Theor Appl Genet (1999) 98: 985–994

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RAPD variation within and among natural populations of outcrossing willow-leaved foxglove (*Digitalis obscura* L.)

Received: 7 September 1998 / Accepted: 28 November 1998

Abstract Random amplified polymorphic DNA (RAPD) markers were used to assess levels and patterns of genetic diversity in *Digitalis obscura* L. (Scrophulariaceae), an outcrossing cardenolide-producing medicinal plant species. A total of 50 plants from six natural populations on the Iberian Peninsula were analysed by six arbitrarily chosen decamer primers resulting in 96 highly reproducible RAPD bands. To avoid bias in parameter estimation, analyses of population genetic structure were restricted to bands (35 of 96) whose observed frequencies were less than $1–3/n$ in each population. The analysis of molecular variance (AMOVA) with distances among individuals corrected for the dominant nature of RAPDs (genotypic analysis) showed that most of the variation (84.8%) occurred among individuals within populations, which is expected for an outcrossing organism. Of the remaining variance, 9.7% was attributed to differences between regions, and 5.5% for differences among populations within regions. Estimates of the Wright, Weir and Cockerham and Lynch and Milligan F_{ST} from null-allele frequencies corroborated AMOVA partitioning and provided significant evidence for population differentiation in *D. obscura*. A non-parametric test for the homogeneity of molecular variance (HOMOVA) also showed significant differences in the amount of genetic variability present in the six populations. UPGMA cluster analyses, based on Apostol genetic distance, revealed grouping of some geographically proximate populations. Nevertheless, a Mantel test did not give a significant correlation between geographic and genetic distances. This is the first report of the partitioning of genetic variability within and between populations of *D. obscura* and provides important baseline data for optimising sampling strategies and for conserving the genetic resources of this medicinal species.

Key words *Digitalis obscura* · AMOVA · HOMOVA · Population genetics · RAPD

Communicated by P. M. A. Tigerstedt

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Introduction

Digitalis species are the main industrial source of cardenolides, widely used for the treatment of cardiac insufficiency in humans. In spite of this, little information is available on the extent, distribution and nature of the genetic variability in *Digitalis*. To-date, genetic resources in the genus *Digitalis* have been characterised by analysing phytochemical traits (Castro-Braga et al. 1997; Nebauer et al. 1999). Nevertheless, the effectiveness of this approach is limited since accurate information on both the amount of diversity present in gene pools and the spatial distribution of diversity in relation to ecogeographic factors is lacking. Information of this nature will allow breeding programs and strategies to be designed for maximum gain from artificially imposed selection regimes. Collection missions can also be directed to areas possessing maximum levels of diversity.

The patterns of genetic variability within and among populations can be influenced by mutation, genetic drift, mating system, gene flow, and selection (Slatkin 1987). Allozyme studies on natural populations often report low levels of genetic divergence within populations in inbreeding species, whereas outbreeders

present higher levels of variation (Schoen and Brown 1991). On the other hand, species with extremely small and disjunct geographic ranges are expected to diverge to a larger extent due to a lower interpopulation gene flow and a larger influence of drift (Lesica and Allendorf 1994). Furthermore, genetic distance tends to increase among populations with increasing geographical distance. Many studies have shown the non-random distribution of genetic variation in populations and emphasised the importance of understanding its spatial structure (Loveless and Hamrick 1984).

Random amplified polymorphic DNA (RAPD) analysis via the polymerase chain reaction (PCR) has profoundly increased the potential to easily, quickly, and inexpensively detect genetic polymorphism among organisms, particularly for those in which DNA sequence information is unknown (Williams et al. 1990; Hadrys et al. 1992). Although RAPD markers must be carefully interpreted because of their dominance (Isabel et al. 1995), this technique has been successfully used to describe population genetic structure in plants (Huff et al. 1993; Le Corre et al. 1997; Palacios and González-Candelas 1997; Aagaard et al. 1998). During recent years, several strategies have been proposed (Lynch and Milligan 1994; Apostol et al. 1996; Stewart and Excoffier 1996) to minimise the effects of RAPD dominance, providing estimates of fixation indices that are similar in nature to those obtained with co-dominant-diploid or haploid data.

Digitalis obscura L. (willow-leaved foxglove) is an outcrossing cardenolide-producing perennial shrub plant commonly found in the thermo- and meso-mediterranean bioclimatic belts of the south, south-east and central Iberian Peninsula. Our previous studies (Nebauer et al. 1999) indicated that variability in the cardenolide content of *D. obscura* is primarily genotype-dependent. To-date, however, no genetic data have been reported for *D. obscura*, making it impossible to generalise from other species. Information on genetic diversity would provide a much improved basis for the appropriate management and conservation of this medicinal plant species.

