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Molecular and morphological differentiation in *Limonium dufourii* (Plumbaginaceae), an endangered Mediterranean plant

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Abstract

We have analyzed the morphological and molecular variation in individuals from a *Limonium dufourii* population in which we had previously described the presence of two markedly different molecular haplotypes by means of RAPDs and AFLPs. Ten different morphological variables were scored in each of 72 individuals and their molecular haplotype group was established by RAPD analysis. The variation observed in the 10 morphometric variables was explained by four dimensions in a principal components analysis, and a plot of each individual in the plane defined by the two first dimensions did not show any significant grouping until the molecular haplotype was incorporated into the plot. A discriminant analysis performed using the molecular haplotype as the grouping variable resulted in 88.9% of correctly classified cases, thus reflecting a high correlation between morphometric and molecular variation in these individuals. We discuss the relevance of this correlation for the conservation strategy previously proposed for this species.

Introduction

One consequence of the application of molecular biology techniques in evolutionary biology is the questioning of the relevance of neutral molecular variation for adaptive evolution, and most specifically for taxonomy, in the light of the many discrepancies among detected levels of molecular and morphological and physiological variation. The most prominent of these disagreements are probably found in the lack of congruence between phylogenetic reconstructions obtained from molecular data and by means of more traditional characters (Bremer and Struwe 1992; Patterson et al. 1993; Miyamoto 1996). However, another important area where this discrepancy is very relevant is conservation genetics (Milligan et al. 1994; Lynch 1996).

Most population genetics models employed for the assessment and design of strategies for the preservation of genetic variation assume, more or less implicitly, that most genetic variants are neutral or nearly so. Nevertheless, this neutral or nearly neutral variation is of little relevance for the maintenance of adaptive variation which, ultimately, will be responsible for the ability of the species to cope with the challenges derived from changing environmental conditions. The lack of connection between both kinds of genetic variation hampers the application and use of simple population genetic markers for the protection of endangered species, for many of which there is simply no information, neither time to gather it at a sufficiently detailed scale, to use more realistic models.

Our research group has analyzed genetic variation and structuring in *Limonium dufourii* (Girard) O. Kuntze (Plumbaginaceae) populations using different molecular markers (Palacios and González-Candelas 1997; Palacios et al. 1999) in an attempt to use this information in the design of a conservation strategy for this species. *L. dufourii* is a triploid species, with

obligate apomictic reproduction and hybrid origin, inhabiting a few coastal marshes and cliffs in the Valencia region (Spain) of the Mediterranean sea. At the beginning of our work, only 4 locations were known for this species, none with more than a few tens of individuals. However, during our sampling work, two new, very close locations were found in Marjal del Moro (Valencia), each housing several hundred individuals. In these two locations we detected the presence of a portion of individuals considerably larger than the usual size for this species. Nevertheless, on the basis of other morphological traits, they were identified as genuine *L. dufourii*. This identification is relevant as this genus is filled with hybrid taxa and hybridization usually involves apomictically reproducing species.

In the analyses of genetic variation with RAPDs and AFLPs, we detected the existence of two relatively divergent groups of individuals, living in sympatry in these two and a nearby third locations. These were denoted as groups A and B and were clearly identified by distinct patterns both with RAPDs and AFLPs (Palacios and González-Candelas 1997; Palacios et al. 1999). Substantial interindividual variation was found with these markers despite the asexual reproductive system of the species.

Since little is known about the selective neutrality of molecular variation, in general, and of the above described variants in *L. dufourii*, in particular, we decided to study whether both kinds of variants, morphological and molecular, were correlated, since such a relationship is of relevance for the conservation of this species. In this paper we report the results of a joint analysis of morphological and molecular variation in a sample of individuals from one of the above mentioned populations.

