Historical biogeography of a disjunctly distributed, Spanish alpine plant, *Senecio boissieri* (Asteraceae)

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Key words: alpine plant, cpDNA, genetic diversity, plant dispersal, population fragmentation, RAPD.

ABSTRACT

Little is known about the historical biogeography of alpine plants that are disjunctly distributed across the mountains of the Iberian Peninsula. Randomly Amplified Polymorphic DNA (RAPD) and chloroplast microsatellite (cpSSR) variation was surveyed in the Spanish alpine endemic, *Senecio boissieri*, to help resolve the causes of its disjunct distribution in the southern Sierra Nevada and Baza, centrally located Sierra Guadarrama, and northern Cordillera Cantábrica. RAPD analysis identified two divergent genetic groups, one containing individuals from the Cordillera Cantábrica and another comprising individuals from the three other mountain ranges. In contrast, chloroplast DNA variation was much more limited with only one of 42 cpSSR loci examined showing polymorphism. At this locus the same allele occurred at high frequency in material from each mountain range. One possible reason for Cantabrian material showing RAPD divergence from other material is that it is derived from plants that survived the Last Glacial Maximum in a northern refugium isolated from the main distribution of the species which spanned the area between the southern and central Spanish sierras. Postglacial fragmentation of the species' main distribution in southern and central Iberia would have resulted in the current disjunction of genetically similar populations in southern and centrally located mountains.

INTRODUCTION

Climatic oscillations during the Pleistocene imposed important range shifts on the Palaeoarctic biota and contributed decisively to shape the demographic

history and genetic diversity of the European flora. The ice sheets of the Northern Hemisphere first developed about 2.5 Myr ago, and a series of major climatic oscillations occurred during the last 700 Kyr with dominant 100 Kyr glacial-interglacial cycles causing the European biota to experience marked and repeated climate change (Taberlet & al., 1998). Cooling of the climate during glaciations forced temperate species to retreat into fragmented distribution ranges in the southern peninsulas of Iberia, Italy and the Balkans, and also in the Caspian/Caucasus region. These areas acted as refugia, and many temperate species now show taxonomic and genetic diversity within and among these regions (Hewitt, 1996; 2004). Conversely, alpine plants exhibited a more continuous distribution during the long glacial stages and in the Mediterranean Basin became isolated in high-altitude mountains during warm (interglacial) periods (Vargas, 2003). The distribution patterns exhibited by components of the alpine flora in southern Europe are likely to be complex due to their different patterns of survival and migration throughout the Pleistocene. For example, Vargas (2003) has proposed different demographic histories for seven alpine species according to their present-day diversification patterns in the Iberian Peninsula and other parts of Europe.

For eastern Spain, there is palaeoclimatic and palaeoecological evidence for the presence of extensive cold, dry steppe-tundra during the last glacial maximum (LGM) linking the high mountain ranges in the south (Sierra Nevada and Sierra de Baza) to those located centrally (Sierra Guadarrama), in the north (Cordillera Cantábrica), and the east (Pyrenees) (Kaiser, 1969; Iversen, 1973; Tichy & al., 2001). Thus, it is feasible that many alpine plant species were more or less continuously distributed at lower altitudes across these mountain ranges

and also the intervening lowland areas during the LGM and earlier glaciations of the Pleistocene. However, with the onset of warming during interglacials and the current postglacial period, the geographic ranges of these species would have become fragmented as such plants became restricted to refugia in the high mountains. Palaeoclimatic data for south-western Europe suggest that postglacial warming progressed from south to north (de Beaulieu & al., 1994) and, therefore, it might be expected that populations in southern high mountain ranges became isolated from the main distribution of a species at an earlier stage than did populations in high mountain ranges to the north. In support of this hypothesis, Kropf & al. (2006) recently showed that the present-day Alpine, Pyrenean, and Iberian populations of three alpine plant species (*Kernera saxatilis*, *Silene rupestris* and *Gentiana alpine*) can be interpreted as vicariant relicts of ancestral forms that were widely distributed in intervening lowland areas during cold periods of the late Pleistocene. For each of these species genetic distance was greater between Sierra Nevada and Pyrenean populations than between Pyrenean and south-western Alps populations. However, for a fourth species (*Papaver alpinum*) no difference was found.

Until now, there has been no detailed survey of molecular diversity which compares variation within and between populations of an alpine species that is restricted to the high mountains of southern, central and north-western parts of Spain. Such an analysis would establish the likelihood of whether this geographical distribution is due to vicariance and/or long-distance dispersal events, and also whether southern populations are more genetically distinct from those in the central and northern mountain ranges than are central and northern populations from each other. The latter would be expected if vicariance

proceeding in a step-wise manner from south to north is responsible for the present-day distribution of such species and the postglacial warming model of de Beaulieu & al. (1994) holds across the whole of the Iberian Peninsula.

