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An assessment of genetic relationships within the genus *Digitalis* based on PCR-generated RAPD markers

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Abstract RAPD markers were used to study inter-specific variation among six species of the genus *Digitalis*: *D. obscura*, *D. lanata*, *D. grandiflora*, *D. purpurea*, *D. thapsi* and *D. dubia*, and the hybrid *D. excelsior* (*D. purpurea* × *D. grandiflora*). A total of 91 highly reproducible bands amplified with four arbitrarily chosen decamer primers were obtained. Homology of the co-emigrating RAPD markers was tested by blot hybridisation and sequencing of selected bands. The application of a range of statistical approaches for RAPD data analysis, including distance and parsimony methods, family clustering and the analysis of molecular variance (AMOVA), indicated that these molecular markers were taxonomically informative in *Digitalis*. The species relationships revealed were fully consistent with those previously obtained using morphological affinities. The hybrid *D. excelsior* seems to have stronger affinity to the section *Digitalis* than to *Grandiflorae*. This is the first known report of the application of RAPD markers for the study of genetic relationships among species of the genus *Digitalis*.

Key words *Digitalis* spp. · AMOVA · Genetic relationships · RAPD · *Scrophulariaceae*

Introduction

Effective conservation and the use of genetic resources in plant breeding programs requires information on genetic diversity and relationships within and between the species of concern. Although several molecular techniques have been employed to assess genetic diversity and relationships, random amplified polymorphic DNA (RAPD) data can be generated faster and with less labour than other methods, such as RFLP (restriction fragment length polymorphism) and microsatellites (Williams et al. 1990; Hadrys et al. 1992). For the study of interspecific genetic relationships, the use of RAPDs has been criticised for revealing unreliable phylogenies because of a possible lack of homology of co-migrating bands (Brummer et al. 1995; Rieseberg 1996; Bachmann 1997). However, RAPD markers have been successfully employed to reveal relatedness at the section level, or above, in several plant genera (Hoey et al. 1996; Chan and Sun 1997; García et al. 1998; Shen et al. 1998; Ahmad 1999; Cao et al. 1999; Rodríguez et al. 1999).

The genus *Digitalis*, a member of the Scrophulariaceae, encompasses 19 species native throughout the Mediterranean area, Europe and central Asia (Werner 1964), but some of them have become naturalised or casual world-wise. Several *Digitalis* species are used therapeutically, as they are the main source of cardiac glycosides (Wichtl et al. 1987), and most of them are of great ornamental value.

In spite of their economic importance, little information is available on the genetic relationships between *Digitalis* spp. Compatibility studies within the genus *Digitalis* showed that interspecific (inter- and intrasectional) crosses can be made without difficulty (Lichius 1991). This provides opportunities to exploit the wide germplasm of *Digitalis* by looking at other species for phytochemical traits of interest in their breeding endeavours. Then, knowledge of genome relatedness among these species is important for the selection of parental strains in interspecific hybridisation. The suitability of RAPDs to assess levels and patterns of genetic diversity in natural populations of *D. obscura* has been reported recently (Nebauer et al. 1999).

To-date, phylogenetic relationships among *Digitalis* species have been investigated mainly by morphological

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studies, and systematics within the genus have been subject to disagreement (Ivanina 1955; Werner 1964). Thus, molecular data may provide a solution to the taxonomic problems, as well as information about the possible evolutionary relationships of sections and species within the genus.

The aim of the present work was to evaluate the suitability of RAPD markers to determine the relationships between six species of the genus *Digitalis*: *D. obscura* L., *D. purpurea* L., *D. dubia* Rodr., *D. thapsi* L., *D. grandiflora* Mill., *D. lanata* Ehrh., and the hybrid *D. excelsior* (*D. purpurea* × *D. grandiflora*). The assumption of the homology of RAPD markers of the same size was tested by blot hybridisation, using labelled probes made from single amplification products, and the sequencing of some selected bands.

Materials and methods

Plant material

Six *Digitalis* species belonging to four sections (see Werner 1964) of the genus *Digitalis*: *D. obscura* L. (Section *Frutescentes* Benth.); *D. thapsi* L., *D. dubia* Rodr., and *D. purpurea* L. (*Digitalis*); *D. grandiflora* Mill. (*Grandiflorae* Benth. em. Werner); *D. lanata* Ehrh. (*Globiflorae* Benth.) and the hybrid *D. excelsior* (*D. purpurea* × *D. grandiflora*), were used in this study.