Methods

Population sampling

One of the two natural, nearby populations of *L. dufourii* in which previous studies had shown the coexistence of two different molecular haplotypes (Palacios and González-Candelas 1997; Palacios et al. 1999) and markedly different in size individuals was chosen for this study. The population is located in the Marjal del Moro (Valencia, Spain), and it was chosen on the basis of ease of access and abundance of the species. It was sampled as four more or less spatially isolated subpopulations, 20–50 metres apart. In each subpopulation, plants were collected regularly along a 30 m transect. Plants were flowering at the time of collection. A total of 72 individuals were used in the study, and the numbers of individuals sampled and census estimate for each subpopulation are shown in Table 1.

Table 1. Sample and census sizes for each of the four *Limonium dufourii* subpopulations analyzed in this study

Subpopulation	Census	Area (m^2)	Individuals analysed
1	530	250	15
2	573	475	20
3	695	700	30
4	497	75	7
TOTAL	2.295	1.500	72

Morphometric analysis

Ten morphometric traits were scored in each individual. Traits were chosen based on previous works on morphometric variation in genus *Limonium* (Ingrouille 1984; Ingrouille and Stace 1986). The following characters were scored in all the individuals (Figure 1): maximum spike length (MSL), maximum number of spikelets per spike (MNSS), distance between the first two spikelets in the previous spike (S12D), inner bract length (IBL), inner bract width (IBW), outer bract length (OBL), outer bract width (OBW), calyx length (CL), petal length (PL), and petal width (PW). All traits except MSL were measured in the lab, after removal of a few leaves and spikes from each individual.

RAPDs analysis

Two or three small leaves were taken from each individual. Samples were kept at –80 °C until DNA extraction. DNA was extracted using a modified CTAB protocol as described in Doyle (1991). Further details of DNA extraction are summarized in Palacios and González-Candelas (1997). DNA yields were estimated by direct comparison with standard DNA concentrations in 0.8% agarose gels stained with ethidium bromide (0.5 μg/mL). After quantification, DNAs were diluted to a final concentration of about 1 ng/μL in distilled water.

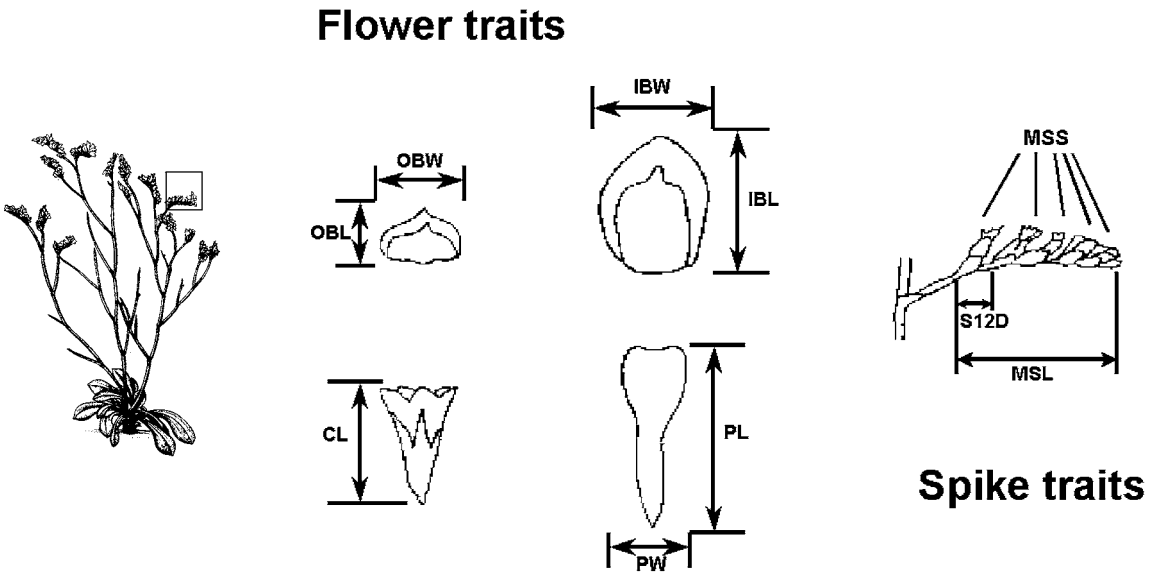

Figure 1. Detail of the 10 morphological characters studied in each *L. dufourii* individual.