Senecio boissieri DC. is a long-lived, lignified, perennial orophilous plant (Boissier, 1839), originally described from high mountain, dry pastures of the Sierra Nevada in southern Spain (De Candolle, 1838). Currently the breeding system of the species is unknown, however its floral traits would suggest that it is insect pollinated and reproduces through outcrossing. De Candolle (1837) placed *S. boissieri* within serie *Incani* DC. [section *Incanae* (DC.)], which included taxa distributed around the Mediterranean Basin. Chater and Walters (1976) retained *Senecio boissieri* in section *Incanae* (DC.) O. Hoff.; however, following molecular phylogenetic analysis, the species has been transferred to section *Jacobaea* where it falls within a group of other closely related southern European mountain plants referred to as *Incani sensu lato* (Pelser & al., 2003, 2004). Section *Jacobaea* has recently been proposed as a separate genus from *Senecio* L., i.e. *Jacobaea* Mill., and therefore *Senecio boissieri* DC. has been renamed *Jacobaea boissieri* (DC.) (Pelser & al., 2006, 2007). In this paper, however, we continue to refer to the species as *S. boissieri* to avoid confusion when referring to the existing literature on this species and its close relatives. The species is currently distributed over several mountain ranges in Spain (Losa & Montserrat 1952, Rivas-Martínez 1956, Sagredo 1975, Molero Mesa & al., 1992) and across two different biogeographic regions as defined by Rivas-Martínez & al. (2002, 2007). In the Eurosiberian region, *S. boissieri* occurs only in the high mountains of the Cordillera Cantábrica (Atlantic European province) (Losa & Montserrat, 1952). In the Mediterranean biogeographic region it is

present in the Sierra Guadarrama (West Iberian Mediterranean province) (Rivas-Martínez 1956) and also in different mountain ranges of the Sierra Nevada National Park, Betic province (Sierras de Baza, la Sagra, and Nevada; Sagredo 1975, Molero Mesa & al., 1992, Fig. 1). Chromosome counts of material from Sierra Nevada (Küpfer & Favarger, 1967; Favarger & Küpfer, 1968; Küpfer, 1968; Blanca, 1992) and Sierra Guadarrama (Castroviejo & Nieto Feliner, 1986) show the species to be tetraploid (*2n* = 40). Losa & Montserrat (1952) have stated that plants from the Cordillera Cantábrica are morphologically identical to Andalusian material.

The highly disjunct distribution of the species across different mountain ranges in Spain could be explained by dispersion through a pre-existing barrier or fragmentation of a once continuous distribution following the origin of a dispersal barrier. Different phylogenetic results are expected for each of these two hypotheses: colonization by long-distance dispersal predicts that lineages from one disjunct area will nest within those from another disjunct area, while fragmentation will result in lineages from disjunct areas being reciprocally monophyletic (Kropf & al., 2006). A recent study by Bettin & al. (2007) provided molecular evidence for the survival of another alpine *Senecio*, *S. halleri*, in high altitude, ice-free refugia (nunataks) in the Alps during the LGM. *Senecio boisierri* and *S. halleri* are similar in gross morphology and both are members of the *Incani* s.l. group, section *Jacobaea,* although occur in different sub-clades of this group (Pelser & al., 2004). It is feasible, therefore, that *S. boissieri* also survived the LGM at high altitude, but in the mountains of the Iberian Peninsula, as well as in intervening lowland areas. Thus, it is possible that present-day

populations of *S. boissieri* are vicariant relicts of what was a more continuous distribution of the species during the LGM.

Here we report a survey of randomly amplified polymorphic DNA (RAPD) and chloroplast DNA microsatellite (cpSSR) variation within and between populations of *S. boissieri* to provide an indication of whether this species' present-day distribution is the product of fragmentation of a once continuous distribution of the species or the result of long-distance dispersal events from a particular source area. Both RAPD and cpSSR variation have been used successfully in previous analyses of the population genetic structure and evolutionary history of plant species (e.g., Coleman & Abbott, 2003; Renau-Morata & al., 2005; Chapman & Abbott, 2005; James & Abbott 2005; Williams & al., 2005).

MATERIALS AND METHODS

RAPD variation. ---

Plant material was collected from populations at nine different locations in Spain: three from the Picos de Europa in the Cordillera Cantábrica, two from Sierra Guadarrama, one from Sierra de Baza, three from Sierra Nevada (Figure 1, Table 1). These populations cover the present-day geographic distribution of *S. boissieri*. The total sample size for RAPD analysis was 128 individuals with at least ten individuals examined per population (mean equals 14.22±5.21 individuals per population). From 13 arbitrary 10-mer primers tested (Operon Technologies Inc. primers belonging to kits B and C), ten were selected for analysis based on their ability to produce clear and reproducible banding patterns in samples of repeated amplifications. The selected primers were

OPB1, OPB4, OPB6, OPB7, OPB9, OPB10, OPB11, OPB12, OPC11, and OPC12.

Total DNA was extracted from dried leaves with cetyltrimethylammonium bromide (CTAB) as outlined in Comes & Abbott (2000). Amplifications were carried out in a total volume of 25 µl, containing 18.1 µl of deionized sterile water, 1.5 µl of template DNA (±7.5 ng), 1 µl of 50mM $MgCl_2$, 2.5 µl of reaction buffer (supplied with the enzyme), 0.5 µl each of 2 mM dNTPs, 0.2 µl of 10 µM primer and 1 U (0.2 µl) of Taq polymerase (Bioline Ltd, London, UK). Amplifications were performed in a thermal cycler (G-STORM; GS-1, Genetic Research Instrumentation Ltd, Essex, UK) programmed for 3 min at 94 °C followed by 45 cycles of 30 s at 94 °C, 45 s at 36 °C, 1.5 min at 72 °C, and a final elongation step of 4 min at 72 °C. Samples were kept at 4 °C until they were loaded and run through 1.5 % agarose gels in 0.5 x TBE buffer (90 mM Tris-borate, 2 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0) for 3 h at 100 V. The DNA was visualized with ethidium bromide and photographed under UV light.

