes are reported in Table 1. One population of the closely related and sympatric species *Lupinus aschenbornii* S.Schauer (voucher no. 1297311) was also sampled on the IZ volcano to provide an outgroup for analysis.

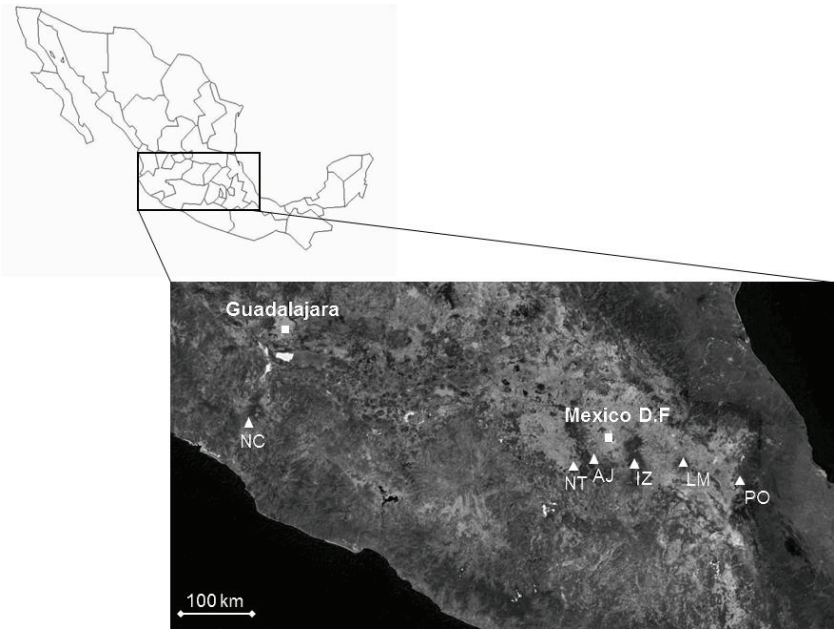

Figure 1. Map of the sampling zone. For details within each site, see the information summarised in Table 1.

Table 1. Populations of *Lupinus montanus* var. *montanus* subjected to ISSR analysis.

Location code	Volcano	Population	Altitude (m)	Latitude (N)	Longitude (W)	Sample size	Voucher number
NC	Nevado de Colima (L. Pal.)	NC2	3787	19°35′3″	103°37′28″	27	Pending
		NC3	3624	19°33′6″	103°36′31″	25	Pending
NT	Nevado de Toluca (L. Pleisto.)	NT1	3940	19°7′28″	99°46′49″	24	Pending
		NT2	3727	19°8′1″	99°47′31″	17	Pending
AJ	Ajusco (Ol-Pl)	AJ1	3551	19°11′60″	99°16′36″	23	1343896
		AJ2	3419	19°11′30″	99°19′58″	26	Pending
IZ	Iztaccihuatl (Ol-Pl)	IZ2	3882	19°8′26″	98°38′54″	27	1297279
		IZ4	4174	19°8′29″	98°38′35″	26	1343914
		IZ5	3581	19°5′11″	98°39′42″	29	432147
LM	La Malinche (Ol-Pl)	LM4	3101	19°16′26″	98°2′32″	17	Pending
		LM6	3690	19°15′6″	98°1′44″	21	1344446
PO	Pico de Orizaba (L. Pleisto.)	PO2	3981	18°59′19″	97°17′56″	23	Pending
		PO3	4387	19°0′28″	97°17′4″	19	Pending

Abbreviations: L. Pal. = Late Paleocene (65 Myr to 50 Myr BP); Ol-Pl = Oligocene-Pleistocene (30 Myr to 1.7 Myr BP); L. Pleisto. = Late Pleistocene (126,000 years to 11,700 years BP).

For DNA extraction, fresh leaves of each individual were collected and dried in silica gel until DNA extraction.

Herbarium voucher material was collected from 3 flowering individuals per population and deposited at the MEXU and ENCB herbaria.

2.2. Total DNA extraction

Dried plant material was ground using a tissue lyser (QIAGEN) at a frequency of 30 revolutions/s and transferred to a 1.5-mL Eppendorf tube. DNA isolation was performed using DNeasy Plant Minikits (QIAGEN)

according to the manufacturer's instructions. DNA concentration was determined with a spectrophotometer at 260 nm.

2.3. ISSR-PCR amplification

Intersimple sequence repeat PCR was performed using 6 different primers (Table 2). Reaction volumes were 25 µL and contained 30 ng of DNA template, 1 µL of primer (50 µM), 1 µL of dNTP (10 mM), 2.5 µL of 5X PCR buffer, 3 µL of $MgCl_2$ (25 mM), and 2.5 units of Taq DNA polymerase (M830A, Promega). Amplifications were carried out using the following program: 4 min of denaturation at 94 °C and 39 cycles of 45 s at 94 °C, 45 s of annealing at 56 °C, 2 min of extension at 72 °C, and a final extension cycle of 10 min at 72 °C.