RAPD profiles of each *L. dufourii* individual were generated as described in Palacios and González-Candelas (1997). The primer OPA-08 was chosen from the previous RAPD survey on the basis of band reproducibility and easy differentiation between the two groups of haplotypes generated. This primer generated 8 scorable fragments in the 250–3000 bp range.

Data analysis

The 10 morphometric variables were tested for deviations from a normal distribution using a Kolmogorov-Smirnov test and log transformed as needed to fulfil the normality condition. Outliers were identified by means of Grubbs' test (Grubbs 1969) and were replaced by the closest adjacent value (Sokal and Rohlf 1995).

NTSYS-PC v. 1.8 (Rohlf 1993) was used for principal components analysis with the ten morphological variables. SPSS v. 9.0 (SPSS Inc.) was used for discriminant analysis including the molecular phenotype, group A or B following the nomenclature in Palacios and González-Candelas (1997) and Palacios et al. (1999), of each individual as the classification variable.

Results

Morphometric measurements were obtained for each individual without knowledge of their molecular pattern. Only in the discriminant analysis was the molecular information used in conjunction with the morphological variables. The analysis of RAPDs included two individuals with known molecular pattern from our previous study (Palacios and González-Candelas 1997) as controls, in which the amplification patterns obtained two years later matched perfectly the original ones. A total of 21 individuals belonged to group A of molecular patterns and the remaining 51 had a RAPD pattern corresponding to that of group B. With the primer used in the amplifications, the two groups differed in four (two present and two absent in each pattern) out of eight bands scorable in the gels.

For the morphological variables, the analysis of outliers revealed 3 atypical values for the variables MSL, S12D and OBW, in individuals 48, 45, and 39, respectively. The corresponding values were replaced by the closest non-significant value of the variable, thus allowing to use all the sampled individuals in the analyses. Nine of the ten morphological variables fitted a normal distribution, and the remaining one (MNSS) did not do so even after a logarithmic transformation. The remaining analyses were performed with the original and the replaced outlier values and no significant differences were found except for a small

Table 2. Coefficients for each morphological variable (MNSS was log transformed) for the 4 significant principal components obtained

Variable	Component			
	First	Second	Third	Fourth
MSL	0.869	–0.037	0.011	0.213
MNSS	0.560	–0.525	0.241	0.250
S12D	0.298	0.534	–0.502	0.412
IBL	0.754	0.144	0.053	–0.083
IBW	0.786	–0.145	0.144	0.013
OBL	0.669	0.116	–0.182	0.020
OBW	0.821	–0.204	–0.099	–0.027
CL	0.437	0.106	0.036	–0.821
PL	0.301	0.760	0.313	–0.042
PW	–0.063	0.196	0.856	0.223
Variance explained (%)	37.5	12.8	12.1	10.1

Table 3. Summary of the discriminant analysis. The discriminating function results in a correct classification in 88.9% of the cases. The standardized coefficients of the canonical discriminant function are: OBW (0.098), IBW (0.960), PW (–0.442), S12D (0.024), OBL (0.235), IBL (0.201), CL (0.161), MSL (–0.294), MNSS (–0.655), and PL (0.381), and the associated eigenvalue is 0.913.

Actual group	Assigned group		Total
	A	B	
A	19	2	21
B	6	45	51

change in the relative position or values of the three involved individuals.

Principal components analysis of the morphological variables yielded 4 statistically significant components, which explained 72.6% of the total variance. The coefficients for each variable and significant component are shown in Table 2. As it is very often the case for morphological variables, the first component is clearly related to overall size of the individual plant, whereas the second and third component reflect the shape of the flowers. Analyses of variance did not show significant differences among subpopulations for the original variables.