Presence or absence of RAPD bands in each individual was determined by visual inspection and scored as 1 or 0. Only bands showing a clear and easily detectable signal were scored. Estimates of genetic diversity within populations, including percentage of polymorphic loci, Shannon's index (Shannon & Weaver, 1949), and Nei's gene diversity assuming random mating (Nei, 1973), were calculated using the software POPGENE (Yeh & al., 1997). Data were then analysed with the program STRUCTURE (Pritchard & al., 2000) which employs a model-based Bayesian clustering procedure to determine the number of divergent groups (K) in a dataset. Six independent runs were

undertaken with K varying from 1–12. Each run was performed with 100,000 MCMC (Markov chain Monte Carlo) repetitions following a burn-in of 100,000 iterations. The analyses used no prior information, assumed correlated allele frequencies and no admixture. Posterior probabilities were calculated for each value of K, and the estimated ΔK (Evanno & al., 2005) was used to choose the optimal K value. Following analysis by STRUCTURE, a principal coordinate analysis (PCoA) was conducted on a Euclidean similarity matrix (Excoffier & al., 1992) constructed from the primary data set using the program PAST (version 1.54, Hammer & al., 2006). In addition, cluster analysis was performed on a matrix of distance values between individuals calculated using the RESTDIST option implemented in PHYLIP (version 3.6, Felsenstein, 2004). Clustering was conducted by UPGMA (unweighted pair group method with arithmetic averages) as implemented in the NEIGHBOR program of PHYLIP. The tree was bootstrapped 1000 times using the SEQBOOT option in PHYLIP.

Data were also subjected to analysis of molecular variance (AMOVA) using ARLEQUIN version 3.1 (Excoffier & al., 2005) to examine the level of differentiation within and among different groupings of populations defined *a posteriori*. A Mantel test was performed with ARLEQUIN to examine the relationship between genetic distance, expressed as F_{ST} and geographic distance (km). This relationship was further examined using the program BARRIER (version 2.2 Manni & al., 2004) which implements Monmonier's maximum difference algorithm to test for the presence of genetic barriers among groups

Chloroplast DNA variation. ---

Chloroplast (cp) DNA variation in *S. boissieri* was examined across a large number of cpSSR loci, after a PCR-RFLP screen of several cpDNA consensus regions (ATP, QS, DJ, and SMF; Desmesure & al., 1995; Dumolin-Lapegue & al., 1997; Grivet & al., 2001) revealed no polymorphism (data not shown). Initially, a representative set of samples comprising four plants from each mountain range was screened for variation at 53 cpSSR loci (Weising & Gardner, 1999; Chung & Staub, 2003) in the manner described by Arroyo-García & al. (2002). PCR reactions were carried out in a 20 µl final volume, containing 12.35 µl of deionized sterile water, 2 µl of template DNA (±25 ng), 1 µl of 50mM $MgCl_2$, 2 µl of 10x reaction buffer (supplied with the enzyme), 0.5 µl of each 2 mM dNTPs, 0.2 µl of 5 µM each primer and 0.5 U (0.1 µl) of Taq polymerase (Invitrogen, Foster City, CA, USA). Amplifications were conducted in a Perkin Elmer 9600 Thermocycler (PE Applied Biosystems, Foster City, Calif.) set for 5 min at 94 °C followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 5 min. An equal volume of denaturing loading buffer (98% w/v formamide, 10 mM EDTA, 0.05 % w/v bromophenol blue, and 0.05 % w/v xylene cyanol blue, pH 8.0) was added to each sample before they were denatured by heating for 3 min at 94 °C. Samples were loaded on to 1 % agarose gels to check the amplification reaction. Following this, 5 µl of each sample were loaded on to 6% polyacrylamide gels (19:1 acrylamide: bisacrylamide, 7.5 M urea and 1x TBE) and electrophoresed at 1800 v and 65 w for 3h. Gels were fixed and stained with a standard silver staining protocol (20 min acetic acid 1 % ethanol 10 %, 3min nitric acid 1.5 %, 20 min silver nitrate 2 g/L), and developed in sodium

carbonate 30 g/L and 0.27 ml/L formaldehyde. Gels were washed in deionized water after each step.

Successful amplification was achieved for 42 cpSSR loci, but polymorphism was detected at only one locus, *ccmp1*. Further analysis therefore focussed on only this locus. A total of 96 individuals, comprising 24 from each of the four main sampling areas (Cantabrian, Guadarrama, Baza and Sierra Nevada) were surveyed for variation at the *ccmp1* locus using the same template DNA as used in the RAPD analysis.