Digitalis obscura seeds were harvested from plants growing in their natural habitats. For all other species, seeds were obtained from a commercial source (Chiltern Seeds, England). In all the cases, seeds represent bulk collections from single or multiple sites of wild populations. Seeds were sown in flats of commercial potting soil and maintained under greenhouse conditions. The flats were regularly surface-irrigated with half-strength Hoagland and Arnon's (1950) nutrient solution. After approximately 3 months, 50 mg of fresh leaf tissue of six individual genotypes from each species were collected separately in microcentrifuge tubes for DNA extraction.

DNA extraction and amplification

Genomic DNA extractions were carried out as described by del Castillo-Agudo et al. (1995). Polymerase chain reaction (PCR)-amplifications were performed according to Williams et al. (1990). Each RAPD reaction (50 µl final volume) contained 0.5 U of *Taq* polymerase (Pharmacia Biotech), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of primer (Operon Tech., Calif., USA) and 10–30 ng of genomic DNA. Amplifications were carried out in a GeneAmp PCR System 9600 Thermal Cycler (Perkin Elmer) programmed for 1-min denaturation at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s annealing at 39°C and a 90-s extension at 72°C, with a last step of an additional 7-min extension at 72°C.

Primers were initially screened to identify well-amplified, polymorphic bands among species. Individual *Digitalis* spp. DNA samples were appropriately diluted and bulked by species to screen 60 decamer primers (Series OPA, OPB and OPC from Operon Tech). Four primers from the initial screening process (OPA10, OPA13, OPB7 and OPC5) that exhibited a high polymorphism and showed the best readability were chosen for further study of the 42 individual genotypes.

Fragments generated by amplification were separated by size on a 1% agarose (SeaKem LE, FMC Bioproducts) gel run in TBE, stained with ethidium bromide, and visualised by illumination with UV light. To aid the interpretation of band identity between gels, each contained *Eco*RI- and *Hin*dIII-digested λDNA molecular-weight markers.

RAPD profiles were photographed in a Gel System Printer (TDI, Spain), and images were captured using a transmission Image Master DTS (Pharmacia-LKB) scanner. The molecular weight of the bands was determined using Diversity One software (version 1.0, PDI Inc). The reproducibility and repeatability of amplification profiles were tested for each primer. Control samples containing all the reaction material except DNA were used to test that no self-amplification or DNA-contamination occurred. Only those bands consistently reproduced in different analysis were considered. At least two replicates per sample were amplified and DNA from all the individuals was extracted twice.

Southern blotting of PCR products

To check that the same-size products were homologous among species, hybridisation on Southern transfer of some RAPD gels was first performed. Selected RAPD fragments were excised from the agarose gel, eluted with Nanosep MF microconcentrators (Pall Filtron) by centrifugation in a microfuge, and labelled with digoxigenin-11-dUTP (DIG DNA Labelling and Detection Kit, Boehringer Mannheim) following the manufacture's instructions. The amplified DNA products were separated on a 1% agarose gel, transferred by Southern-blotting onto positively charged nylon membranes (Boehringer Mannheim) and hybridised with the digoxigenin-labelled probes.

Subcloning and sequencing of PCR products

PCR products were eluted from gel slices as indicated above and ligated into the pGEM-T (Promega) vector for subsequent transformation in *Escherichia coli* strain DH5α. Transformed cell colonies were selected by blue/white colony screening, and appropriate plasmids were recovered by alkaline lysis, phenol-chloroform extraction, and polyethylene glycol precipitation (Sambrook et al. 1989). The DNA was sequenced with an automated ABI 373 Sequencer (Servicio de Secuenciación, SCSIE, Universidad de Valencia) using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). To minimise sequencing errors, at least two independent clones of each amplified RAPD fragment were sequenced.

Multiple alignments of nucleotide sequences were obtained using the CLUSTAL W version 1.6 program (Thompson et al. 1994). Nucleotide diversities among sequences (Pi ± SD) were calculated using the program DnaSP 3 (Rozas and Rozas 1999).

RAPD analyses

Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single biallelic locus (Williams et al. 1990). Amplified fragments, named by the primer used and the molecular weight in base pairs (bp), were scored in terms of the presence (1) or absence (0) of homologous bands, and a matrix of the different RAPD phenotypes was assembled. A pairwise distance matrix was computed based on Nei's coefficient of similarity (Nei and Li 1979), using the RAPDPLOT program of Black (1998). Dendrograms were then created with UPGMA (unweighted pair group method with arithmetic averaging) and NJ (neighbor-joining) cluster analysis as implemented in Neighbor from the Phylip 3.57c package (Felsenstein 1993). To give a measure of the variability in the data, the original matrix was bootstrapped 100-times via RAPDPLOT and a consensus tree was generated using the Neighbor and CONSENSE programs in Phylip. A Permutation Test Probability (PTP) analysis was also performed, using RAP-Distance (Armstrong et al. 1996) software, to test whether the resulting tree reflects an actual tree-like signal in the data or merely an artefact of the algorithm (Faith and Craston 1991).