A dispersion plot of all the individuals in the plane defined by the two first principal components (Figure 2) did not show any significant grouping. However, when information on the molecular phenotype of each individual was considered, it became obvious that individuals with pattern A tended to cluster closer to each other than those with pattern B. A similar pattern is found in PC1-PC3 and PC1-PC4 plots (Figure 2), where the first principal component is again the main grouping factor.

The canonical discriminant analysis with the molecular haplotype as the grouping variable produced a classification function that correctly predicted the molecular pattern group to which an individual belonged, based on its morphological values, in 64 of the 72 cases (Table 3). Hence, with an expected success rate of 88.9%, it is possible to predict the molecular pattern of an individual, as characterized by RAPDs, merely by examining the morphological variables considered in our study. The 8 misclasified individuals are evenly distributed between the two molecular phenotypes and subpopulations. Two group B individuals from subpopuulation 1 are incorrectly assigned to group A, one individual from each group in subpopulation 2 is assigned to the other, four B individuals from subpopulation 3 are assigned to group A, and one individual from subpopulation 4 is misassigned to group B. Only two incorrectly assigned individuals (64 and 65) from subpopulation 3 are next to each other, but there are no close individuals from the other molecular group, and their phenotypic values are quite distinct (see Figure 2B). This high success rate is thus a reflection of a correlation between variation at molecular marker loci and those loci determining variation at the quantitative traits investigated.

The spatial distribution along transects and subpopulations of the studied individuals is shown in Figure 3. There is a clear departure from a random distribution of individuals with the two different molecular phenotypes in the four subpopulations, as only subpopulation 2 shows a relatively even number of individuals from both types, and the others are practically monomorphic for group A (subpopulation 4) or B (subpopulations 2 and 3).This is further confirmed by a contingency analysis (results not shown). Nevertheless, given the size of the sampling site, there were no appreciable differences in conditions among the different subpopulations. This observation, along with the lack of grouping of misclassified individuals, supports the main conclusion that there is a high correlation between molecular genotype and morphometric phenotype without a hidden environmental effect acting on both variables.