RESULTS

RAPD variation.---

Seventy-five polymorphic RAPD bands were amplified and scored for presence or absence in each individual surveyed. The number of fragments scored per primer ranged from 5 (OPC11) to 11 (OPC12) with an average of 7.5 ±1.98. Fragment size varied between 0.2 and 1.8 Kbp. All 128 individuals surveyed possessed a unique multilocus RAPD phenotype. No bands were specific to a particular population; however, one band (OPC12-1) was specific to all Mediterranean samples, and was absent from Eurosiberian samples. Another band (OPB9-10) was present in all Eurosiberian samples, but exhibited a low frequency in the Baza (0.25) and Sierra Nevada populations (zero to <0.1), and was absent from Sierra Guadarrama populations. Finally, one band was present in Sierra Nevada and Baza populations with a frequency ranging from 0.2 to 0.36, but was absent from all other material surveyed. The proportion of polymorphic loci over all populations averaged 62.5%, and ranged from 46.6% to 57.3% in Cantabrian populations, and from 61.3% to 76% in populations from

the other three mountain ranges (Table 1). Cantabrian populations were also shown to contain significantly lower levels of genetic diversity based on comparisons of Shannon's index and Nei's gene diversity values with the other populations (Mann Whitney tests, U = 18, p = 0.0238), but there were no significant differences for either estimate of diversity between the Sierra Guadarrama, Baza and Nevada populations (Table 1).

The Bayesian-clustering method employed in STRUCTURE demonstrated that two main clusters (K = 2) were present in the RAPD data set (Fig. 2). One cluster contained all 42 individuals from the Eurosiberian biogeographical area (Cantabrian range), while the second cluster included all individuals from the remaining populations (Fig. 2). The membership frequency in each cluster of individuals from each mountain range (Table 2) shows that assignment to one or other cluster was always greater than 90%. The same two major groups were also evident in a plot of the first two principal coordinates extracted from the principal coordinate analysis (Fig. 3). The two groups were separated along principal coordinate 1, while variation along principal coordinate 2 indicated some degree of separation between samples from Sierra Guadarrama and Sierra Nevada, with samples from Baza overlapping those from both of these ranges. A UPGMA dendrogram (not shown) also separated samples into the same two major groups, but with low bootstrap support (<50%).

Hierarchical analysis of molecular variance (AMOVA) showed that the difference between the two main groups (representing Eurosiberian *vs* Mediterranean biogeographical areas) was highly significant and accounted for approximately 17% of the total variance (Table 3a). There was also significant

variation among populations within these two main groups accounting for 15.5% of the total variance. The remaining variation was due to that present within populations (67.5%). A second AMOVA was conducted to examine variation within and between the two provinces of the Mediterranean biogeographic region, i.e. Mediterranean West Iberian province (comprising material from Sierra Guadarrama) and the Betican province (comprising samples from Sierra Nevada and Baza). This showed that the difference in RAPD phenotype between these two provinces was not significant and accounted for only 2.72% of the total variance (Table 3b). A third AMOVA performed using the three disjunct mountain ranges included in the Mediterranean area as groups also showed no significant genetic differentiation among these groups (Table 3c). Finally, an AMOVA (Table 3d) of the Mediterranean ranges, which excluded Baza samples, showed no significant difference in RAPD phenotype between the Sierra Guadarrama and Sierra Nevada groups.

A Mantel test revealed that the correlation between genetic distance (F_{ST}) and geographical distances between all pairs of populations was positive and highly significant (r = 0.63 and p = 0.002). However, a separate test on population pairs from only the Mediterranean area resulted in a reduced correlation that was not significant (r = 0.34 p = 0.083), indicating that the significant correlation obtained between all pairs of populations was largely due to the inclusion of Cantabrian material. A clear barrier separating the Eurosiberian samples from those collected in the Mediterranean area of Spain was detected using Monmonier's maximum difference as implemented in the Barrier 2.2 software in a comparison of F_{ST} values and geographic distances.

Chloroplast DNA microsatellite variation.---

Three different alleles were recorded at the *ccmp1* locus among the 96 individuals surveyed: 136, 137 and 138 (named according to size in bp). The most frequent allele was 137 (82.8% of total) and was detected in all populations examined. Allele 138 occurred at an overall frequency of 9.7% and was present in populations from each mountain range except Sierra de Baza where it was replaced by allele 136, which reached a frequency of approximately 30% in the single Baza population surveyed (Fig. 1).

DISCUSSION

The survey of RAPD variation within and among populations of the alpine Spanish endemic *Senecio boissieri* showed that two different genetic groups comprise the species. One group consists of material from the Cordillera Cantábrica in northern Spain, which is placed in the Eurosiberian biogeographic region of the Iberian Peninsula, while the other group is composed of material sampled from populations in the Sierra Guadarrama (central Spain), Sierra de Baza and Sierra Nevada (southern Spain), which are located within the Mediterranean bigeographic region. These two distinct genetic groups were clearly defined by a Bayesian model-based clustering method employed in the program STRUCTURE, by principal coordinate analysis (PCoA), by UPGMA analysis, and by AMOVA. Furthermore, a clear barrier separating Eurosiberian from Mediterranean populations was detected using Monmonier's maximum difference algorithm in a comparison of F_{ST} values and geographical distances. Interestingly, in contrast to its clear genetic difference, Cantabrian material is reported to be morphologically identical to Andalusian material (Losa &

Montserrat, 1952) and therefore treated as taxonomically equivalent. Additionally, the populations of *S. boissieri* sampled for present study didn´t show morphological variability, although a higher frequency of yellow tubular florets could be appreciate in northern localities. However, both yellow and reddish florets can be found in single populations along broad distribution of the species, so this feature should not be used as taxonomical character.

