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Intersimple sequence repeat (ISSR) variation in *Lactoris fernandeziana* (Lactoridaceae), a rare endemic of the Juan Fernández Archipelago, Chile

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Abstract

Sixteen populations and 89 individuals of *Lactoris fernandeziana* were examined for variation in intersimple sequence repeat (ISSR) banding patterns. The species is a rare endemic of Masatierra Island in the Juan Fernández Archipelago, and is the only member of the endemic family Lactoridaceae. Five populations showed a single genotype whereas the other 11 populations had from two to 16 multilocus genotypes. Over 73% of the ISSR diversity occurred across populations, with only about 27% within populations. Diversity among populations results from the presence of different subsets of loci within each population rather than unique loci within populations; only two populations displayed novel loci, with one and three in each. Levels of differentiation at ISSR loci among populations are not correlated with geographic distance on Masatierra; rather, the pattern of variation is mosaic. The presence of differentiated local populations is concordant with the geitonogamous breeding system of the species and suggests low levels of long distance pollen or seed dispersal. The mosaic pattern of ISSR variation on Masatierra may result, in part, from drift and inbreeding in small populations following fragmentation of a once more continuous distribution of *Lactoris* with the formation of canyons by erosion. Also, the generation of new ISSR loci by mutation could occur with rare, sporadic gene flow among populations accounting for the mosaic pattern of variation and the paucity of unique alleles within populations. The ISSR results for *Lactoris* suggest that studies of morphological, ecological and physiological features may elucidate differentiation among populations of *L. fernandeziana*. Field studies have demonstrated that plants occur both in the dense forest understory and in the full sunlight in forest openings.

Keywords: conservation biology, intersimple sequence repeat, Juan Fernández Islands, *Lactoris*, rare plant.

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Introduction

Lactoris fernandeziana Phil., the sole member of the family Lactoridaceae, has received considerable attention for three reasons. First, the species is distinct and unusual morphologically and, hence, there has been discussion and debate on its phylogenetic affinities. Second, *Lactoris* is of interest in discussions of early angiosperm evolution (Stuessy *et al*. 1998). Third, the species, despite its possibly archaic nature within flowering plants, is rare and endemic to a volcanic island only four million years old in the Pacific Ocean (Stuessy *et al*. 1984). A comprehensive review of the evolutionary relationships of the family, including data from morphology, palynology, wood anatomy and DNA sequences was presented recently by Stuessy *et al*. (1998). The reader is referred to that publication for details and primary literature citations. Almost all the data suggest that *Lactoris* is sister to elements of Aristolochiaceae (Stuessy *et al*. 1998) or nested within the family (Qiu *et al*. 1999). However, Stuessy *et al*. (1998) emphasized that even though both morphological and molecular data place *Lactoris* in the same general phylogenetic position in the flowering plants (at least among the extant taxa sampled to date), all data sets likewise suggest that it is highly divergent, and it seems likely that extinctions have led to the present isolation of *Lactoris* among extant flowering plants. Molecular phylogenetic studies with additional taxon sampling may provide more refined insights into the closest living relatives of *Lactoris*.

The isolation and rarity of *Lactoris* on the island of Masatierra in the Juan Fernández Archipelago make it an important species to study in the context of conservation biology because the species represents a very distinctive element within the flowering plants. Whereas it was once thought to be extinct (Skottsberg in Carlquist 1964), more recent field studies have documented over 500 plants and suggest that possibly 1000 are extant on Masatierra (Crawford *et al*. 1994; Stuessy *et al*. 1998; Bernardello *et al*. 1999). Previous attempts to study genetic variation in *L. fernandeziana* using allozymes (Crawford *et al*. 1994) and random amplified polymorphic DNA (RAPD) markers (Brauner *et al*. 1992) provided little or no useful information on genetic diversity within and among populations. No allozyme diversity was detected in *Lactoris*, which is consistent with the low to non-existent variation reported for other rare plant species (Moran & Hopper 1983; Waller *et al*. 1987; Crawford *et al*. 1988; Lesica *et al*. 1988; Rieseberg *et al*. 1989; Hickey *et al*. 1991; Les *et al*. 1991; Soltis *et al*. 1992). Although more diversity is usually reported for RAPD markers than allozymes in rare plant species (Peakall *et al*. 1995; Szmidt *et al*. 1996; Ayres & Ryan 1997; Brunell & Whitkus 1997; Crawford 1997; Esselman *et al*. 1999), there was not sufficient RAPD variation to be helpful in *Lactoris* despite the use of 16 primers (Brauner *et al*. 1992). Given that this very distinctive species of phylogenetic interest is known only to Masatierra, it is important to know whether populations in different canyons and/or different areas of the island are differentiated. If a molecular marker demonstrates geographic differences, any plan to conserve maximum diversity in this enigmatic species should take these data under advisement.