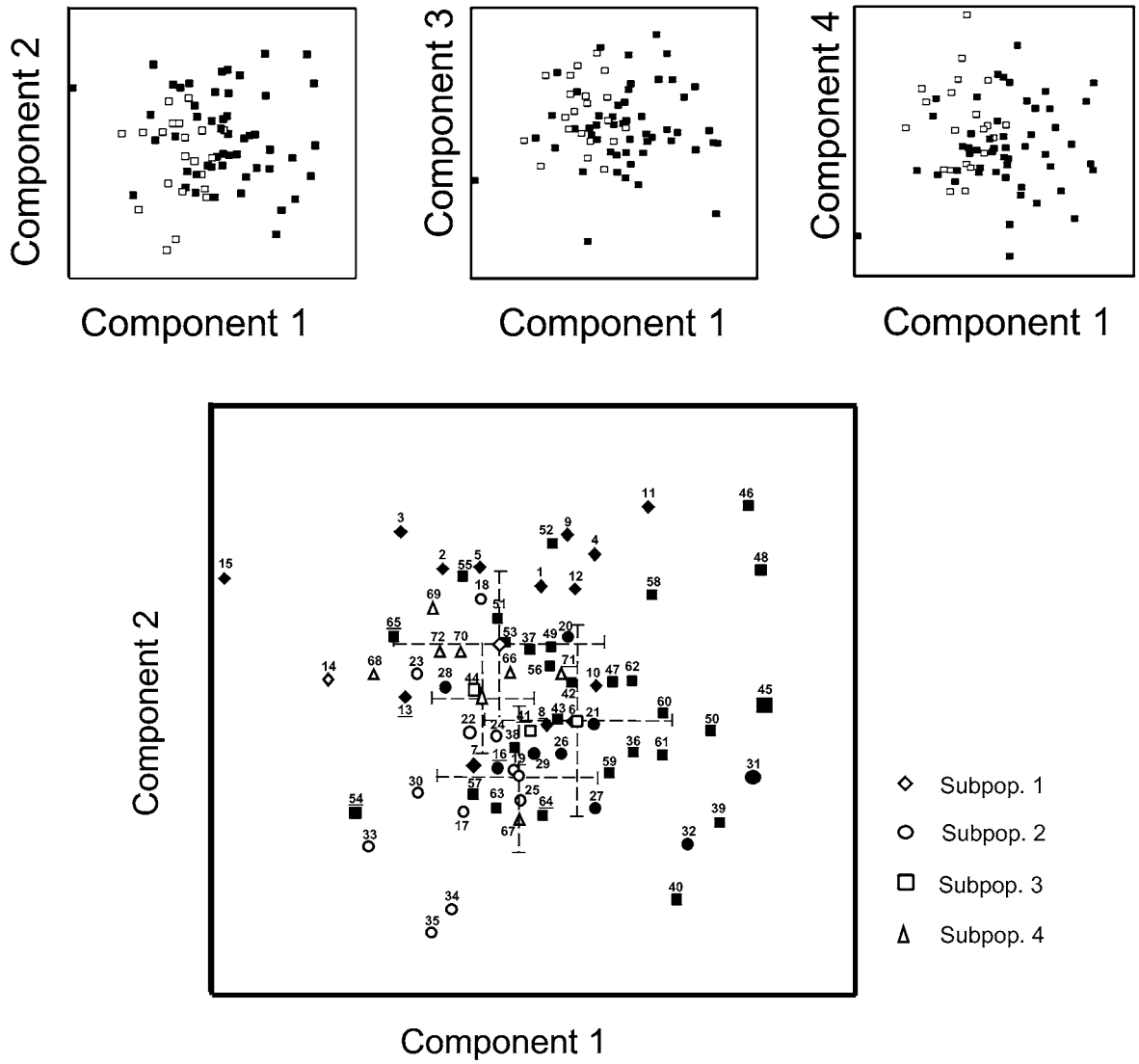

Figure 2. **(A)** Principal components plots (first vs second, third and fourth dimensions, respectively) of the *L. dufourii* individuals studied for the 10 morphological characters. The molecular haplotype (group A or B, estimated by RAPD analysis) of each individual is also indicated by open or filled symbols. **(B)** Detailed plot of first vs. second principal components. Each individual is identified by number, subpopulation and molecular haplotype (open symbols for group A). Individuals incorrectly assigned in the discriminant analysis are indicated by underlining. Average and standard error values of the two variables for each subpopulation are represented by hatched lines.

Discussion

It is evident that many factors determine the abundance or rarity of plant species. What biological information is most critical for the conservation of rare species has been debated for the last 20 years and no consensus has been reached (Franklin 1980; Frankel and Soulé 1981; Schonewald-Cox CM et al. 1983; Soulé 1987; Falk and Holsinger 1991; Hoelzel 1992). Most scientists advocate an approach that is either ecological or genetic in emphasis (Caughley 1994). Proponents of a population genetic approach argue that understanding the organization of genetic diversity and its maintenance are key to the long-term survival of species, since genetic variation is a requisite for evolutionary adaptation (Berry 1971; Lande and Barrowclough 1987; Vrijenhoek 1987; Hamrick et al. 1991), and may also have short-term fitness consequences (Huenneke 1991). This view accounts for the current emphasis on genetic studies in