Within the Mediterranean biogeographic region, material was sampled from two biogeographic provinces – the West Iberian Mediterranean province (Sierra Guadarrama) and the Betican province (Sierra de Baza and Sierra Nevada) and was found not to be genetically different. There was a suggestion from the principal coordinate analysis that material sampled from Sierra Guadarrama might be genetically different from that sampled from Sierra Nevada; however, an AMOVA revealed that the difference between them was not significant and accounted for only 5.72% of the total variance. Taken overall, it was evident that a large proportion of RAPD variation within the species was due to differences within populations and each individual possessed a unique multilocus phenotype.

In contrast to the relatively high level of RAPD variation recorded within and between populations of *S. boissieri*, our survey of cpDNA variation revealed that only one of 42 cpSSR loci analysed showed polymorphism. This very low level of cpSSR diversity within the species might reflect a relatively recent origin of *S. boissieri* in Spain followed by a range expansion during which it colonised the different mountain ranges where it is currently found. This range expansion could have occurred during the last glaciation or a previous glaciation of the Pleistocene, when cold conditions may have favoured the migration of the

species between the high mountain ranges across lowland areas. A modification of this scenario would be that the species is of more ancient origin, but underwent a severe genetic bottleneck in the recent past, possibly during a recent interglacial, before subsequently undergoing a marked range expansion. A molecular phylogeny of *Senecio* section *Jacobaea* based on chloroplast DNA and nuclear ITS sequences placed *S. boissieri* in an unresolved clade with two other species, *S. abrotanifolius* and *S. adonidifolius* (Pelser & al., 2004). All three species were joined in a polytomy at a node towards the tips of the tree indicating a relatively recent origin; however, the tree was not dated and because of this, and also the unresolved nature of the clade, it is not yet possible to estimate when *S. boissieri* is likely to have originated. A third possible reason for the low level of cpSSR diversity within *S. boissieri* is that the chloroplast genome of the species was subjected to a selective sweep relatively recently, which resulted in neutral diversity being lost from the genome. In the absence of other evidence, it is not yet possible to determine which of these three possible explanations is the correct one.

At the single cpSSR locus found to be polymorphic, one allele occurred at high frequency throughout the species distribution, whereas a second, less frequent allele, found in material from the Cantabrian, Sierra Nevada and Guadarrama ranges, was replaced by a third allele in the Sierra de Baza population examined. The variation at this locus, therefore, indicates a difference in some Baza material that was not apparent from the RAPD analysis. This latter difference could have arisen during the geographical isolation of Baza material in the current postglacial period.

The finding that populations in the Cordillera Cantábrica are genetically distinct from those in other parts of Spain, based on RAPD variation, whereas those from the central and southern mountain ranges are genetically similar, was unexpected in light of the proposal that postglacial warming proceeded from south to north (de Beaulieu *& al*., 1994). Under this scenario, it would be expected that fragmentation of what might have been a more or less continuous distribution of an alpine plant would begin first in southern parts of its range and occur later in more northerly parts. Thus, we may have expected that the Sierra Nevada/Sierra de Baza populations would be genetically distinct from those occurring in the Sierra Guadarrama, which in turn would be relatively similar to those in the Cordillera Cantábrica. Such results would be in keeping with the recent findings of Kropf & al. (2006) which showed that Sierra Nevada populations were more genetically distinct relative to Pyrenean and western Alpine populations in three alpine plant species examined. However, in contrast to expectation, our results for *S. boissieri* do not concord exactly with those predicted from the postglacial warming model of de Beaullieu & al. (1994). A possible reason for this is that although at the LGM the species may have been more or less continuously distributed in areas connecting the southern and centrally located sierras of Spain, further north the species may have been restricted to a refugium such that Cantabrian material remained isolated from material to the south. Broadly equivalent levels of RAPD diversity were present within populations surveyed from the southern and central sierras, whereas a lower level of diversity occurred within Cantabrian populations indicating possibly a smaller population size for this material at the LGM. In support of the hypothesis that Cantabrian material was isolated from the main distribution of *S.*

boissieri at the LGM, it is worth noting that in two other alpine species, *Androsace vitaliana* and *Saxifraga pentadactylis,* ITS variation indicates that samples from Eurosiberian localities in northern Spain are phylogenetically separated from samples from other mountain ranges in Spain (Vargas, 2003), suggesting that alpine material from the Cantabrian range has been isolated for a long period of time. In the case of *Senecio boissieri*, habitat specifity could play an important role in its isolation. The high windy and sunny places in which this plant lives remember steppe-tundra habitats that dominated eastern face of Iberian peninsula during LGM, explaining a continuous past distribution of the species. However, similar ecological conditions can be found in periglacial landforms, to which most of the plant communities related to *S. boissieri* in cantabrian mountains can be related, either in basic or acid lithologies. Furthermore, current distribution of *S. boissieri* in northern Spain link very well with the more intensively glaciated areas in cantabrian mountains during LGM, suggesting a possible survival of the plant in periglacial habitats, and an earlier isolation.

Thus, from the distribution of RAPD variation in S. boissieri, it is feasible that postglacial fragmentation of a once more or less continuous geographical distribution explains the present-day disjunct distribution of the species in the central and southern sierras of Spain, whereas Cantabrian material remained isolated from the rest of the species distribution throughout the LGM. Whether this proposed distribution pattern also prevailed during previous glaciations of the Pleistocene, or whether at one glacial stage the species had a more or less continuous distribution from north to south, remains unknown. Additional phylogeographic studies are now required on other high mountain endemics

that are distributed across the mountain ranges of southern, central and northern Spain to broaden our understanding of the evolutionary history of these species. and their individual responses to glacial and postglacial cycles. Molecular investigations of the population genetic structure of such species together with increased palaeobotanical evidence should lead to an improved understanding of the historical biogeography of these plants during the Pleistocene.