In the present study the genetic diversity in six natural populations of *D. obscura* was examined using RAPD. The major objectives were to quantify the amount and distribution of genetic variation within and among these populations using genetic diversity measures, F-statistics and spatial-correlation statistics.

Materials and methods

Plant material

Six populations of *D. obscura* L. (Scrophulariaceae) were sampled throughout the natural distribution of the species in Spain. These populations are 1 km to approximately 400 km apart (Table 1). The Huesa population was sampled as two isolated subpopulations. A total of 50 plants were sampled (the sample size for a given population varied from 6 to 13 plants, representing more than 50% of the total number of plants per population).

DNA extraction and RAPD amplifications

DNA was extracted from fresh leaf tissue collected in the six populations. The extractions and PCR-amplifications were carried out as described in Del Castillo-Agudo et al. (1995), and accomplished in duplicate. Fragments generated by amplification were separated according to size on a 1% agarose (SeaKem LE, FMC Bioproducts) gel run in TBE, stained with ethidium bromide, and visualised by illumination with UV light.

RAPD profiles were photographed in a Gel System Printer (TDI), and images were captured using a high-resolution scanner (ImageMaster DTS, Pharmacia LKB). The size of each amplification was automatically estimated using Diversity One software (v 1.0, PDI Inc.).

The reproducibility and repeatability of amplification profiles was tested for each primer. Only those bands which were clear and consistently reproduced were considered. Negative controls were routinely used to check for possible contamination. The bands with the same molecular weight and mobility were treated as identical fragments.

Statistical analysis

Amplified fragments, named by the primer used and the molecular weight in base pairs (bp), were scored for the presence (1) or absence (0) of homologous bands and a matrix of the different RAPD

Table 1 Sampled populations of *D. obscura* with abbreviations, bioclimatic conditions, sample size, geographical location and distances between populations (in km)

Population	Province	Bioclimatic belt[a]	Size	Latitude (N)	Longitude (W)	Distances between populations (km)				
						A	O	L	S	U
Aiora (A)	València	Meso	8	39°03′	1°03′					
Oliete (O)	Teruel	Meso	6	40°59′	0°40′	215				
Llanorell (L)	València	Meso	13	39°19′	0°48′	82	135			
Segart (S)	València	Thermo	8	39°41′	0°22′	94	140	60		
Huesa (U)	Jaén	Meso	7	37°45′	3°04′	230	410	290	320	
Huesa (H)	Jaén	Meso	8	37°45′	3°04′	231	411	291	321	1

[a] Meso: Meso-mediterranean belt with dry ombrotypes. Thermo: Thermo-mediterranean belt with dry ombrotypes

phenotypes was assembled. Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single bi-allelic locus (Williams et al. 1990). Pairwise distance matrices were computed based on both the Euclidean metric (Excoffier et al. 1992) and Apostol (Apostol et al. 1993) coefficients using RAPDistance (Armstrong et al. 1996) software. A Mantel test (1967) showed a complete correlation ($r = 1$) when both matrices were compared in NTSYS-pc (Rohlf 1993). The former distance was chosen due to its adequacy for AMOVA (see below) and the latter because it uses both presence and absence of matches, providing more information regarding the phenotypic similarity among pairs of individuals (Apostol et al. 1993).

The relationships among RAPD phenotypes were assessed as follows. First, the Apostol distance matrix was used to produce a dendrogram using the unweighted pair-group method with arithmetical averages (UPGMA) as implemented in NEIGHBOR from the PHYLIP 3.57c package (Felsenstein 1993). To give a measure of the variability in the data, bootstrap analysis was conducted and 500 similarity matrices were produced using RAPDPLOT (Black 1998). The NEIGHBOR and CONSENSE programmes in PHYLIP were employed to generate the 500 trees that were then used to produce a consensus tree. Permutation test probability (PTP) analysis was also performed, using RAPDistance, to test whether the resulting tree reflects an actual tree-like signal in the data or merely an artefact of the algorithm (Faith and Cranston 1991). We have also used the STATGRAPHIC Plus for Windows (v 2.1 Statistical Graphics Corp, USA) program to study the relationships among the RAPD phenotypes employing multivariate data analysis.

Since DNA fingerprinting techniques may overestimate the relatedness among individuals (Lynch 1988), relationships among RAPD phenotypes were studied following the recommendations of Lynch and Milligan (1994).

The distance matrices between RAPD patterns were used to calculate the pairwise genetic distances between populations. These distance matrices were then employed to construct dendrograms using the UPGMA method. The relationships between matrices of genetic and linear geographic distances were examined with a Mantel (1967) test in NTSYS-pc. The resulting r values were interpreted as correlation coefficients.