The present study used intersimple sequence repeat (ISSR) markers to assess genetic diversity in *Lactoris*. In this PCR-based method, primers are designed for the common simple sequence repeats (SSR or microsatellite) present in the plant genome (e.g. CT_n, Ca_n and GT_n). A one- or two-nucleotide anchor is used on one side of the SSR to prevent strand slippage during PCR amplification (Wolfe & Liston 1998; Wolfe *et al*. 1998b). When a given SSR motif occurs on opposing strands at distances short enough for amplification, bands of different fragment sizes will be generated for visualization in agarose or acrylamide gels (Wolfe & Liston 1998; Wolfe *et al*. 1998b). Two plants with the same SSR and anchoring sequences will produce the same (homologous) band that is scored as a diallelic (present/absent) locus. While few studies of natural populations have been carried out, available data suggest that ISSR markers may have certain advantages over the more commonly used RAPD markers for assessing genetic variation within and among populations of the same and closely related species. These include a higher reproducibility of amplified bands because the longer primers allow more stringent annealing conditions (Moreno *et al*. 1998; Ratnaparkhe *et al*. 1998) and a higher diversity (Moreno *et al*. 1998; Wolfe & Liston 1998; Wolfe *et al*. 1998a,b; Esselman *et al*. 1999).

The purposes of the present study were: to determine the level and apportionment of ISSR diversity within and among populations of *L. fernandeziana*, and compare the results to allozymes and RAPD markers; to examine the spatial structure of ISSR diversity on Masatierra Island; discuss the ISSR results relative to the reproductive biology and distribution of *L. fernandeziana*; and consider possible conservation implications of the ISSR results.

Materials and methods

Field collections

Populations of *Lactoris* used as sources of DNA are given in Table 1 and their locations are shown in Fig. 1. Voucher specimens of Stuessy *et al*. were deposited in the herbaria at the Ohio State University (OS) and the Universidad de Concepcíon (CONC), and those of Anderson at the University of Connecticut (CONN). These populations occur throughout the known distribution range of the species on Masatierra (Fig. 1), and are likely to represent

Table 1 Populations of *Lactoris fernandeziana* examined for intersimple sequence repeat (ISSR) variation. All from Masatierra Island, Juan Fernández Archipelago, Chile

*Collection no.	Population	Location designation	Population size	No. plants examined
11 178	1	Corrales de Molina: W side of canyon at bottom of trail. 340 m	7	2
11 591	2	Puerto Francés: La Piña. 580 m	3	2
11 666	3	Cerro Agudo: fourth canyon. 550 m	7	2
11 784	4	Villagra: from Mirador S into valley. 590 m	5	2
11 897	5	Cerro Agudo: first canyon. 600–650 m	100–200	2
11 908	6	Cerro Agudo: fourth canyon. 600–650 m	20	3
11 950	7	Puerto Francés: La Piña. 490 m	6	3
12 027	8	Corrales de Molina: down from Damajuana ridge on south side. 430 m	100	3
12 141	9	Corrales de Molina: up from Pangal, then down into canyon. 640 m	11	2
15 133	10	Cerro Agudo: first canyon. 630 m	Several hundred estimated	10
15 134	11	Cerro Agudo: second canyon. 640 m	6	6
15 135	12	Cerro Agudo: second canyon. 640 m	2	2
15 169	13	Corrales de Molina: over and down from Pangal. 630 m	3	3
15 174	14	Corrales de Molina: over and down from Pangal. 630 m	2	2
2041	15	Puerto Francés. 540 m	25	15
2053	16	Corrales de Molina. 400 m	300–500	30

*Collection numbers are those of Stuessy *et al.*, except 2041 and 2053, which are of Anderson.