ACKNOWLEDGEMENTS

The authors wish to thank Daniel Sánchez-Mata, Mario Ruíz-Girela for their help in the sampling work, Sierra Nevada and Picos de Europa National Parks, Mr Dave Forbes and Mr Eduardo Cires for their help during the development of this work, and Peter Comes and José Carrión for comments on an earlier version of the manuscript. Elena L. Peredo was in receipt of a FPU grant.

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Figure legends.

Fig 1 Map of the locations of populations (♦) of *Senecio boissieri* investigated. The Eurosiberian region is represented in grey and the Mediterranean region in white. Also illustrated are 'pie diagrams' representing the relative frequencies of each cpDNA haplotype (136 -- black, 137 -- grey, 138 -- white) in material sampled from each mountain range.

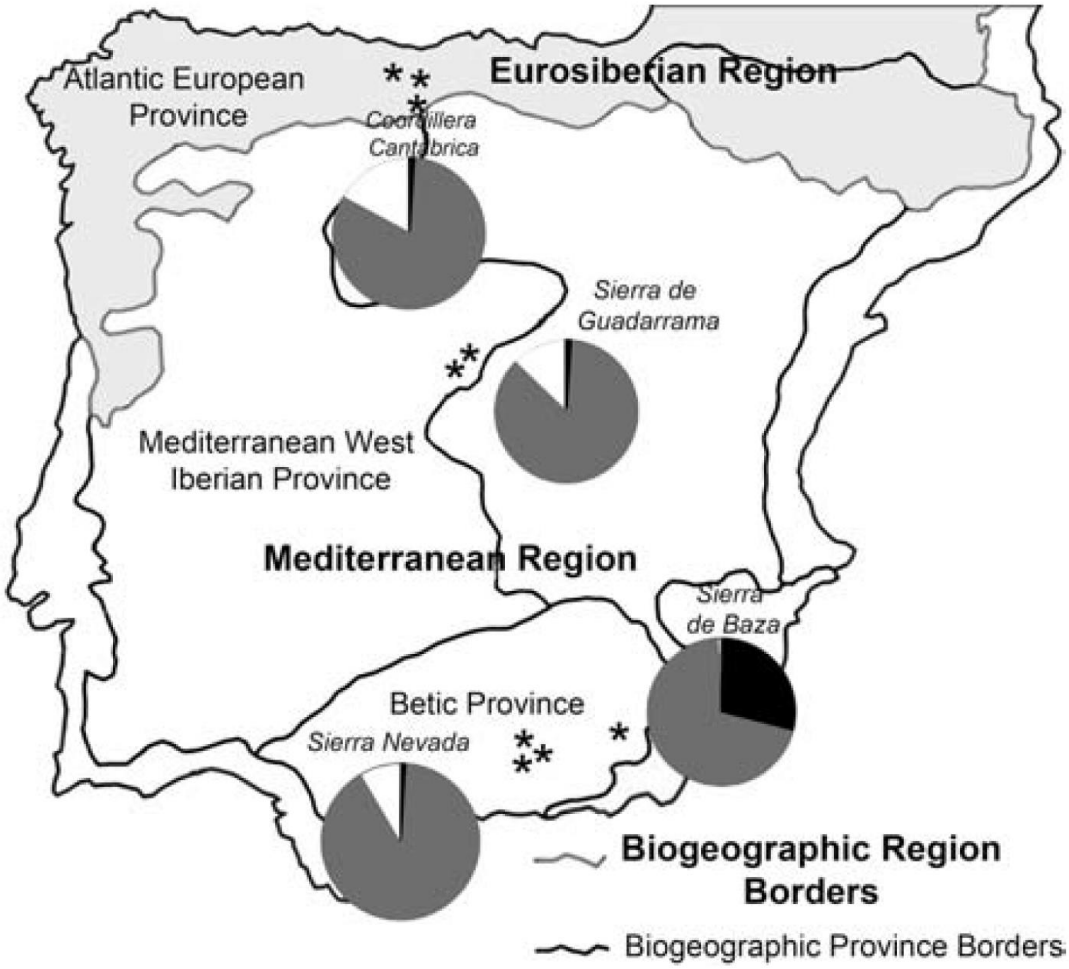

Fig 2 Left: Bar plot of the proportion of an individual's genome belonging to one or other cluster inferred by the STRUCTURE analysis. The Eurosiberian cluster is represented in light gray and includes individuals from sampled sites in the Cordillera Cantábrica while the Mediterranean cluster is represented in dark gray and includes individuals from sampled sites in the Guadarrama, Baza and Sierra Nevada ranges. Right: Plot of ΔK for each K value calculated as described in Evanno & al. (2005) where K is the number of clusters.