The analysis of population genetic structure with RAPD data is hampered by the lack of complete genotypic information resulting from dominance, since this enhances the sampling variance associated with single loci as well as inducing bias in parameter estimation. To avoid such bias, those bands (61 of 96) with extremely low recessive frequencies ($q^2 < 3/n$) were excluded from the analysis (Lynch and Milligan 1994). Assuming that all populations are in Hardy-Weinberg equilibrium, we estimated $q_j(i)$, the frequency of the null allele a at locus i ($i = 1, \ldots, 35$) in population j ($j = 1, \ldots, 6$), as $q_j(i) = [x_j(i)]^{1/2}$, where $x_j(i)$ is the frequency of null recessive homozygotes in population j at locus i.

Two types of analysis were performed to study the genetic structure of *D. obscura* populations. First, we used the extension of the analysis of molecular variance (AMOVA, Excoffier et al. 1992) developed by Stewart and Excoffier (1996) which allows the estimation of population genetic parameters at the genotypic level with RAPD profile data. Initially, the 35 selected bands were analysed directly as phenotypes, as done by Huff et al. (1993), using the Euclidean distance matrix of Excoffier et al. (1992). Since a population structure was evident, the previously estimated null-allele frequencies were employed to generate a genotypic distance matrix assuming random mating ($S = 0$). The nested AMOVAs were used to estimate the partitioning of total genetic diversity in variance components among individuals within populations, among populations and among regions. Regions were selected on geographical differences because there were no evident phenotype differences between the groups. AMOVAs were also performed for the individuals within each of the two selected regions: Huesa (U and H populations) and Levante (A, O, L and S).

A non-parametric test for the homogeneity of molecular variance (HOMOVA), based on Barlett's (1937) statistic, was also applied to test whether populations have different amounts of RAPD variation. Both AMOVA and HOMOVA were performed using the WINAMOVA 1.5 program (available from L. Excoffier, Genetics and Biometry Laboratory, University of Geneva, Switzerland).

To corroborate the AMOVA results, we used F-statistics as a second approach to study population genetics in *D. obscura*. F_{ST} was estimated from our 35 selected RAPD bands using RAPDFST from RAPDPLOT (the corresponding statistical methods and equations are given in Apostol et al. 1995). This program estimates F_{ST} according to Lynch and Milligan (1994) and Wright (1951), and as θ (Weir and Cockerham 1984). When appropriate, a subsequent chi-square value was calculated to determine if these estimates of F_{ST} varied from zero (significant population differentiation). Pairwise values of F_{ST} between populations were also calculated using RAPDDIST from RAPDPLOT, applying the Lynch and Milligan (1994) correction when estimating allele frequencies. The resulting distance matrices were compared to test their correlation with the Φ_{ST} distance matrix from AMOVA, as described above. The corresponding UPGMA dendrograms were implemented in NEIGHBOR from PHYLIP.

Results

Fingerprinting of *D. obscura* populations

Sixty primers (Series A, B and C, Operon Technology) were initially tested against ten plants randomly selected from three (Aiora, Llanorell and Segart) of the studied populations. A set of 42 decamers gave amplification bands: 12 of the series A, 10 of the series B, and 20 of the series C. On the basis of the high reproducibility of the patterns, the signal intensity and an adequate number of bands (between 6 and 15), six primers (OPA10, OPA13, OPB7, OPC5, OPC7 and OPC8) were used to screen the 50 genotypes. These oligonucleotides generated a total of 96 consistently well-amplified bands, ranging in size from 350 to 4000 bp. Most of these bands (90.6%) were polymorphic among populations (Table 2). Reflecting this high level of genetic polymorphism, no individuals had the same band pattern over all studied primers (data not shown). Figure 1 illustrates a typical example of the band patterns generated.

The distribution of RAPD bands among populations appeared to be highly constant and both origin and population-size independent (Fig. 2). Very few bands were differentially present in the populations or exclusive to a single individual. Thus, population-specific bands were only found for Segart (OPB7, 950-bp fragment), Aiora (OPA13-3100), and Huesa (OPB7-500, OPA10-1610, -1550 and -660). Genotype-specific markers were detected in Segart (S1, 5 and 8: B7-880, A10-810 and A13-370 fragments respectively) and Oliete (O3: C7-1500 and -3200 fragments).

Relationships among RAPD phenotypes

An unrooted UPGMA dendrogram based on the 96 RAPD phenotypes clustered the 50 individuals within

Table 2 Primers employed and the number of RAPD markers obtained

Primers	Sequence (5′ → 3′)	Size (bp) min–max	Number of bands		
			Polymorphic	Monomorphic	Total
OPA10	GTGATCGCAG	620–2810	15	0	15
OPA13	CAGCACCCAC	350–3200	7	5	12
OPB7	GGTGACGCAG	480–3100	19	0	19
OPC5	GATGACCGCC	750–3600	11	3	14
OPC7	GTCCCGACGA	650–3100	21	0	21
OPC8	TGGACCGGTG	750–4000	14	1	15