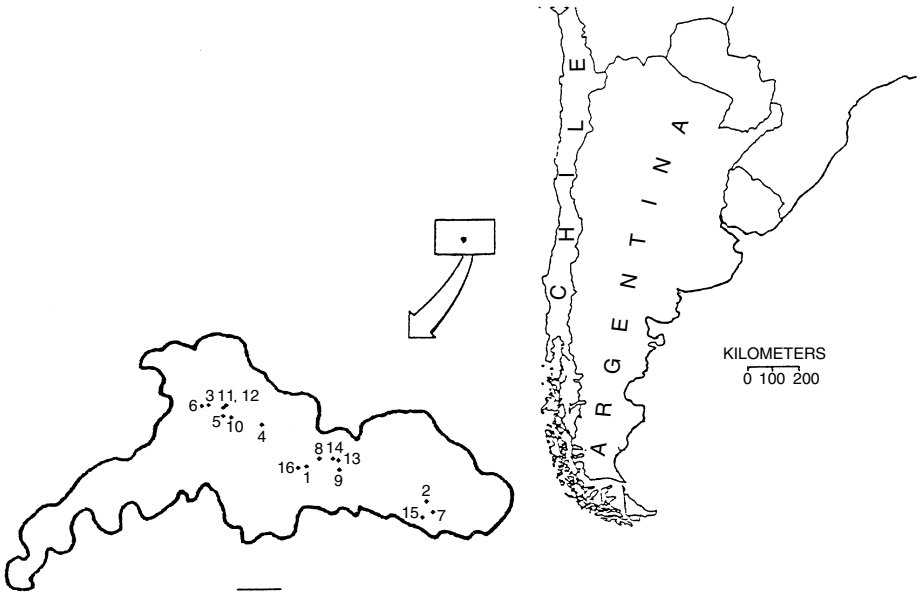

Fig. 1 Map showing the location of Masatierra Island and the populations of *Lactoris fernandeziana* examined for intersimple sequence repeat (ISSR) variation. Population numbers are the same as in Table 1. Line below Masatierra indicates 1 km scale.

the majority of extant populations because there are few other areas with proper habitat that have not been explored during our seven expeditions to Masatierra. In several instances, populations were collected from the same general areas but in different canyons, or at different locations in the same canyon. Leaves were collected from natural populations and either kept fresh at 4°C until returned to the laboratory in Columbus or dried by placing in sealable plastic bags with silica gel.

DNA extraction and ISSR analysis

Total DNA was extracted from leaf material using a modification of the miniprep technique of Doyle and Doyle

(1987) as described by Esselman *et al*. (1999). The ISSR amplifications were likewise done using the methods described in Esselman *et al*. (1999). The three ISSR primers were: 844 $(CT)_8RC$; 17 899 $(CA)_6RG$; and 17 901 $(GT)_6YR$. Bands amplified by PCR were resolved in 1.0% agarose gels in TAE buffer, stained for 30 min with ethidium bromide and destained for 1 h. The ISSR profiles were recorded digitally as TIFF (tagged information file format) files using an Alpha Innotech imaging system (Alpha Innotech Corporation, San Leonardo, CA, USA). The digital imaging files were transferred to a PowerMac 7500 and analyzed with the BioMax 1D image analysis software (Eastman Kodak Company, Rochester, NY, USA). Fragment sizes were estimated based on 100-bp ladder size standards (Gibco BRL, Rockville, MA, USA) according to the algorithm provided in the BioMax 1D software. Each unique band size was designated as a locus for each primer and scored as diallelic (present = 1, absent = 0). The Jaccard coefficient was employed to calculate pair-wise similarities of bands for all 89 plants as described by Esselman *et al*. (1999). Average similarity values were calculated for plants from the same and different populations. Distances were calculated as 1 – (similarity value). Given the high similarity values (0.90 or higher for more than 80% of the pair-wise comparisons of individuals), the relationships between similarities and distance can be regarded as linear. Unweighted pairgroup arithmetic average clustering (UPGMA) and neighbor-joining analyses were carried out on the matrix of pair-wise distances between all individuals. A bootstrap with 100 replicates was performed using neighbor-joining analysis.

The number of multilocus genotypes was determined for plants from each population and these values were divided by the number of plants sampled in a population to give the proportion of distinguishable genotypes per population (Ellstrand & Roose 1987). The number of variable loci and the percentage of variable loci were determined for each population. The ISSR locus diversity was calculated with the Shannon–Weaver information statistic with the Brilliouin formula for eliminating the bias of the finite sample size (Peet 1974; Whitkus *et al*. 1998). Diversity was estimated within each population and across all populations. The apportionment of diversity within and among populations was determined according to Lewontin (1972).