The same presence/absence data matrix was used to compute the uniqueness of species relative to the amplified DNA fragments. Wagner parsimony (Eck and Dayhoff 1966; Kluge and

Farris 1969) analysis was performed to derive a cladogram using the MIX program in PHYLIP. Bootstrap analysis (1000 replicates) of the most-parsimonious tree was also conducted.

The distance matrix between RAPD patterns was also used to calculate the pairwise genetic distances between species applying Nei's (1978) unbiased distance [with or without Lynch and Milligan's (1994) correction when estimating allele frequencies] as implemented in RAPDDIST from RAPDPLOT, and UPGMA dendrograms were then generated.

Finally, two types of analysis were performed to corroborate the relationships among the seven *Digitalis* species. First, we used the method of Apostol et al. (1993) to evaluate whether the 42 individual genotypes actually cluster according to their sectional groups. The corresponding analysis was performed using MCALC and FINGERS programs from RAPDPLOT. The AMOVA procedure (analysis of molecular variance; Excoffier et al. 1992) was implemented as a second approach to study the relationships between the taxa. The resulting variance components were used as estimates of the genetic divergence among the species. Significance levels for variance-component estimates were computed by nonparametrical permutational procedures (5000 permutations). The distance between species was used to generate a dendrogram. The AMOVA was performed using Arlequin ver. 1.1 (Schneider et al. 1998).

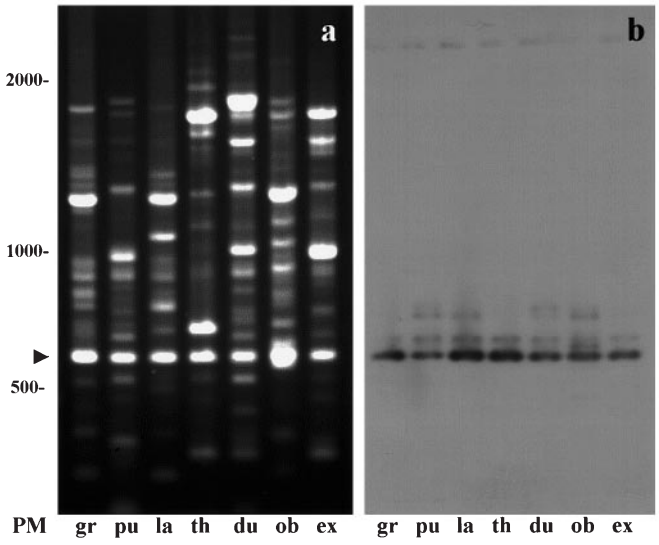

Fig. 1 **a** RAPD profiles in *D. grandiflora* (*gr*), *D. purpurea* (*pu*), *D. lanata* (*la*), *D. thapsi* (*th*), *D. dubia* (*du*), *D. obscura* (*ob*) and *D. excelsior* (*ex*) individuals using primer OPA10. The fragment in the second lane, marked with an arrow, and corresponding to *D. purpurea*, was used as a probe. **b** Southern blot of the RAPD profile analysis showing the hybridisation of this probe with all fragments of similar size

Results

Band homology

RAPDs can be of great potential to gain useful phylogenetic information among plant species. Nevertheless, before using this information, it must be confirmed whether bands which co-emigrate in different RAPD profiles represent homologous sequences, or whether they are simply fortuitous bands of similar size (Bachmann 1997). In our study, homology was corroborated through Southern-blot analysis of selected PCR gels and sequencing of some amplification products.

Bands used as digoxigenin-labelled probes were either monomorphic for individuals of the seven species (OPA10–610 isolated from *Digitalis purpurea*) or present only in some of the species (OPB7–500 and OPB7–1100 isolated from *D. purpurea* and *Digitalis dubia* respectively, detected in *D. purpurea*, *Digitalis excelsior*, *Digitalis thapsi* and *D. dubia*). Probe OPA10–610 hybridised with similar-size fragments in the seven species (Fig. 1). In contrast, probes OPB7–500 and OPB7–1100 recognised co-emigrating fragments in *D. purpurea*, *D. excelsior*, *D. thapsi* and *D. dubia*, but did not hybridise with fragments from *D. obscura*, *Digitalis lanata* and *Digitalis grandiflora* (data not shown).