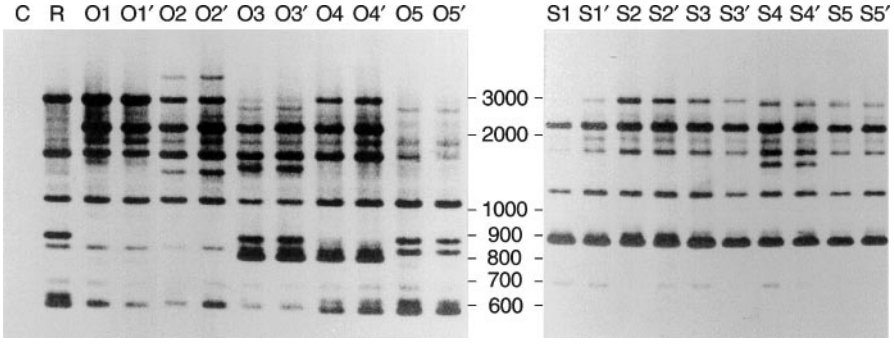

Fig. 1 Examples of RAPD profiles in *D. obscura* individuals from Segart (S1–S5′) and Oliete (O1–O5′) populations using primer OPC7. Each individual is represented by two lanes. *C* = negative control; *R* = reference lane (individual A1 from the Aiora population)

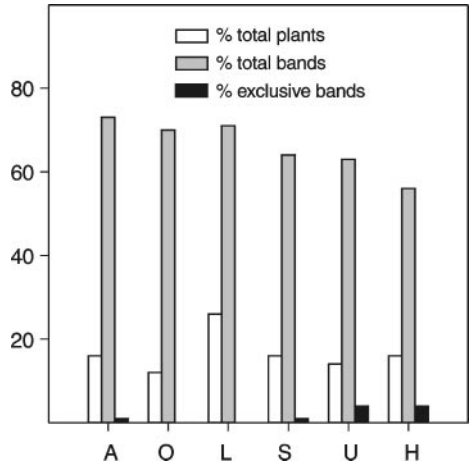

Fig. 2 Distribution of plants and RAPD bands by geographical location. The percentages of the total number of plants in each population, the percentages of all the known bands present in each population, and the percentages of bands being exclusive to each population are indicated

distinct groups according to their geographical origin. Two major clusters were formed: one including both Huesa populations (H and U), the other including the Levante populations (A, O, L and S). In this second group, all four populations formed minor clusters of their own. A majority rule consensus tree derived from the 500 replications revealed similar relationships (data not shown). Furthermore, the PTP test gave a Z-value of 64.94, suggesting that the probability of this tree to occur by chance is almost nil. Individual plants were also separated by their area location using Factor analysis of the original data. The first three factors accounted for 42, 15 and 8% of the total variation respectively and identify two main groups: Huesa (H and U) and Levante (A, O, L and S). The second factor discriminated the Llanorell population from the three remaining populations, while the Segart population is differentiated from the other two by the third factor. The fourth factor separated the Aiora and Oliete genotypes (data not shown).

When the UPGMA analysis was restricted to those bands (35) whose observed frequency was less than $1–3/n$, there was not a complete concordance between the individuals and their respective geographical location. Also the consensus tree from the 500 bootstrapped samples indicated a lower level of support for the distinctness of the six populations (Fig. 3). Although most individuals grouped according to their geographic origin, phenotypes O2, O3, L4, L5, L6, U5, L13 and U3 clustered out of their corresponding population. The PTP test gave a Z-value of 10.96.

Divergence at the population level

The matrices of interpopulation distances obtained using the Apostol coefficient are shown in Table 3. The dendrograms constructed with the UPGMA method (Fig. 4) showed a tendency for the populations to cluster according to their geographical location when the 96 RAPD phenotypes were analysed. Corroborating this, a Mantel's test showed that the genetic distance between populations was correlated with the