DNA electrophoresis used 2% agarose gels (280 mL of 1X Tris acetate, 5.6 g of agarose, and 14 µL of ethidium bromide). PCR products (7 µL) were mixed with 3 µL of bromophenol blue and deposited into prepared wells. The run time was about 2 h at 120 V. Bands were visualised and photographed under UV light.

2.4. Data analysis

Only clear, unambiguous, reproducible, and strongly stained bands were taken into account in data analysis, amplified fragments were scored for presence (1) or absence (0), and matrices generated by each primer were assembled.

We used the binary matrix under the Hardy–Weinberg equilibrium to calculate the percentage of polymorphism (P), Nei's gene diversity (h), total Nei's gene diversity (H_e), Shannon's index of diversity (H), the population pairwise F_{ST}, and the level of gene flow (Nm) using PopGene version 1.32 (Yeh et al., 1997). In order to estimate genetic variability within and among populations, the nonparametric test analysis of molecular variance (AMOVA) was carried out using Arlequin software version 3.0 (Excoffier et al., 2005). The correlation between genetic and geographic distances was tested for all populations with Mantel's test (Mantel, 1967) with 10,000 permutations.

2.4.1. Distance analysis

Plant population polymorphisms have frequently been analysed using the UPGMA approach to process genetic band patterns (Li and Ge, 2001; Nan et al., 2003; Sheng et al., 2005; Sica et al., 2005; Qiu et al., 2007). However, this technique does not attempt to make an optimal tree, and successive iterations reducing the matrix do not enable the different evolutionary patterns to be considered.

Therefore, a heuristic search for a dendrogram was carried out by tree-bisection-reconnection (TBR). The distance method is based on the mean character difference; shared bands are not informative in this technique. Distance analysis was performed using PAUP software version 4.0b10 (Swofford, 2001). Dendrograms were visualised with TreeView version 1.5 (Page, 1996). The closely related species *L. aschenbornii* was added to the data set and examined as the outgroup.

The binary data from ISSR fingerprinting were also subjected to principal component analysis (PCA) implemented in R software version 2.14.2 (Ihaka and Gentleman, 1996).

2.4.2. Self-organising map analysis

A further statistical method, the self-organising map (SOM) (Kohonen, 1982), was employed to evaluate differentiation between populations. This artificial neural network uses an unsupervised learning algorithm that achieves the same tasks as conventional statistics but is not biased by the occurrence of rare individuals or nonlinear data (Brosse et al., 2001). The goal of the SOM is to analyse multidimensional data by performing a nonlinear projection of the multidimensional data space onto 2D space (i.e. Kohonen map). The algorithm has an input layer composed of vectors from the data samples and an output layer formed by the Kohonen map or a rectangular grid made up of neurons. The map corresponds to the 2D space in which objects placed in the same neuron are considered similar. Likewise, neighbouring objects in the map are liable to be more similar to each other and belong to the

Table 2. ISSR primers tested for amplification.

Primer	Primer sequence	Amplification pattern	Number of loci
RYCA	RYCACACACACACACA	Smears	13
CARY-T	CACACACACACACART	Unsuccessful amplification	-
GACA3RG	GACAGACAGACARG	Good quality	22
GA8YG	GAGAGAGAGAGAGAGAYG	Good quality	25
G+	WBGACAGACAGACAGACA	Good quality	26
AG8YT	AGAGAGAGAGAGAGAGYT	Good quality	20

Abbreviations: B = C + G + T; R = A + G; Y = C + T; W = A + T.

same cluster. To determine the number of groups of output neurons, we applied hierarchical cluster analysis (the Ward linkage method). The SOM method was performed using the SOM toolbox in MATLAB (MathWorks, 2001). Here, the SOM was trained with different map sizes to find the optimum one to classify individuals. Topographic errors were calculated for each map size as an indicator of topology relevance. Output neurons were then classified into different groups according to the hierarchical cluster analysis. For more details about the SOM method used in this study, see the work of Roux et al. (2007).

2.4.3. Bayesian method

To explore the genetic partitioning of populations and to look for possible introgression/hybridisation events, all the individual patterns were submitted to a Bayesian admixture process implemented in Structure 2.3.3 (Pritchard et al., 2000). This tool was designed to identify the K populations of unknown origin among the sampled individuals and to assign each individual to one or more populations with a probability (q_i). To determine the most probable number of population units K, the program was run 10 times for each different value of K from 1 to 14 (the value K = 14 corresponds to the number of natural populations of *Lupinus montanus* plus 1 population of *Lupinus aschenbornii*), and parameters were set at 10^5 burn-in period steps and 10^6 run-time steps. We used the admixture model with the correlated allele frequencies model. To identify the most reliable number of population units K, we graphed the tested K values against the mean values of the posterior probabilities of the data for each K [referred to as Ln p(D) in software output]. A plateau was reached at K = 7. When several values give equal estimates, the smallest K is thought to be the true value (Pritchard and Wen, 2004).