To confirm that bands of the same size are homologous in their nucleotide sequences, OPA10–610 fragments isolated from *D. purpurea*, *D. excelsior*, *D. thapsi*, *D. dubia*, *D. lanata* and *D. grandiflora* were subcloned and sequenced. In all species, the length of sequenced fragment was 597 bp. The alignment of sequences was straightforward and no indels had to be postulated (sequences available upon request). The nucleotide composition of the six sequences showed a high A-T content (63.3% A-T vs 36.7% G-T). There were five polymorphic sites, four of which were parsimony informative. Three haplotypes (*D. purpurea* /*D. excelsior* / *D. thapsi*; *D. lanata* /*D. grandiflora*; and *D. dubia*) with identical sequences were detected. The sequence divergence (Pi ± SD) between species ranged from 0 to 0.0067 ± 0.0033, with an average of 0.00436 ± 0.00087 nucleotide substitutions per site.

DNA sequences of the fragments were examined for homology using the BLAST program (Altschul et al. 1997) in GeneBank (NCBI) and EMBL databases. All three *Digitalis* haplotypes had a high similarity with a 260-kDa hypothetical chloroplast protein described for *Epifagus virginiana* (Accession number P30072), *Nicotiana tabacum* (P09976), *Spinacia oleracea* (P08973) and *Pelargonium hortorum* (M83200) in the databases (data not shown).

Table 1 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	630–3000	27	0	27
OPA13	CAGCACCCAC	450–2700	19	0	19
OPB7	GGTGACGCAG	400–3700	21	1	22
OPC5	GATGACCGCC	650–2300	23	0	23

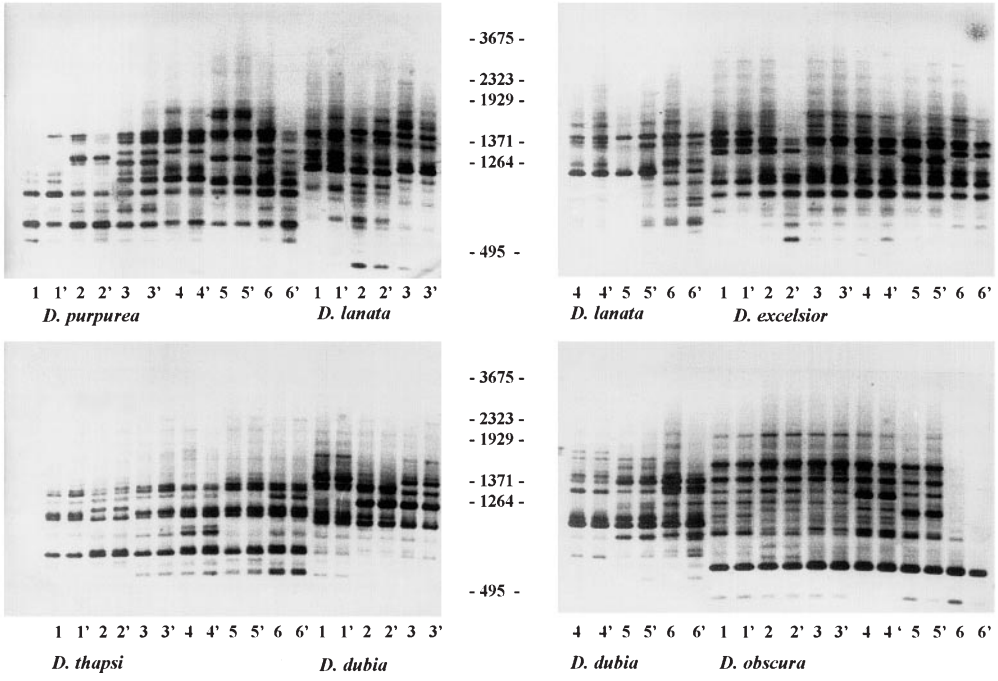

Fig. 2 Examples of RAPD profiles in six individuals from the *Digitalis* species, *D. purpurea*, *D. lanata*, *D, excelsior*, *D. thapsi*, *D. dubia* and *D. obscura*, using the primer OPA13. Each individual appears in duplicate in two lanes