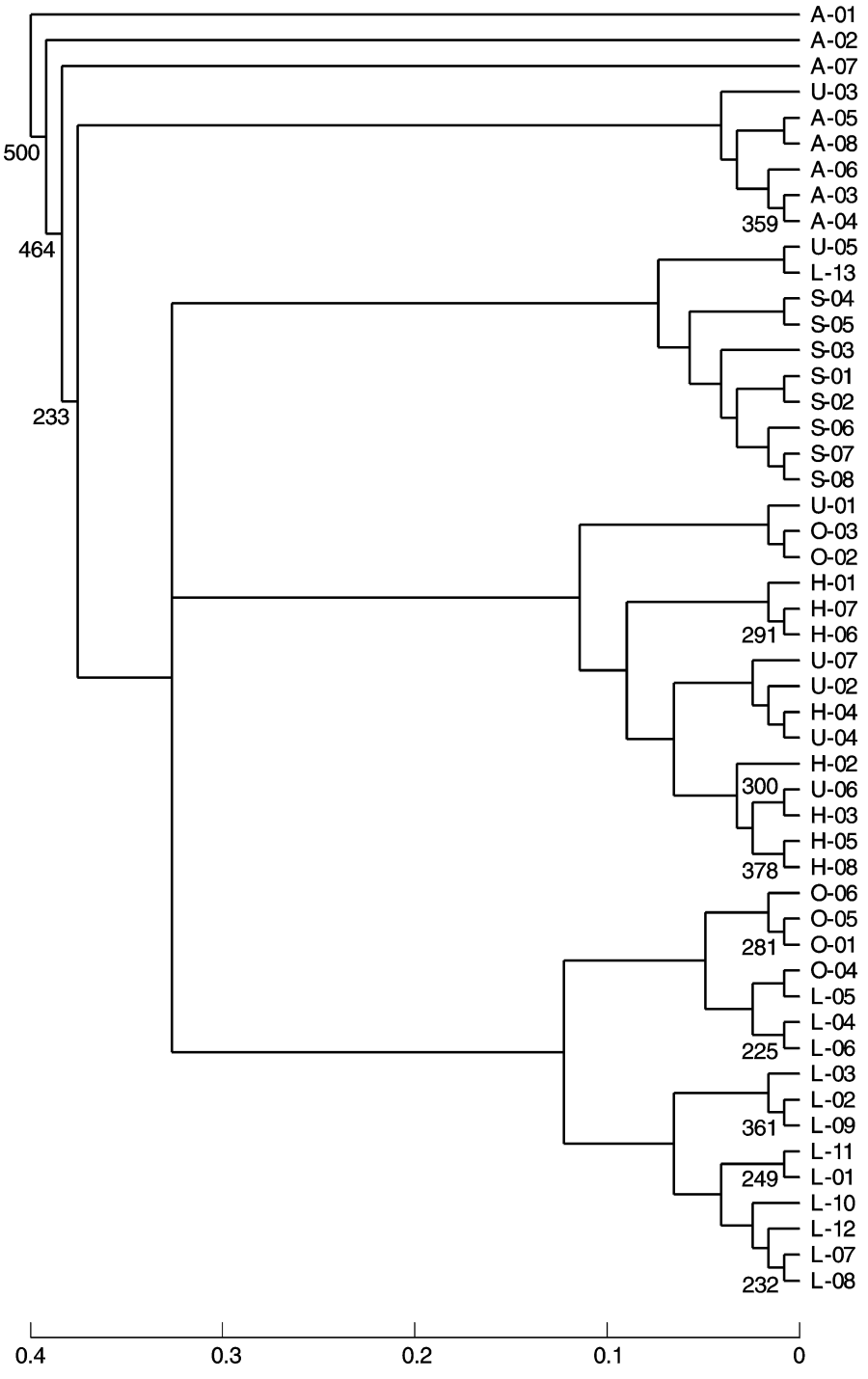

Fig. 3 Bootstrapped UPGMA tree from the Apostol distance matrix using the 50 *D. obscura* individuals and the 35 RAPD markers that fulfil Lynch and Milligan's (1994) criteria. *Numbers* at the nodes indicate the number of trees with that node (omitted if in less than 40% of trees)

Table 3 Interpopulation distances obtained using the Apostol coefficient based on 96 (above diagonal) and 35 (below diagonal) RAPD phenotypes produced in 50 individuals of *D. obscura*

Population	A	O	L	S	U	H
A		0.2752	0.3063	0.3268	0.3501	0.3698
O	0.3274		0.2489	0.3607	0.3517	0.3616
L	0.3264	0.2579		0.3026	0.3549	0.3628
S	0.3063	0.3155	0.2440		0.3733	0.3695
U	0.3296	0.2857	0.2845	0.2806		0.1235
H	0.3063	0.2643	0.2780	0.2589	0.1725	