Individuals were assigned to 1 group if their proportion of membership (q_i) was equal to or higher than 0.80. If not, they were considered as admixture individuals and assigned to 2, or more than 2, populations.

3. Results

Of the 6 primers initially tested, 1 did not produce an amplification pattern, 1 showed smears, and 4 produced clear and reproducible fragments (Table 2). These selected primers gave a total of 93 loci for the 13 populations sampled.

3.1. Genetic diversity and variability

At the species level, total polymorphism was very high at 97.9%. Among sites, polymorphism was 26.6%, and among populations within sites it was 25.7%. At the population level, the mean percentage of polymorphism within populations was 51%, ranging from 38.7% in population AJ2 (Ajusco) to 65.5% in PO3 (Pico de Orizaba).

Total Nei gene diversity was 0.29 ± 0.17, and the Shannon index was 0.44 ± 0.22.

On average, population-paired values of F_{ST} tended to be very high; most were situated around 0.50. All the values for the 3 diversity indexes (P, H, and h) and population-paired values of F_{ST} are summarised in Table 3.

Table 3. Pairwise estimated F_{ST} values among 13 populations of *Lupinus montanus* var. *montanus* and genetic diversity index values within populations.

	IZ2	IZ4	IZ5	LM4	LM6	NT1	NT2	AJ1	AJ2	PO2	PO3	NC2	NC3	P	H	h
IZ2	****													47.3	0.13 ± 0.18	0.21 ± 0.26
IZ4	0.32	****												54.9	0.16 ± 0.18	0.24 ± 0.26
IZ5	0.37	0.28	****											49.5	0.15 ± 0.18	0.23 ± 0.27
LM4	0.54	0.49	0.51	****										54.8	0.17 ± 0.18	0.26 ± 0.26
LM6	0.44	0.33	0.41	0.39	****									60.2	0.20 ± 0.19	0.30 ± 0.27
NT1	0.45	0.43	0.47	0.54	0.45	****								54.9	0.16 ± 0.18	0.24 ± 0.26
NT2	0.42	0.41	0.45	0.50	0.44	0.47	****							55.9	0.18 ± 0.19	0.27 ± 0.27
AJ1	0.53	0.47	0.47	0.53	0.42	0.49	0.47	****						51.6	0.12 ± 0.15	0.21 ± 0.23
AJ2	0.61	0.52	0.54	0.61	0.53	0.60	0.59	0.37	****					38.7	0.09 ± 0.13	0.14 ± 0.21
PO2	0.52	0.43	0.41	0.54	0.40	0.53	0.52	0.35	0.39	****				43.0	0.12 ± 0.17	0.19 ± 0.25
PO3	0.46	0.35	0.38	0.40	0.31	0.43	0.40	0.37	0.45	0.30	****			65.5	0.20 ± 0.19	0.31 ± 0.27
NC2	0.66	0.60	0.59	0.69	0.59	0.60	0.62	0.60	0.67	0.65	0.55	****		46.2	0.10 ± 0.15	0.17 ± 0.22
NC3	0.66	0.60	0.59	0.68	0.58	0.60	0.60	0.59	0.65	0.63	0.55	0.18	****	40.9	0.11 ± 0.16	0.18 ± 0.24

The level of gene flow was estimated at N_m = 0.25. The results of the Mantel test indicated isolation by distance with a significant correlation between geographic and genetic distances (R = 0.71, P-value = 0.003).

The results of AMOVA analysis indicated that 47.7% of the genetic variability was due to differences between individuals within populations.

3.2. Population structure (clustering)

3.2.1. Classical method: distance analysis

The dendrogram showed no clear structure for the 13 populations of *Lupinus montanus* var. *montanus*, except for NC populations, which were gathered in a strongly supported cluster (bootstrap value of 0.92; Figure 2). The much longer branch lengths of the NC populations indicate a greater genetic distance from the other populations. However, segregation seems to emerge between all the populations sampled, because despite nonresolved nodes, individuals were grouped according to their population of origin.

Relationships established by PCA were concordant with distance analysis, showing a clear segregation between NC populations and an overlap including all the eastern populations (Figure 3).