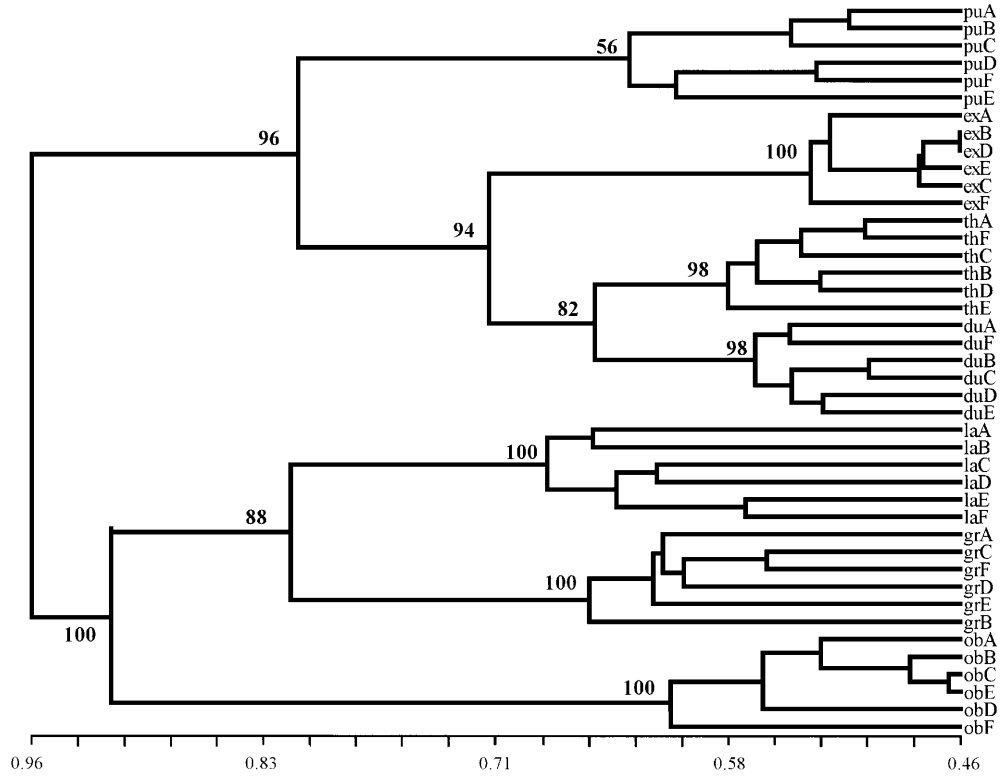

Fig. 3 UPGMA consensus tree (100 replications) from the Nei and Li (1979) distance matrix using the 42 individuals from *D. purpurea* (*pu*), *D. lanata* (*la*), *D, excelsior* (*ex*), *D. thapsi* (*th*), *D. dubia* (*du*), *D. grandiflora* (*gr*) and *D. obscura* (*ob*) species and the 91 RAPD markers. *Numbers* in the principal nodes indicate the number of trees with that node

RAPD fingerprinting

The four primers (OPA10, OPA13, OPB7 and OPC5) used to screen the 42 individual genotypes amplified a total of 91 well-amplified DNA markers, ranging in size from 400 to 3700 bp. The total number of amplified bands per primer varied from 19 (OPA10) to 27 (OPB7). Most of these bands (98.9%) were polymorphic among the species (Table 1). Individuals from the different sections gave rise to characteristic RAPD profiles, which were so obviously different as to allow identification at the section level by eye. Figure 2 illustrates an example of the profiles.

Some of the markers were species-specific: bands OPB7–550, OPB7–670, OPC5–480, and OPC5–630 (for

D. obscura); OPC5–520 (*D. purpurea*), OPC5–1460 (*D. lanata*); OPA13–1920 (*D. dubia*); OPA10–2050 (*D. thapsi*) and OPA10–1030 and OPC5–760 (*D. grandiflora*). Bands OPA10–1000, OPA10–2300 and OPC5–830 were shared for all the individuals of *D. thapsi*, *D. dubia*, *D. purpurea* (integrated in the section *Digitalis*) and the hybrid *D. excelsior*, but were not found in the remaining species.

Interspecific phylogeny

Although distance methods are the most commonly used for RAPD analysis (Clark and Lanigan 1993), RAPD data seems to be also suitable for parsimony (Hadrys et al. 1992; Bachmann 1997). In order to assess the reliability of the signal in our RAPD data, both methods were used to study relationships among *Digitalis* species. First, the 91 RAPD markers were analysed using Nei's coefficient of similarity (Nei and Li 1979). UPGMA or NJ generated from the distance matrix revealed similar relationships among the 42 individuals studied; therefore, only the UPGMA dendrogram is presented. The tree revealed a clear differentiation of two main groups. One group included individuals restricted to the section *Digitalis* (*D. purpurea*, *D. thapsi* and *D. dubia*) and the hybrid *D. excelsior*. The other main group included individuals from the section *Globiflorae* (*D. lanata*), *Grandiflorae* (*D. grandiflora*) and *Frutescentes* (*D. obscura*). In the dendrogram, the 42 individuals grouped into seven main clusters, as would be expected from their morphological characters: *D. purpurea*, *D. excelsior*, *D. thapsi*, *D. dubia*, *D. lanata*, *D. grandiflora* and *D. obscura*. The bootstrapped dendrogram (Fig. 3) confirmed the reliability of the differences between species and the grouping of individuals within their own species. Furthermore, the PTP test gave a Z-value of 57.6, suggesting that the probability of this tree to occur by chance is almost nil.