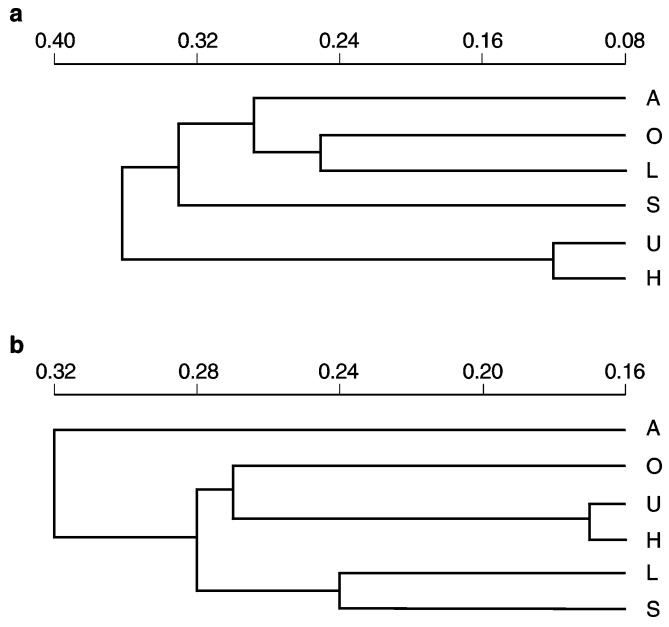

Fig. 4 UPGMA trees for the six *D. obscura* populations using the Apostol distance from 96 (**a**) or 35 (**b**) RAPD phenotypes. The latter fulfil Lynch and Milligan's (1994) criteria

geographical distance (Tables 1 and 3, $r = 0.68$, $P = 0.04$). This, however, did not hold true ($r = 0.21$, $P = 0.18$) when the pairwise distance matrix was constructed from the 35 RAPD phenotypes that fulfil the criteria of Lynch and Milligan (1994). Note that the dendrogram produced some clusters, specially U–H, of geographically proximate populations. Furthermore, factor analysis on the estimated null-allele frequencies of the 35 selected bands substantiated these results since the first two factors (39.5% and 23.3% respectively of the total variance explained) discriminated Huesa from the rest of populations (data not shown).

Population genetic structure

Of the 96 markers scored, 35 (36.5%) of them were in frequencies which fulfilled the Lynch and Milligan (1994) condition for obtaining unbiased estimates of population-genetic parameters. This set of selected polymorphic markers was used, therefore, in the following analyses.

AMOVA and HOMOVA analysis

AMOVA analysis from the phenotypic distance matrix (DP) permitted a partitioning of the overall variation into three levels (Table 4). Although most of this variation was found within populations (63.2%), there was also evidence for a significant phenotypic structure of the populations ($\Phi_{ST} = 0.326$, $P < 0.0003$). The remaining phenotypic diversity was distributed between regions (14.2%) and between populations (22.6%). For the Huesa-region analysis, all of the diversity was attributable to differences among individuals within populations (95.7%) since the among-population

Table 4 Summary of the AMOVA and HOMOVA analyses. Population statistics were estimated according to different assumptions of the data. DP and DG: analyses based on the matrices of phenotypic and genotypic distances, respectively, between individuals (see text for their definitions). Statistics include: degrees of freedom (*df*), sum of squares (SSD), variance-component estimates (CV), percentages of the total variance (% Total) contributed by each component, and Bartlett's test (B)

Analysis	Source of variation	*df*	SSD	CV	% Total	B
DP				*		
Huesa vs Levante	Among groups	1	28.7	0.75	14.15***	1.04***
	Among populations within groups	4	52.7	1.19	22.61***	1.59***
	Within populations	44	147.0	3.34	63.24***	
Huesa	Among populations	1	3.8	0.13	4.33NS	0.92*
	Within populations	13	36.8	2.83	95.67	
Levante	Among populations	3	48.9	1.50	29.69***	0.42*
	Within populations	31	110.3	3.56	70.31	
DG						
Huesa vs Levante	Among groups	1	49.3	1.41	9.67***	1.91***
	Among populations within groups	4	77.1	0.81	5.52***	2.44***
	Within populations	44	552.4	12.56	84.81***	
Huesa	Among populations	1	2.3	−0.80	−10.67NS	0.88*
	Within populations	13	107.3	8.25	110.67	
Levante	Among populations	3	74.2	1.21	7.83***	0.32***
	Within populations	31	445.1	14.36	92.17	

*** $P < 0.001$; * $P < 0.01$; NS = not significant (significance tests after 3000 permutations)

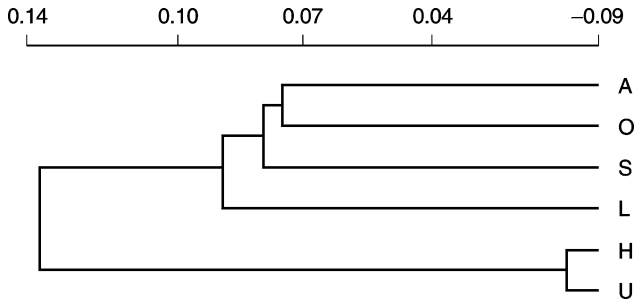

Fig. 5 UPGMA tree for the six *D. obscura* populations analysed using pairwise comparisons derived from genotypic AMOVA analysis (Stewart and Excoffier 1996)

partitioning was not significant (Table 4). This variance partitioning was also evident when the Levante region was analysed. Note however that the geographical structure among these populations was maintained (29.7% of population differences within this region).