The Spearman correlation coefficient was used to determine if the percentage of variable loci, proportion of distinguishable genotypes, or locus diversity are correlated with population sizes, sample sizes or proportion of plants sampled in populations. Population locations were plotted on a map of Masatierra and the locations converted to X-Y coordinates that were used to create a matrix of Euclidian distances among the populations. NTSYS-pc (Rholf 1997) was used to perform a Mantel test of the hypothesis that there is a random association between geographic and ISSR distances between populations.

Results

A total of 62 loci was scored for the three primers: 17 for primer 844, 24 for 17 899, and 21 for 17 901 (Fig. 2). The number of genotypes detected within populations varied from one in five populations to 16 genotypes in population 16 (Table 2). The proportion of distinguishable genotypes ranged from 0.33 to 1.00, and the percentage of variable loci ranged from 0.00 to 37.3 (Table 2). Unique loci were detected in two of the 16 populations. Three plants in population 13 had unique loci, and in population 15 three of the 15 plants possessed unique loci. Total locus diversity across populations was 0.08 with 73.6% of this diversity apportioned among populations. The proportion of distinguishable genotypes, percentage of variable loci, and locus diversity in populations were not significantly (5% level) correlated with population size, sample size or proportion of plants sampled from populations.

Mean distances between individuals within populations varied from 0.00 to 0.10, whereas values for plants from different populations ranged from 0.02 to 0.18. The mean distance within populations was 0.03 and for different populations it was 0.10.

The neighbor-joining and UPGMA trees were very similar; only the former will be presented and discussed. In the neighbor-joining tree, individuals from populations 1, 3, 4–9, 13 and 14 form distinct groups with no plants from other populations included in each group (Fig. 3). All 10 groups have bootstrap support of 63% or higher and six populations enjoy support higher than 75% (Fig. 3). Eight of 15 plants from population 15 group together and 25 of 30 individuals from population 16 cluster

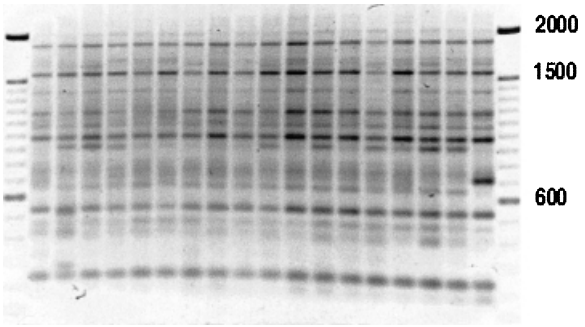

Fig. 2 Ethidium bromide-stained gel showing ISSR profiles generated for 18 plants of population 16 (Table 1) using primer 17 899. Outside lanes are 100-bp ladders with the sizes of several fragments indicated at the right margin.

Table 2 Proportion of intersimple sequence repeat (ISSR) variation within populations of *Lactoris*. Population designations same as in Table 1

Population	No. genotypes detected	No. plants examined	Proportion of distinguishable genotypes	No. variable loci	No. loci in population	Percentage of variable loci
1	2	2	1.00	2	45	4.40
2	2	2	1.00	6	46	13.00
3	2	2	1.00	2	47	4.30
4	1	2	0.50	0	45	0.00
5	1	2	0.50	0	46	0.00
6	2	3	0.66	1	43	2.30
7	1	3	0.33	0	46	0.00
8	1	3	0.33	0	46	0.00
9	1	2	0.50	0	48	0.00
10	9	10	0.90	8	44	18.20
11	2	6	0.33	1	45	2.20
12	2	2	1.00	1	46	2.20
13	3	3	1.00	9	47	19.10
14	2	2	1.00	2	43	4.70
15	14	15	0.93	19	51	37.30
16	16	30	0.53	12	51	23.50