Wagner parsimony analysis of the raw presence/absence matrix confirmed the distinctness among the species. All individuals of any given *Digitalis* taxon clustered together and were distinct from all other taxa. The results showed seven different clades synonymous with the seven *Digitalis* taxa studied. Bootstrap analysis provides strong support for these main clades (Fig. 4).

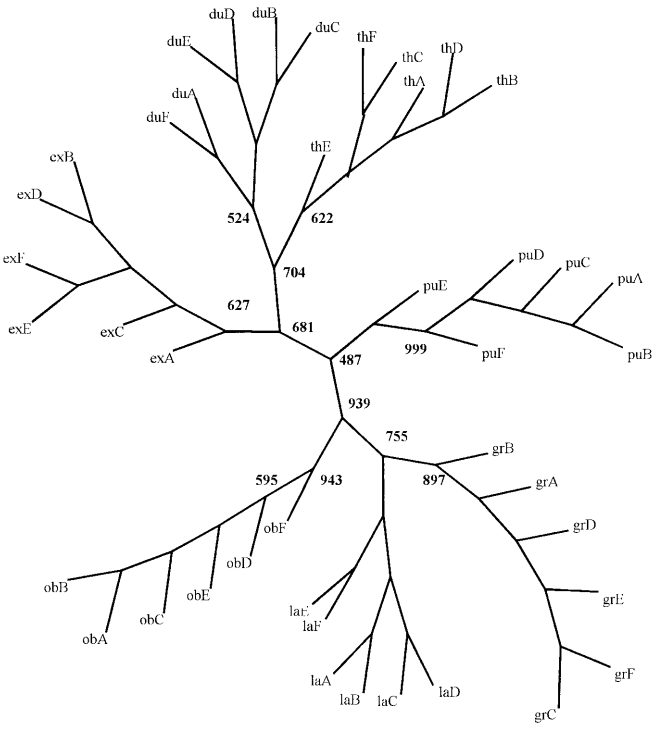

Fig. 4 Unrooted consensus tree (1000 replications) from the parsimony analysis using the 42 individuals from *D. purpurea* (*pu*), *D. lanata* (*la*), *D. excelsior* (*ex*), *D. thapsi* (*th*), *D. dubia* (*du*), *D. grandiflora* (*gr*) and *D. obscura* (*ob*) species and the 91 RAPD markers. *Numbers* in the principal nodes indicate the number of trees with that node

To further evaluate the relationships between species, grouped data analysis was performed using Nei's (1978) unbiased distance. The application of the Lynch and Milligan (1994) correction did not substantially vary the results obtained. The between-groups similarity matrices (Table 2) showed that the higher divergences among species appear when either *D. obscura*, *D. grandiflora* or *D. lanata* are compared with each of the other species. In contrast, the similarity coefficients among *D. purpurea*, *D. dubia*, *D. thapsi* and the hybrid *D. excelsior* were higher, especially between *D. thapsi* and *D. dubia*. These results suggest that the hybrid *D. excelsior* has stronger affinity to the section *Digitalis* than to the section *Grandiflorae*. The UPGMA dendrogram derived from

Table 2 Similarity coefficients obtained using Nei's (1978) unbiased distance with and without Lynch and Milligan's (1994) correction (below and above diagonal respectively) for the 42 individuals of *Digitalis* species: *D. purpurea* (pu), *D. lanata* (la), *D. excelsior* (ex), *D. thapsi* (th), *D. dubia* (du), *D. obscura* (ob) and *D. grandiflora* (gr)

Species	pu	la	ex	th	du	ob	gr
pu		0.4980	0.2977	0.3325	0.3041	0.4633	0.4918
la	0.4948		0.5510	0.4866	0.5449	0.3381	0.2410
ex	0.2975	0.5503		0.2715	0.2100	0.5642	0.4494
th	0.3349	0.4832	0.2740		0.1063	0.4792	0.4804
du	0.3046	0.5401	0.2122	0.1057		0.5705	0.5240
ob	0.4561	0.3364	0.5655	0.4742	0.5654		0.4979
gr	0.4940	0.2405	0.4533	0.4826	0.5258	0.4942	