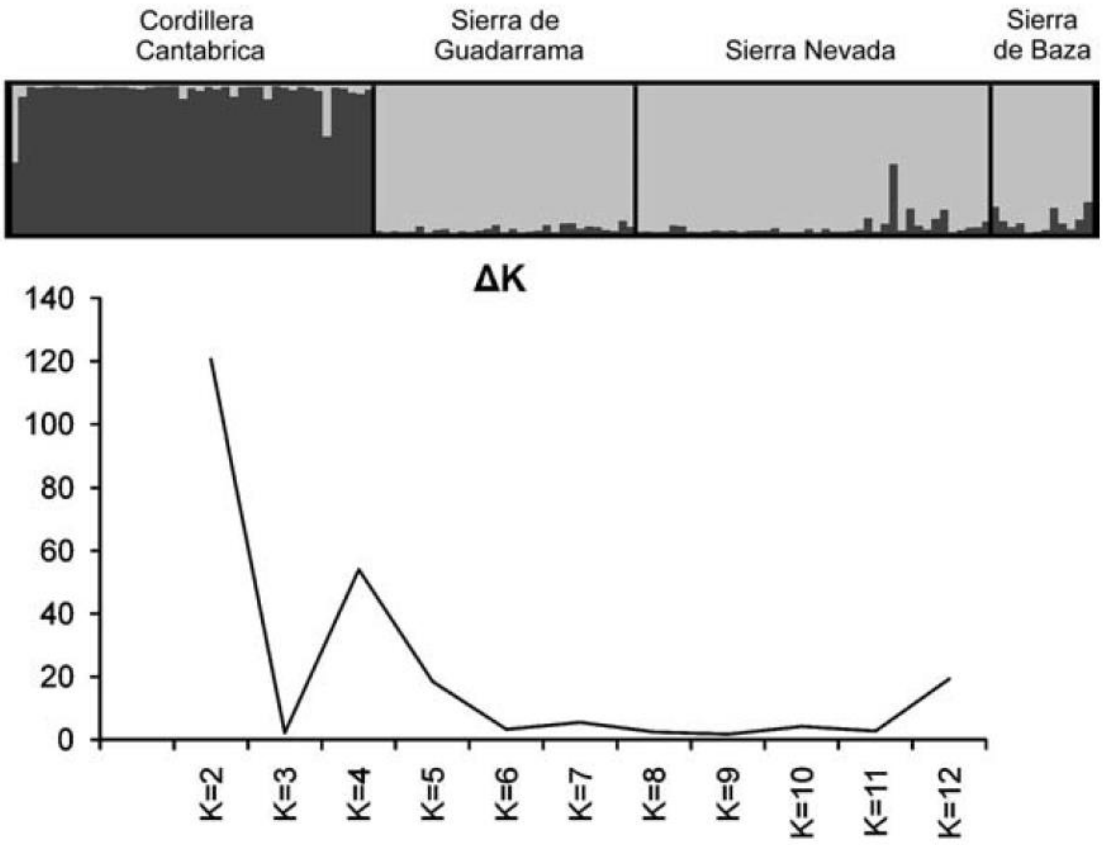

Fig 3 Plot of principal coordinates 1 *vs* 2. Open squares represent individuals from the Cantabrian range, open rhombus from the Baza range, full squares from the Guadarrama range, the crosses from the Sierra Nevada range.