These significant results allow us to compute RAPD-site frequencies separately for each population and to generate a genotypic distance matrix (DG). The AMOVA analysis of this matrix strongly corroborated the outbreeding behaviour of *D. obscura*. As shown in Table 4, the proportion of variation attributable to within-population differences was very high (84.8%). Nevertheless, this analysis also indicated a significant population differentiation ($\Phi_{ST} = 0.152$, $P < 0.0003$). Furthermore, except for the Huesa populations, all pairwise comparisons of population variance were significant (data not shown). The UPGMA dendrogram made with the data of net divergence among populations is shown in Fig. 5. For the within-region analysis, we emphasise the absence of population structure in the Huesa region ($\Phi_{ST} = -0.107$) and a significant population differentiation ($\Phi_{ST} = 0.078$, $P < 0.0003$) in the Levante region (Table 5).

Bartlett's test for the homogeneity of variance indicated significant levels of RAPD variation (both phenotypic and genotypic) among populations (Table 4). The lowest level of genetic variation (intrapopulation variances for genotypic AMOVA) was observed in the Huesa populations, followed by the Llanorell, Segart, Oliete and Aiora populations, respectively (data not shown).

Estimation of F_{ST}

Estimates of F_{ST} and θ averaged over the 35 loci are shown in Table 5. The correlations across loci between all three values were high ($r > 0.96$, $P = 0.0000$). Irrespective of the method employed, the estimated F_{ST} and θ were significantly different from zero, indicating among-population genetic differentiation. This also holds true for the Levante populations, but not for Huesa region, where the low value of F_{ST} indicates an absence of population structure. Interpopulation distances obtained using Φ_{ST} from AMOVA and Lynch and Milligan's F_{ST} or θ showed a significant positive correlation ($r > 0.97$, $P = 0.002$). This correlation was slightly lower with Wright's F_{ST} pairwise distances ($r = 0.78$, $P = 0.006$). All these results corroborated those above described in the extension of the AMOVA analysis (Stewart and Excoffier 1996), suggesting that this technique, first employed for inbreeding species, can be successfully applied to outbreeders.

Discussion

Despite their pharmaceutical interest and economic importance, the genetic variation of *Digitalis* spp. over their natural range has not been previously investigated. A systematic evaluation and quantification of the variability available in these species will make their exploitation as a source of cardenolides easier. This is the first study reporting genetic variation within and among natural populations of *D. obscura*, an outcrossing insect-pollinated cardenolide-producing perennial shrub plant. Although self-incompatibility mechanisms have not been described for *D. obscura*, floral dichogamy (protandry) promotes cross-fertilization in this species (Kampny 1995).

The dominant RAPD markers caused severe biases and discordant estimations of population genetic parameters, and especially decreased the expected heterozygosity and inflated the among-population differentiation (Isabel et al. 1995). To avoid such bias, all fragments at high frequencies were discarded to assess the population genetics of *D. obscura*. The need to increase the number of individuals per locus, a requisite to improve the estimates of allele frequencies derived from fragment frequencies (Lynch and Milligan 1994), seems to be less important in our study because

Table 5 Estimates for *D. obscura* populations of F_{ST}, θ and Φ_{ST} according to Wright (1951), Weir and Cockerham (1984), Lynch and Milligan (1994) and Stewart and Excoffier (1996)

Item	F_{ST} Wright	θ Weir and Cockerham	F_{ST} Lynch and Milligan	Φ_{ST} Stewart and Excoffier
Overall	0.167***	0.214***	0.210***	0.152***
Levante region	0.150***	0.186***	0.173***	0.078***
Huesa region	0.049NS	0.028NS	0.011NS	−0.107NS

*** $P < 0.001$, NS = not significant

sampling sizes always represented more than 50% of the total number of *D. obscura* plants per population. Although some problems of bias cannot be completely eliminated when working with RAPDs (Lynch and Milligan 1994), the high correlation obtained between the two approaches used to assess the population genetics of *D. obscura* [the AMOVA extension of Stewart and Excoffier (1996) and *F*-statistics] suggests that our estimates of fixation indices are similar in nature to those obtained with co-dominant-diploid or haploid data. Although genetic variation studies at allozyme loci in *Digitalis* have not been reported, estimates of fixation indices in other Scrophulariaceae, with the same mating system (Godt and Hamrick 1998), are close to those obtained by us. Furthermore, several studies have demonstrated that RAPDs and allozymes can exhibit similar levels of diversity and differentiation among plant populations (Bucci et al. 1997; Le Corre et al. 1997; Aagaard et al. 1998) if a high proportion of loci with a high frequency of null alleles is selected for analysis.