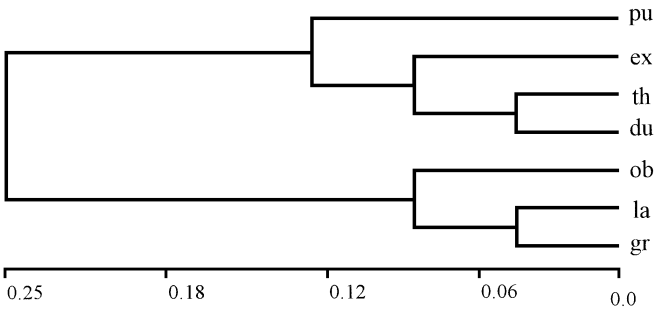

Fig. 5 UPGMA tree for the seven *Digitalis* species using pairwise comparisons with Nei's (1978) unbiased distance and applying Lynch and Milligan's (1994) correction for the 91 RAPD markers. *pu*: *D. purpurea*; *ex*: *D. excelsior*; *th*: *D. thapsi*; *du*: *D. dubia*; *ob*: *D. obscura*; *la*: *D. lanata*; *gr*: *D. grandiflora*

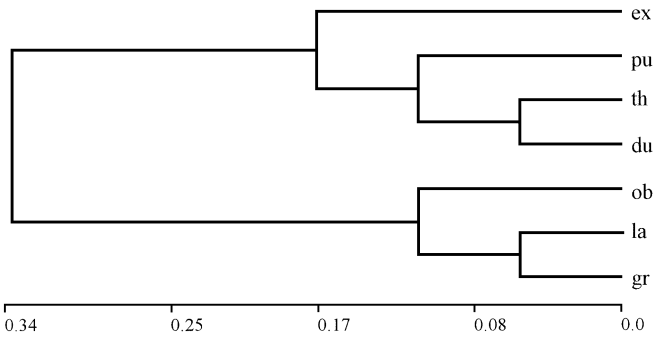

Fig. 6 UPGMA tree for the seven *Digitalis* species using pairwise comparisons derived from AMOVA. *ex*: *D. excelsior*; *pu*: *D. purpurea*; *th*: *D. thapsi*; *du*: *D. dubia*; *ob*: *D. obscura*; *la*: *D. lanata*; *gr*: *D. grandiflora*

the pairwise distance matrix with the Lynch and Milligan (1994) correction is shown in Fig. 5.

Family clustering

Apostol et al. (1993) described a procedure based on PCR-markers to accurately predict the number and size of full-sibling families in a collection of families. Under the hypothesis that individual genotypes belonging to the same taxonomic section might behave as a full-sibling family, we used this method to test whether the 36 individual genotypes of the six *Digitalis* species studied cluster according to the taxonomic sections of Werner (1964). This statistical approach would also give additional information on the affinities of the hybrid *D. excelsior*. The results obtained partially corroborated the hypothesis, since a total of five families were detected and the average family size was 8.4 individuals. Note that three species families (*D. obscura*, *D. lanata* and *D. grandiflora*) clustered according to their respective sectional groups (*Frutescentes*, *Globiflorae* and *Grandiflorae*) and do not contain members of other families, nor do members of these families fall in with other families. However, the 18 individuals belonging to the section *Digitalis* (*D. purpurea*, *D. thapsi* and *D. Dubia*) formed two different families with five (*D. purpurea*) and 13 (*D. purpurea*, *D. thapsi* and *D. dubia*) individuals respectively. The latter family also included the six *D. excelsior* hybrids. All these results are in agreement with those obtained in the interspecific phylogeny study.

AMOVA analysis

The AMOVA converts a phenotypic distance matrix into an equivalent analysis of variance (Excoffier et al. 1992), and has been previously used to optimise some aspects of the application of RAPDs for the assessment of genetic relationships among species (Guirao et al. 1995). The AMOVA method was first performed to determine the variance component accounted for in the among-species variations. Of the total phenotypic diversity (Table 3), about 65% was attributable to among-species differences ($P < 0.0001$).