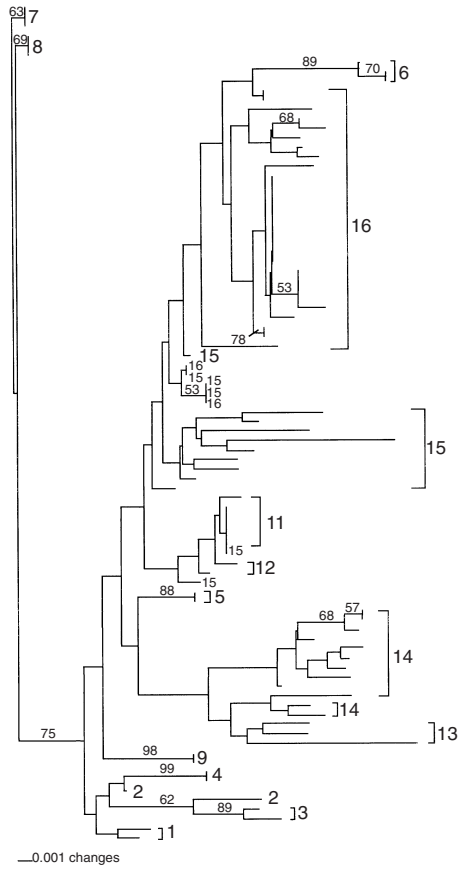

Fig. 3 Neighbor-joining tree based on distances calculated from intersimple sequence repeat loci for 89 individuals of *Lactoris fernandeziana*. Population numbers same as in Table 1. Bootstrap values above 50 are shown above the nodes.

together. Different populations from the same canyon do not cluster together, and those from the same geographic areas of Masatierra occur in different large clusters.

The normalized Mantel statistic z (= matrix correlation) for the ISSR and geographic distances was 0.082, yielding an approximate Mantel *t*-value of 0.857, with a one-tail probability (random z = observed z) of 0.21. Thus, the null hypothesis for a random association between geographic and ISSR distances cannot be rejected.

Discussion

Lactoris fernandeziana is of particular interest because it appears to be the remnant of an old angiosperm lineage (Qiu *et al*. 1993) yet it is endemic to Masatierra, an island about four million years of age (Stuessy *et al*. 1984). A better understanding of the biology of the species, including reproductive biology, demography, and genetic variation within and between populations, would be useful in formulating conservation plans for its long-term survival. Bernardello *et al*. (1999) have shown that the species is self-compatible and probably anemophilous. The orientation of flowers on the branches and the distribution of hermaphroditic and female flowers indicate that geitonogamy is common. The seeds are small (ca. 1 mm long), and seed set and viability are very high (Crawford *et al*. 1994; Stuessy *et al*. 1998). Information on seed dispersal is lacking, but its small size suggests wind as the likely vector. However, the plants occur primarily in the forest understory, which would seemingly minimize the effectiveness of wind as a means of long distance dis-

persal. On the basis of the available data, it is difficult to see how seeds could be moved between different canyons or, indeed, between localized populations within a canyon. No other possible seed dispersers of *Lactoris* have been identified during several years of field studies (Anderson *et al*. unpublished data).

Previous studies of *Lactoris* with molecular markers (Brauner *et al*. 1992; Crawford *et al*. 1994) failed to detect sufficient variation for inferences to be made about the apportionment of diversity within and between populations. No allozyme diversity was found at the 22 presumed loci examined in 83 plants from 12 populations (Crawford *et al*. 1994). An earlier study of *Lactoris* using another PCR-based marker (i.e. RAPD), sampled fewer plants (27 individuals from 15 populations) but 16 primers were used (Brauner *et al*. 1992) compared with three ISSR primers in the present investigation. Despite using over five times as many primers in the earlier study, less than 25% of the loci were polymorphic as compared to 53% in this study, and 12 of the 27 individuals (44%) had identical genotypes for RAPD markers, whereas in this study, no more than five individuals shared the same ISSR genotype. These results of higher diversity per primer with ISSR than with RAPD markers are consistent with other studies (Yang *et al*. 1996; Nagaoka & Ogihara 1997; Parsons *et al*. 1997; Esselman *et al*. 1999).

There is little information on ISSR diversity within natural populations and species of wild plants, but the results for *Lactoris* can be compared with several other taxa. At the specific level, *Lactoris* has 53% variable loci and the values within populations vary from 0% to 37.3%. The value for *L. fernandeziana* as a whole is comparable to the 58% found in *Myrceugenia fernandeziana* (Crawford *et al*. unpublished data), an abundant tree species also endemic to Masatierra. However, the values for *Lactoris* are low compared with species of *Penstemon* where the range is 72–95% (Wolfe *et al*. 1998b). At the populational level, the range of percent variable loci (0–37.3%) is larger than the aforementioned Masatierra endemic *Myrceugenia fernandeziana,* which has a populational range of 13% to 28% (Crawford *et al*. unpublished). Four populations of the rare and highly clonal *Calamagrostis porteri* ssp. *insperata* have percentages of variable ISSR loci ranging from 10% to 21% (Esselman *et al*. 1999). Although sufficient data are not available for extensive comparisons, it appears that ISSR diversity in *Lactoris* is comparable to other endemic or rare species, but is less than that encountered in common continental taxa.