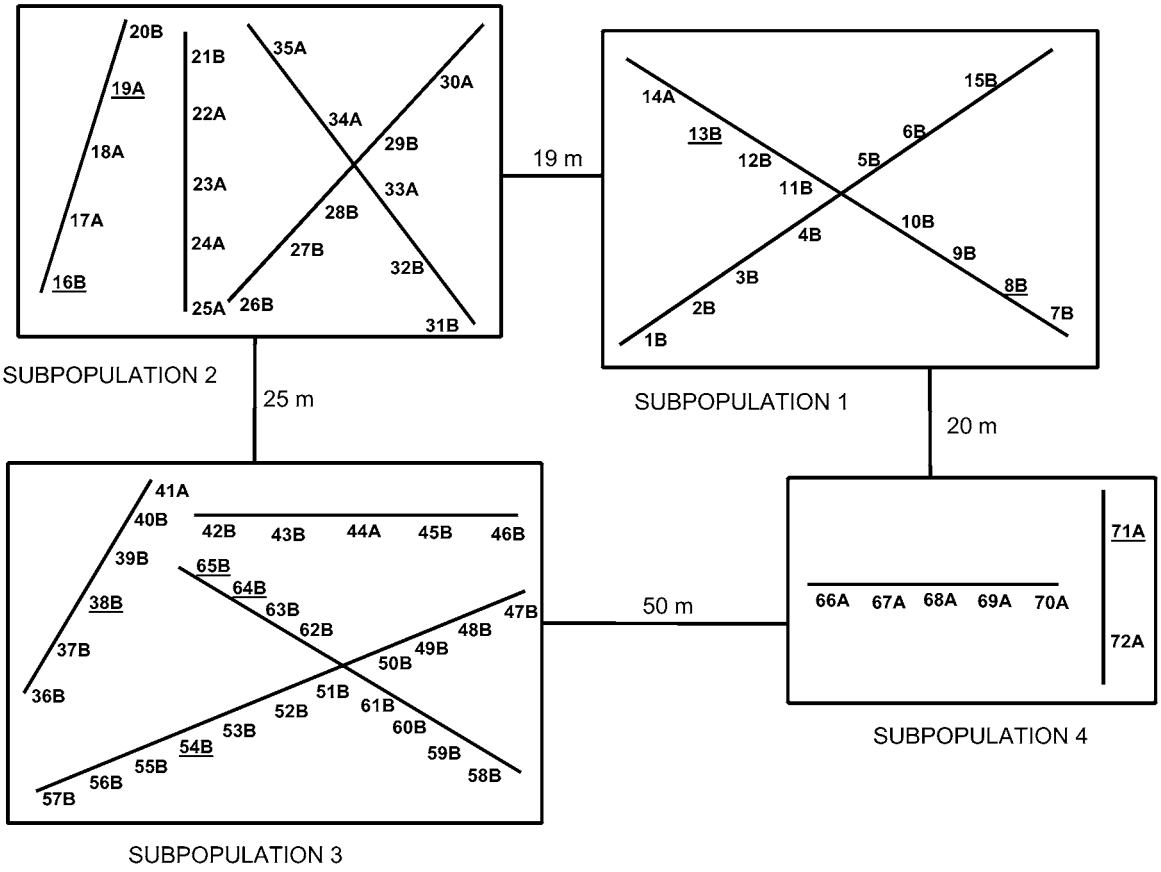

Figure 3. Scheme of the spatial distribution of individuals and their corresponding molecular haplotype in the four subpopulations analysed. Individuals incorrectly assigned in the discriminant analysis are indicated by underlining. Subpopulations are drawn at approximate scale.

conservation biology. However, one central question in population genetics is the relative degree to which random processes and natural selection lead to genetic differentiation. Neutral loci may provide estimates of the amount of gene flow and genetic drift. Molecular markers, as well as some morphological ones, are commonly considered to be selectively neutral, but their adaptive value often remains unknown or questioned (Heywood and Levin 1985; Nevo et al. 1986; Nevo et al. 1991; Allard et al. 1993; Lönn 1993; Begun and Aquadro 1994).