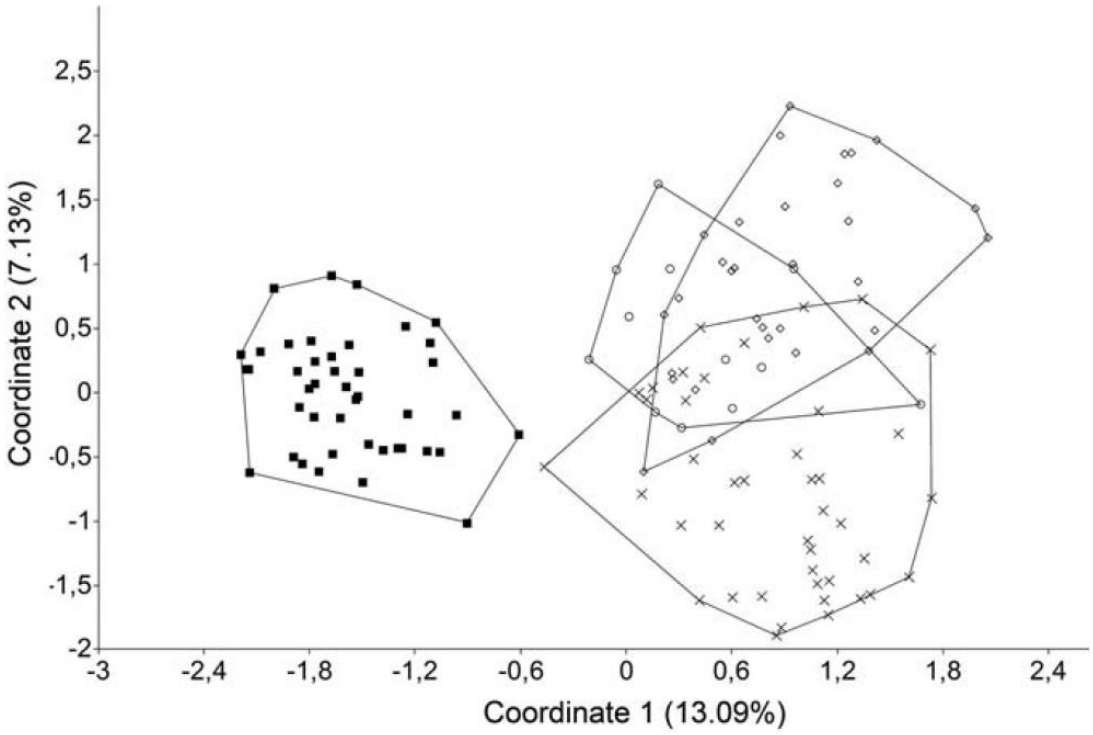

Table 1. Estimates of genetic diversity based on RAPD variation within populations and also after pooling samples across populations within each mountain range. (n = number of samples analysed, N = approximate number of individuals in population, *P* = Percentage polymorphic loci; H_E = Nei's gene diversity; *I* = Shannon's index; sd = standard deviation). Voucher reference (when exits) and UTM coordinates of localities: **G1**- JBAG-331; *30T 418734 / 4521445*; **G2**- *30T 419428 / 4516650*; **B1**- JBAG-369; *30T 514150 / 4137374*; **S1**- JBAG-395; *30T 466249 / 4104492*; **S2**- *30T 465795 / 4105763*; **S3**- *30T 463004 / 4107578*; **P1**- JBAG-249; *30T 358271 / 4765504*; **P2**- JBAG-167; *30T 361830 / 4784489*; **P3**- JBAG-152; *30T 349892 / 4785412*.

Locality	**Altitude (m)**	**n (N)**	***P***	***H_E* (sd)**	***I* (sd)**
Guadarrama Range					
G1- Collado Peña Lara	2200	19 (150)	69.33	0.2457 (0.2053)	0.3656 (0.2864)
G2- La Cuerda	2200	12 (200)	62.67	0.2091 (0.1940)	0.3169 (0.2760)
Pooled across populations		31	68	0.2697 (0.1939)	0.4028 (0.2667)
Mean		15.5	66	0.2274 (0.1996)	0.3412 (0.2812)
Baza Range					
B1- Santa Bárbara	2100	12 (250)	72.00	0.2319 (0.1842)	0.3543 (0.2596)
Sierra Nevada					
S1- Veleta	2600	22 (200)	70.67	0.2214 (0.1857)	0.3387 (0.2633)
S2- Albergue Universitario	2500	10 (50)	61.33	0.2401 (0.2113)	0.3520 (0.2989)
S3- Pradollano	2200	10 (150)	62.67	0.2255 (0.1940)	0.3382 (0.2800)
Pooled across populations		42	81.33	0.2676 (0.1916)	0.4020 (0.2614)
Mean		14	64.89	0.229 (0.197)	0.3429 (0.2807)
Cordillera Cantábrica					
P1- Cubil del Can	2200	22 (200)	57.33	0.1746 (0.1960)	0.2664 (0.2763)
P2- Alto del Hoyo Oscuro	2100	11 (400)	46.67	0.1489 (0.1848)	0.2280 (0.2680)
P3- Jou Negro	2200	10 (300)	49.33	0.1814 (0.2023)	0.2699 (0.2909)
Pooled acros populations		43	61.33	0.1919 (0.1883)	0.2981 (0.2622)
Mean		14.33	51.09	0.1683 (0.1943)	0.2547 (0.2784)
Total		128		0.2943 (0.1812)	0.4418 (0.2407)

Table 2. Proportion of individuals from each mountain range assigned to each of the two clusters inferred by the STRUCTURE analysis (Pritchard & al., 2000) based on RAPD phenotype.

	Inferred Clusters		
Given groups	**1**	**2**	**Nº individuals**
Cantabrian range	0.041	0.959	43
Guadarrama range	0.976	0.024	31
Sierra Nevada range	0.960	0.040	42
Baza range	0.928	0.072	12

Table 3. Hierarchical analyses of molecular variance (AMOVA) for RAPD variation in Senecio boissieri (N = Sierra Nevada; B = Sierra de Baza; G = Sierra Guadarrama; C = Cordillera Cantábrica populations).

a	**N, B, G vs C**	d.f.	Sum of squares	Variance components	% of variation
	Among groups	1	142.61	1.87 Va	16.96
	Among populations within groups	7	217.30	1.71 Vb	15.50
	Within populations	119	891.08	7.48 Vc	67.54
			FST :0.32460	FSC :0.18670	FCT :0.16956
			Vc and FST p<0.0001	Vb and FSC p<0.0001	Va and FCT **p = 0.0127***
b	**N, B vs G**				
	Among groups	1	54.427	0.30948 Va	2.72
	Among populations within groups	4	147.281	2.08557 Vb	18.32
	Within populations	79	710.233	8.99029 Vc	78.96
			FST :0.21036	FSC :0.18830	FCT :0.02718
			Vc and FST p<0.0001	Vb and FSC p<0.0001	Va and FCT p = 0.26295
c	**G vs N vs B**				
	Among groups	2	95.276	0.40001 Va	3.52
	Among populations within groups	3	106.432	1.96570 Vb	17.31
	Within populations	79	710.233	8.99029 Vc	79.17
			FST :0.20832	FSC :0.20832	FCT :0.03522
			Vc and FST p<0.0001	Vb and FSC p<0.0001	Va and FCT p = 0.22581
d	**G vs. N**				
	Among groups	1	55.626	0.56820 Va	5.72
	Among populations within groups	3	91.723	1.69986 Vb	17.11
	Within populations	68	521.514	7.66933 Vc	77.18
			FST : 0.22824	FSC :0.18143	FCT :0.05718
			Vc and FST p<0.0001	Vb and FSC p<0.0001	Va and FCT p = 0.10068

*statistically significant