In *Lactoris*, individuals within populations have a lower average distance at ISSR loci than plants from different populations. The greater among compared with the within-population distance is also evidenced by the fact that over 73% of the diversity within the species resides among populations. Furthermore, for 10 of the 16 populations, individuals from each population form distinct groups in the neighbor-joining tree, and all have moderate to strong bootstrap support. The lower ISSR distances within populations result from different combinations of loci present in the species. That is, populations have subsets of loci found in the species as a whole, with only two of the populations having unique loci. Despite relatively low diversity within as compared to among populations, ISSR markers still exhibit more intrapopulational variation than allozymes or RAPD markers. At the interpopulational level, results of the Mantel test show that higher ISSR distances among populations are not correlated with spatial distances among them, and that populations within the same canyon may be just as dissimilar as those from different canyons. In the UPGMA, neighbor-joining and principal coordinates analyzes populations from the same canyons and the same areas of the island do not group together.

The lower level of diversity within populations compared with among populations of *Lactoris* may be viewed from the perspective of the breeding system of the species. While *Lactoris* is anemophilous (no flower visitors were ever seen during extensive field studies), it is self-compatible and appears to be highly geitonogamous (Bernardello *et al*. 1999). Outcrossing to more distant plants in other populations within the same canyon cannot be ruled out, but it may be rare because plants are often in the understory of the forest and effective dispersal of pollen by wind for more than a few meters seems highly improbable (Bernardello *et al*. 1999). No data on seed dispersal are available, but, as with the pollen, wind is suspected to be the primary agent because the seeds are very small and light and there is no evidence of any other dispersal agent (Anderson *et al*. unpublished data). The observed greater ISSR diversity among populations compared to within populations is what would be expected given the localized gene flow. The six instances where all individuals of a population do not occur in the same group (Fig. 3) could be the result of otherwise rare seed or pollen dispersal. Alternatively, recombination resulting from outcrossing within populations could produce arrays of loci that make genotypes in one population appear more similar to genotypes from other populations.

The overall pattern of diversity on Masatierra shows no correlation between genetic distances and spatial separation of populations. Rather, a mosaic pattern of variation is evident (cf. Figs 1,3). The factors responsible for this pattern are not known, but there are several possibilities. Some of the diversity now seen in *Lactoris* may result from variation brought to Masatierra by its ancestor. There may have been an initial and rapid radiation of the species into open habitats over much of the island, with populations subsequently isolated as the canyons were formed and widened as a result of erosion. Thus, the array of loci now

present in each population could result, in part, from drift and inbreeding, with loci being lost or fixed. This interpretation is concordant with 11 of the 16 populations having less than 5% variable loci. It is also possible that the generation of new ISSR loci via mutation could occur, with rare gene flow between populations a factor accounting for the paucity of loci unique to populations. On the basis of ISSR markers, *Lactoris* appears to consist of small isolated populations.

The present study, together with the results of earlier investigations (Brauner *et al*. 1992; Crawford *et al*. 1994; Bernardello *et al*. 1999), have conservation implications for *Lactoris fernandeziana*. The species appears to consist of a series of populations among which there is little gene flow. The species is self-compatible, wind-pollinated and contains a high seed set. Furthermore, there is seedling recruitment in natural populations and thus the species does not, at present, appear to be threatened by low reproductive success. However, as emphasized by Bernadello *et al*. (1999), seed germination is not easy. The pattern of variation at ISSR loci, with most of the diversity found among populations, indicates that the preservation of maximum genetic diversity with *L. fernandeziana* requires the conservation of most of the known populations. Furthermore, the lack of correlation between genetic similarity and spatial proximity of populations means that conservation of a population from the same geographical area of Masatierra, or even any given population within the same canyon, will not preserve the majority of diversity present within the area. Detailed studies have not been carried out on *L. fernandeziana* to determine whether there are morphological, ecological or physiological differences between populations at different elevations within the same canyons or different areas on Masatierra. Field studies have revealed that while plants occur primarily in the forest understory, they may also be found exposed to full sunlight in canopy openings. Low variation at single locus markers such as allozymes, and PCR-based RAPD and ISSR markers should not be taken a priori as evidence for lack of variation at quantitatively inherited variation (Booy *et al*. 2000). Our ISSR data suggest that investigations of variation in morphology, ecology and physiology would be rewarding, and would provide a better understanding of the biology of this remarkable relict species on a young volcanic island.

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