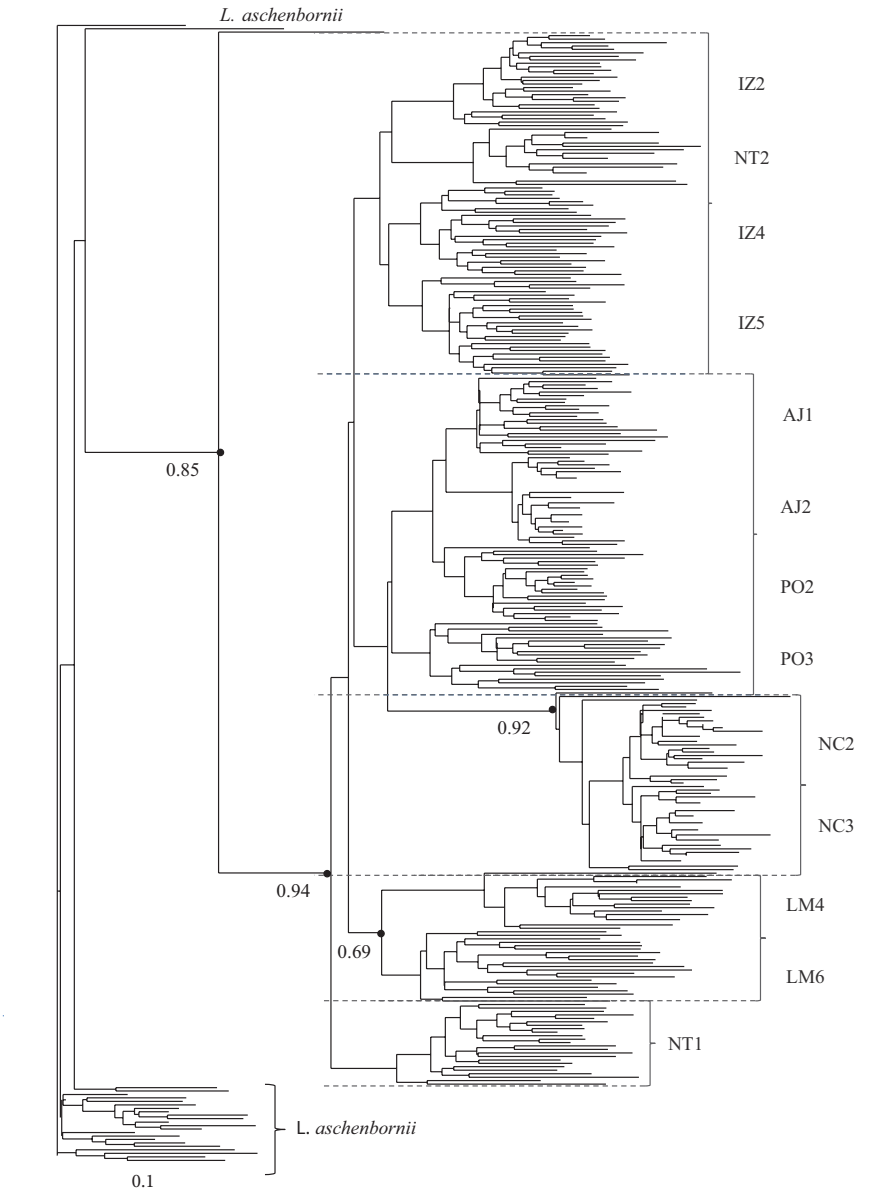

Figure 2. Dendrogram obtained by distance analysis of the 13 populations of *Lupinus montanus* var. *montanus*. The outgroup is the *Lupinus aschenbornii* population.

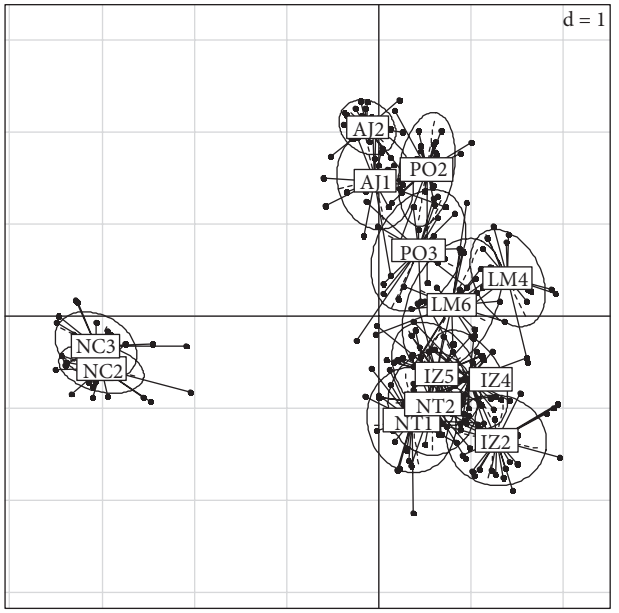

Figure 3. Scatter plots of all individuals of *Lupinus montanus* var. *montanus* based on the first and the second components of PCA using ISSR data.