All the means of detecting genetic variation based on survey of proteins or DNA treat the various genotypes as markers of particular regions of the genome, rather than as genotypes specifically related to the traits involved in either adaptation or individual fitness. The underlying assumption is that the level of variation detected at marker loci directly reflects the level of variation that influences future adaptation or individual fitness. But the different types of genetic variation may respond to small population sizes in different ways (Lewontin 1984; Lande and Barrowclough 1987; Polans 1989). For example, neutral variation at marker loci may require many thousands of generations to recover following an extended bottleneck (Nei et al. 1975), whereas recovery of variation for quantitative traits may be achieved in only hundreds of generations. Therefore these two types of genetic variation have different evolutionary dynamics and will respond to rarity or fluctuation in population size in different ways. This difference is relevant to conservation genetics because much, if not most, adaptive evolution is based on polygenic traits that exhibit meristic or continuous variation, not on single locus polymorphisms. As a result, marker locus variation may not provide a reliable measure of the ability of a population to adapt to future conditions or of the fitness of individuals inbred with

respect to the markers. Empirical data also suggest that these relationships may be less straightforward than commonly assumed. For instance, it is not necessary for increased homozygosity to result in decreased fitness, although many examples of the phenomenon exist, with several known examples of no correlation between heterozygosity and fitness (Hutchings and Ferguson 1992; Eguiarte et al. 1992; Whitlock 1993). The central assumptions underlying the direct importance of genetic variation at marker loci to conservation biology, i.e the existence of relationships between marker locus variation and both the potential for adaptation and individual fitness, are not necessarily true in all cases (Milligan et al. 1994).

Given that the evolution of single-locus polymorphism and polygenic variation is not necessarily the same, knowledge of patterns of genetic variation using markers and quantitative traits is required before starting any program of gene conservation (Hamrick et al. 1991; Schaal et al. 1991). The pattern of genetic variation of quantitative traits can be compared with that of molecular or other supposedly neutral genetic markers to determine the relative importance of natural selection and migration in the process of differentiation (Felsenstein 1986; Rogers 1986). However, in a survey of studies using genetic information with a conservation goal (Schemske et al. 1994), in most of the genetic investigations, evolutionary potential was inferred from geographic surveys of isozymes and DNA polymorphisms and only six studies described variation in quantitative characters in rare species (e.g. Meagher et al. 1978), and one considered the importance of inbreeding depression (Karron 1989). Even fewer studies have simultaneously addressed the evaluation of genetic differentiation at marker loci and quantitative traits (e.g. Bonnin et al. 1996).

We have previously reported the existence of multilocus genetic variation and its partitioning within and among populations of *L. dufourii* (Palacios and González-Candelas 1997; Palacios et al. 1999) and this information has helped to establish a conservation strategy for this species. However, given the previously discussed possible lack of correlation between molecular marker and quantitative genetic variation and its relevance for adaptive evolution and conservation of endangered species, the proposed conservation measures might fail to actually preserve most of the relevant genetic variation. In this paper, we have shown that the two more clearly marked variants detected in the largest remnant populations of *L. dufourii*, molecular phenotypes and size variants, are highly correlated in the analysed population. This is also likely the same in the the two other Marjal del Moro populations where both morphometric and molecular variants coexist. Consequently, for this species, preservation of variation detected by multilocus fingerprinting, which can be easily scored and monitored in the future, will also result in the preservation of variation at loci determining morphometric variation. Given the reproductive system of this species, strictly asexual through apomixis, this is true regardless of the actual genetic correlation between both kinds of variation and of .Nevertheless, it is important to note that our recommendations for preservation of genetic variation at the molecular level were not based on surveys of specific, localized loci but on surveys of genome-wide variation.

Unfortunately, the lack of information on the genome organization of *L. dufourii* or its close relatives and the impossibility of performing sexual crosses prevent a more detailed analysis of the relationship between the two kinds of variation described in this work. However, this is not that relevant for our goal in this study, and probably for similar studies with other endangered species with predominantly asexual reproduction, since the main objective in using the information on genetic variation of these species is to identify relevant conservation units and to preserve as many genetic variants as possible so that the adaptive potential of the species is not compromised. Whenever molecular, mostly neutral, and quantitative, possibly adaptive, variation are highly correlated, i.e. in strong linkage disequilibrium due to an asexual reproductive system or strong selection on closely linked loci, for instance, conservation strategies designed for preserving any of them will also contribute to the preservation of the other.

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