Our RAPD survey of the Spanish populations of *D. obscura* demonstrates that this medicinal plant displays a high level of genetic variability. The importance of high levels of genetic variation has been emphasised as a safeguard against co-evolving biotic factors, such as pests and diseases (Namkoong 1986). The distribution of genetic diversity in natural populations of *D. obscura*, with high levels of variation within populations, agrees with the behaviour of an outcrossing species (Hamrick and Godt 1990; Schoen and Brown 1991). The value of RAPDs for detecting this intrapopulation variation is endorsed by a previous study on cardenolide content in the plants used for RAPD analysis, in which the proportion of phytochemical variation attributable to individuals was significantly higher than that attributable to population differences (Nebauer et al. 1999).

The levels of within-population variation found in *D. obscura* (84.8% for the among-regions analysis and 92.2–100% for the two within-regions analysis) were similar to those reported in other outcrossing species such as *Buchloë dactyloides* (Huff et al. 1993), *Populus tremuloides* (Yeh et al. 1995), *Ancistrocladus korupensis* (Foster and Sork 1997), and *Banksia* spp. and *Dryandra* spp. (Maguire and Sedgley 1997). These data suggest that *D. obscura* populations still retain links via gene flow and are thus able to maintain considerable genotypic variability. Such gene flow among the populations would be advantageous since it would act against the detrimental effects of inbreeding depression and genetic drift, thus increasing the effective population size (Ellstrand and Elam 1993).

Our estimates of the fixation indices (Φ_{ST} or F_{ST}) also demonstrate that some structure can be discerned among *D. obscura* populations. Furthermore, HOMOVA analysis showed significant differences in the amount of genetic variability present in the six populations. Obligate outcrossers generally show a weak spatial genetic structure within continuous plant populations in the absence of apparent environmental heterogeneity, with F_{ST} estimates ranging from 0.004 to 0.08 (Heywood 1991). This also holds true for the two Huesa subpopulations where values of F_{ST} given by genotypic AMOVA and RADPLOT (-0.107 to 0.049) are within the range indicated above. As geographic distances between populations increased, so did the fixation indices (0.078 to 0.173 for the Levante populations and 0.152 to 0.214 for the two-region analysis). All these F_{ST} values are close to those given in the literature for the analysis of population structure in mixed and outcrossing species: 0.1 to 0.24 (Loveless and Hamrick 1984), 0.099 to 0.216 (Hamrick and Godt 1990), and 0.03 to 0.31 (Heywood 1991). These results suggest that the subdivisions in *D. obscura* occurred due to the decrease of gene flow with distance between populations, supporting the expected pattern (Slatkin 1993) that genetic differentiation is higher at larger spatial scales. Nevertheless, there was no significant correlation between the genetic and the actual geographical distances among populations. Selection can also lead to differentiation within populations (Falconer and Mackay 1996).

A UPGMA dendrogram, based on the 96 RAPD phenotypes, grouped the 50 individuals according to their geographical origin. It is likely, however, that this analysis overestimates the relatedness among individuals, since the support given by the dendrogram constructed using the 35 selected bands (which fulfilled Lynch and Milligan's assumptions for the analysis of dominant markers) was not strong. Thus, in agreement with Lynch (1988), an accurate estimation of relationships among RAPD phenotypes is dependent upon an accurate estimation of allele frequencies in the populations. Genotypes were also separated by their area location using a multivariate analysis of overall similarity. Here, the whole matrix of RAPD phenotypes seems to be highly effective in distinguishing genotypes from geographically separated areas, suggesting that this technique can be useful in population fingerprinting and germplasm assessment. The capacity to fingerprint individuals within populations is particularly valuable for ecological studies involving parentage analysis, mating systems, pollen flow, and reproductive fitness (Lewis and Snow 1992; Milligan and McMurry 1993). Nevertheless, a high number of available primers is necessary for strain or cultivar identification purposes.

RAPD is an appropriated technique to monitor genetic diversity in plant populations when there are only small amounts of biological materials available for analysis, closely related individuals are being compared, little (or no) DNA sequence information is available for the particular species, and global evaluations of variability are impossible (Williams et al. 1990; Hadrys et al. 1992; Palacios and González-Candelas 1997).

Most of these conditions apply to our study, in which the RAPD technique has been employed to evaluate genetic variation within and among natural populations of the cardenolide-producing plant species *D. obscura*. Estimates of the genetic variation reported herein provide a basis for the conservation and exploitation of genetic resources in *D. obscura*. With a knowledge of the available genetic structure, an appropriate strategy for sampling and propagation may be easily formulated when ex situ conservation is required. Finally, the application of molecular methods to a species which has been classified solely in terms of morphology and geographic origin has the potential to accelerate the accumulation of information in comparison with traditional methods.

Acknowledgements The financial support of DGICYT, Madrid, Spain (Projects PB93-0695 and PB96-0789), is gratefully acknowledged. A FPI Research Fellowship (MEC, Spain) to S.G.N. is also acknowledged. We thank D. Lindsay for his revision of the english version of the manuscript. Experiments carried out for this work comply with the current laws of the country where they were performed.

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