3.2.2. SOM analysis

Data on the presence or absence of 93 loci were presented to the input layer of the SOM. A Kohonen map of 88 output neurons (i.e. a map of 11 × 8 neurons) was used and provided the best classification for the data with a very low value of final topographic error (0.007) (Figure 4).

Hierarchical cluster analysis of the map indicated 6 clusters. Each cluster contains all the populations of 1 site (clusters 4, 5, and 6), while NT and PO populations are gathered in different groups (clusters 1 and 2 for NT and clusters 3 and 4 for PO). As shown in the rooted dendrogram deduced from the Ward algorithm, NC populations are suspected to present a genetic differentiation and constitute cluster 6, a distinct group on a separate branch.

3.2.3. Bayesian method

Based on the membership probabilities given by Structure, all the individuals were arranged into 7 groups (K = 7), showing a gathering of initial populations as evaluated by the Q values (Table 4). The distribution of natural populations into new clusters was good and significant (Q ≥ 0.8), revealing a first association (cluster 1) with all IZ populations, a second association (cluster 2) with all LM populations, a third association (cluster 5) with all AJ populations plus the PO2 population, and a fourth association (cluster 6) with all NC populations. NT populations were separated into 2 clusters (cluster 3 for NT1 and cluster 4 for NT2), and the PO3 population consisted of a mix of clusters 3 and 5 (Figure 5). These results complete

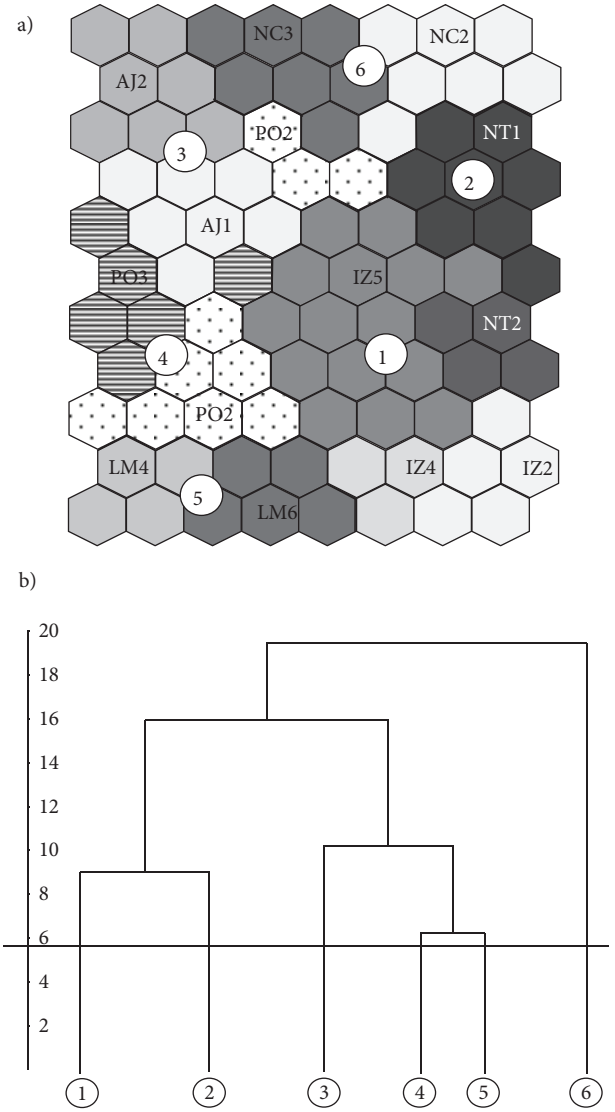

Figure 4. Classification of all individuals from the 13 populations of *Lupinus montanus* var. *montanus* using the self-organising map (SOM) method. a- The pattern SOM map. Individuals are ordinated into the map based on similarities among ISSR bands. Similar individuals are in the same or neighbouring neurons. The 6 clusters from b are differentiated by bold lines and a gradient of greys. b- Hierarchical classification of the SOM map. The bold line shows the level of significance of the clustering.

the SOM analysis and confirm that the 2 localities NT and PO present genetically divergent populations. This analysis allows us to reach a better resolution between populations and significantly discriminate among populations that were mixed using other treatments. The closely related *Lupinus aschenbornii* formed cluster 7, and the Bayesian analysis appears to show that a small part of the genetic pattern of *Lupinus montanus* var. *montanus* was also observed in a few individuals of *Lupinus aschenbornii* (3

Table 4. Probability (Q) of membership of individuals of the 13 initial populations of *Lupinus montanus* var. *montanus* and 1 population of *Lupinus aschenbornii* in each of the 7 inferred clusters determined by Structure. Highest percentage of a sample assigned to 1 cluster, for K = 7, is indicated in bold.

	Clusters						
	1	2	3	4	5	6	7
IZ2	0.019	0.014	0.006	0.003	0.004	**0.940**	0.014
IZ4	0.005	0.015	0.014	0.006	0.003	**0.915**	0.043
IZ5	0.003	0.016	0.007	0.009	0.002	**0.937**	0.026
LM4	0.006	0.004	**0.957**	0.002	0.004	0.013	0.013
LM6	0.005	0.008	**0.834**	0.016	0.004	0.086	0.047
NT1	0.016	**0.934**	0.005	0.003	0.003	0.023	0.016
NT2	**0.938**	0.006	0.006	0.005	0.002	0.024	0.018
AJ1	0.024	0.005	0.011	0.008	0.002	0.020	**0.930**
AJ2	0.002	0.003	0.003	0.003	0.002	0.010	**0.978**
PO2	0.004	0.003	0.011	0.003	0.004	0.078	**0.897**
PO3	0.025	0.019	**0.420**	0.025	0.008	0.102	0.401
NC2	0.002	0.003	0.012	**0.966**	0.002	0.008	0.007
NC3	0.002	0.002	0.003	**0.970**	0.002	0.011	0.010
L. aschenbornii	0.002	0.003	0.003	0.003	**0.959**	0.007	0.022

individuals concerned = 10% of the sampled population, but only a single individual presented a clear admixture of the 2 genomes).

4. Discussion

In this study, the ISSR method was an efficient tool for gaining new insights into the genetic diversity and structure of natural populations of *Lupinus montanus* var. *montanus*. The high value of polymorphism at the species level (P = 97.9%) can be noted, stressing the representativeness of our sample. With only 4 markers used, we confirmed the informative power of this technique, as shown in other studies (Gilbert et al., 1999; Belaïd et al., 2006; Bouzid et al., 2008).

4.1. Genetic diversity of natural populations of *Lupinus montanus* var. *montanus*

In order to characterise the degree of genetic variability, polymorphism at the species level can be compared with results obtained in other studies: 1) in widely occurring plant species such as *Lathyrus cicera* L. (Fabaceae) (P = 87.7%; Belaïd et al., 2006), and 2) in endemic or very local species such as *Ammopiptanthus mongolicus* (Maxim.) S.H.Cheng (Fabaceae) (P = 18.6%; Ge et al., 2005b). Our value of P = 97.9% seems to be concordant with a widely distributed taxon.

Half the genetic variability occurred within populations (47.7%), and the other half was found at a higher geographic scale, among populations and among sites (25.7% and 26.6%, respectively). If we do not consider NT and PO populations (the most divergent populations in this study), the proportion of intersite variability was even higher, reaching 35.8%. This distribution of genetic variability lies between that which is expected for an outcrossing species such as *Myrica faya* Aiton (Myricaceae) (within-population variation of 92.5%; González-Pérez et al., 2009) and a clonal species such as *Psammochloa villosa* (Trin.) Bor (Poaceae) (within-population variation of 12.5%; Li and Ge, 2001). The partitioned variation of *Lupinus montanus* var. *montanus*, mainly visible using Structure, is consistent with a rather low potential for admixture between populations. As a consequence, populations of *Lupinus montanus* var. *montanus* exhibit notable degrees of differentiation with high values of F_{ST} and diversity indexes. This level of differentiation, as well as the fact that half the genetic variance occurs within populations, suggests a combination of recent population isolation and restricted long distance gene flow between populations. Indeed, in this study, the level of gene flow N_m (0.25) was estimated at 1 plant every 4 generations; in theory, 4 migrants per generation are sufficient to prevent genetic

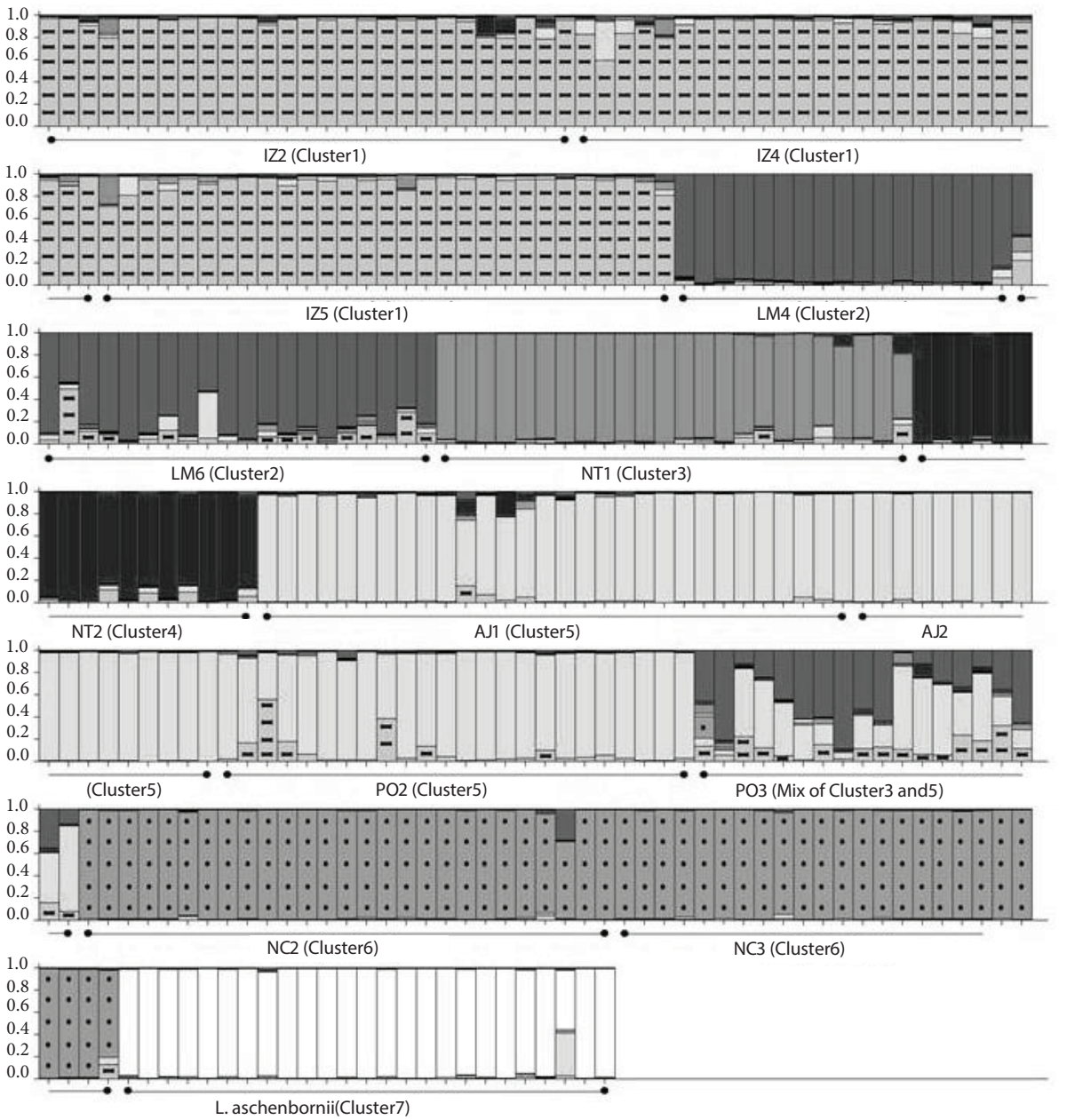

Figure 5. Bayesian analysis of *Lupinus montanus* var. *montanus* performed by Structure 2.3.3 software with K = 7. Each individual is represented by a single vertical line broken into K segments of length proportional to the estimated membership (probability q_i) in the K clusters.

differentiation between populations (Slatkin, 1987). Here, the level of gene flow was too low and is concordant with the possibility that isolation by distance is the main cause of differentiation, as revealed by the significant results of the Mantel test. In California, Drummond and Hamilton (2007) found very similar patterns of genetic variability in 2 other *Lupinus* species. In addition to recent divergence and limited gene flow, they invoked the role of putative selfing and consanguineous mating of individuals, and self-fertilisation is a generalist strategy well-known to be useful in the colonisation of new habitats (for instance, habitats where cross-pollination is minimal or absent) (Pujol et al., 2009), which may be considered to be the case in the present study zone.

4.2. Genetic partitioning of populations

In order to obtain a strong and reliable genetic structuration of the populations, we used 4 complementary classification methods, all of which gave similar results and conclusions,

albeit with different levels of resolution. Classical methods (distance analysis and PCA) emphasised a clear separation between the western populations from Nevado de Colima and the eastern populations from all the other sampled volcanoes. Then, we revealed a substructure for all the populations with genetic diversity indexes and more accurate methods (SOM and Bayesian), in which there is a tendency for a site-dependent effect, except for NT and PO. As revealed by AMOVA, short branches and the PCA overlapping distribution of the eastern populations indicate a very recent divergence that could potentially be attributed to Pleistocene climatic oscillations in Mexico.

The Pleistocene was characterised by a succession of glacial and interglacial periods. For alpine species such as *Lupinus montanus*, the cooling phases induced the displacement of steppe-like habitats from higher to lower elevations (Davis and Shaw, 2001). Consequently, species migrated and colonised the plains during glacial periods, and settled in refuges at higher elevations during the interglacials (Willis and Whittaker, 2000). In Central Mexico, the cooling phases involved glacial advances and occurrences of glaciers on the highest volcanoes of the TMVB (Metcalfe et al., 2000).

Between 2 to 4 glacial periods are described in Central Mexico during the Pleistocene (De Terra et al., 1949; White, 1986; Heine, 1988). During these periods, *Lupinus montanus* var. *montanus* populations may have been in contact with events of dispersion/migration, which generated metapopulations along the TMVB, which would have been fragmented in isolated refuges between each cooling period, thus leading to the island-like distribution. This scenario is supported by the fact that a last glacial episode, allowing a wider distribution of the taxon, was dated from the Early Holocene (<10 kyr BP) (Heine, 1988), with significant warming in the study area as supposed from palynological data (González Quintero and Fuentes Mata, 1980). This suggests that isolation between the sampled populations could be as recent as 8 kyr.

On Nevado de Toluca and Pico de Orizaba, the populations were well differentiated and did not converge following a site-dependent effect. These 2 volcanoes are the youngest of the TMVB, being formed during the late Pleistocene (Hoskuldsson, 1992; Macias, 2007). As a result, NT and PO populations experienced more recent and intense volcanic activity (García-Palomo et al., 2002; Macias, 2007), potentially restricting gene flow among populations even more than among the older volcanoes (LM, AJ, NC, and IZ were formed from the Oligocene to the early Pleistocene; see Marshall and Liebherr, 2000). On average, the pairwise F_{ST} values are higher between populations on young volcanoes than between populations on older volcanoes (0.39 and 0.32, respectively). Matos and Schaal (2000) encountered population segregation of the same magnitude in pine species on the 2 volcanoes NT and PO.

In this study, we demonstrated a clear split between populations of NC and the other populations. Following the distribution of *Lupinus montanus* var. *montanus* depicted by Dunn and Harmon (1977), NC represents the westernmost limit of *Lupinus montanus* var. *montanus*. A similar East-West divergence was demonstrated by Ruiz-Sanchez and Specht (2013) for *Nolina parviflora* Hemsl. (Asparagaceae). The Balsas Basin, a well-identified geographic barrier to gene flow for several taxa (Bryson et al., 2011), was suggested to explain the genetic separation between eastern and western populations and can also be invoked in our study. Secondly, during the Pleistocene the NC volcano was located in the middle of a vast ecological corridor allowing passage from Isthmus Pacific plains to Sonora lowlands, but was strongly isolated from the ecological corridor of the eastern part of the TMVB (Ceballos et al., 2010).

Based on our results, we hypothesise that during the Pleistocene glacial periods described above, the eastern and western parts of the TMVB were not in contact, and that this isolation could be responsible for the greater divergence of NC populations and partly responsible for the positive result of isolation by distance.

4.3. Clues to introgression?

The absence of morphological variability among the populations and individuals sampled is consistent with the recent divergence observed. Indeed, the extreme environmental conditions (alpine conditions) can lead to a stabilising selection on morphology and reduce the morphological changes that can accompany processes of diversification (Bickford et al., 2006). Such patterns of population differentiation have already been described in other widespread plant species (Ruiz-Sanchez and Specht, 2013). Although gene flow among populations is estimated to be low, some evidence of gene flow was found in the PO3 population as well as in a few individuals of the close sympatric species, *Lupinus aschenbornii* (Bayesian analysis). It is notable that the PO3 population grows at the highest elevation among sampled populations and occurs above the documented altitudinal limit for *Lupinus montanus* var. *montanus* (Dunn, 2001). Given that hybrid genotypes occur more commonly at the edges of the populations (low density of individuals, rare partners, and low or null selection against hybrids), the pattern revealed by our study may be the consequence of such gene exchange.

Our study of the genetic diversity of *Lupinus montanus* var. *montanus* has enabled us to gain new insights concerning a widespread taxon that follows an island-like distribution. Results showed that *Lupinus montanus* var.

montanus is genetically highly diverse despite the lack of morphological variability. Such alpine taxa may undergo strong environmental and selection pressures, which maintain phenotypic uniformity (Bickford et al., 2006).

Moreover, the TMVB topography and Pleistocene glacial events appear to have played important roles in the genetic diversification of *Lupinus montanus* var. *montanus*, provoking an East-West separation and also incipient divergence among the eastern populations. These conclusions are similar to results from genetic and phylogenetic studies of other TMVB species (Bryson et al., 2011; Ruiz-Sanchez and Specht, 2013).

The present study provides a useful foundation for investigations into the uncertain morphologically based status of the taxa of the *Lupinus montanus* complex, which, except for *Lupinus montanus* var. *montanus*, have very restricted distributions.

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