Table 3 AMOVA analysis results for differentiation between seven species of *Digitalis*. Statistics include: degrees of freedom (df), sum of squares (SSD), variance-component estimates (CV) and percentages of Total variance (% Total) contributed by each component

Source of variation	*df*	SSD	CV	% Total
Among species	6	498.3	12.69***	64.8
Within species	35	241.0	6.89	35.2
Total	41	739.3	19.58	

*** P <0.001 (5000 replications)

To accurately know the net divergences among species, pairwise AMOVA analyses were also performed (Table 4). The dendrogram made with these data is shown in Fig. 6. The results obtained reproduce those mentioned above in relation to divergence at the species level (Table 2 and Fig. 5). *D. obscura*, *D. lanata* and *D. grandiflora* species were again the most distant species from the other four, whereas *D. purpurea*, *D. dubia*, *D. thapsi* and *D. excelsior* appeared to be the closest. A Mantel (1967) test showed a significant correlation ($r = 0.85$; $P = 0.0004$) when the pairwise AMOVA distance matrix (Table 4) and Nei's (1978) unbiased distance matrix with the Lynch and Milligan (1994) correction (Table 2) were compared with the NTSYS-pc (Rohlf 1993) package. The high level of conformity between both types of analysis demonstrates the reliability of the signal in the RAPD data. Note, however, that AMOVA results must be interpreted with caution due to the dominant nature of RAPDs, which can overestimate the divergence among species (Lynch and Milligan 1994). Nevertheless, the levels of significance were very high, suggesting that the results are robust.

Table 4 Genetic distances matrix derived from the pairwise AMOVA analyses between seven *Digitalis* species. The probability (P) of obtaining a more-extreme genetic distance value by chance than the observed distance is indicated above the diagonal. pu: *D. purpurea*; la: *D. lanata*; ex: *D. excelsior*; th: *D. thapsi*; du: *D. dubia*; ob: *D. obscura*; gr: *D. grandiflora*

Species	pu	la	ex	th	du	ob	gr
pu		0.0000	0.0022	0.0000	0.0020	0.0000	0.0000
la	0.6432		0.0000	0.0000	0.0000	0.0020	0.0020
ex	0.6144	0.7135		0.0026	0.0018	0.0000	0.0018
th	0.6070	0.6772	0.6337		0.0000	0.0000	0.0000
du	0.5862	0.6880	0.5648	0.3983		0.0024	0.0024
ob	0.6961	0.6077	0.7712	0.7401	0.7537		0.0010
gr	0.6068	0.4395	0.6390	0.6440	0.6462	0.6551	

Discussion

To-date, we have almost no knowledge of inter-specific variation in any of the *Digitalis* species that are based on non-morphological characters like molecular markers. The experiments described in this paper constitute the first application of RAPD markers for the study of genetic relationships among species of the genus *Digitalis*. Since bands with similar mobility may represent different DNA sequences, RAPD analysis might overestimate genetic relatedness when interspecific comparisons are considered (Thormann et al. 1994; Bachmann 1997; Castagna et al. 1997). Because of this, we first confirmed that bands which co-migrate in different RAPD profiles represent homologous sequences. This error, however, is more frequent when distantly related genotypes are compared (Castagna et al. 1997).

RAPDs sample polymorphisms across different fractions of the genome and can, therefore, be employed in a wide range of biosystematic studies (Bachmann 1997). Our RAPD survey of seven *Digitalis* species demonstrated that some of the identified RAPD markers were species-specific. Such markers may be important for strain identification and cultivar characterisation, and can be used to detect instances of natural interspecific gene introgression. Nevertheless, further analysis with more species and primers will be required to establish fully the specificity of loci to particular taxa and subsequent interspecific gene flow in *Digitalis*.

The high level of conformity among the different statistical approaches used for RAPD data analysis, demonstrates the suitability of these molecular markers to accurately resolve *Digitalis* species at the section level on the basis of their morphologies. Results herein seem to corroborate the taxonomic classification of Werner (1964), the one most accepted by botanic authorities (Hegnauer 1972; Tutin et al. 1972), which divides the genus *Digitalis* into the sections *Frutescentes* (integrates *D. obscura*), *Digitalis* (*D. thapsi*, *D. dubia, D. heywoodii*, *D. mariana* and *D. purpurea*), *Grandiflorae* (*D. ciliata, D. davisiana, D. atlantica* and *D. grandiflora*), *Tubiflorae* (*D. subalpina, D. lutea, D. viridiflora* and *D. parviflora*) and *Globiflorae* (*D. laevigata, D. nervosa, D. ferruginea, D. cariensis and D. lanata*). Thus, RAPDs can be of great usefulness to clarify the phylogenetic relationships among the different taxa of this economically important genus.

RAPD studies on interspecific hybridisation in natural populations of *Cyrtandra* (Smith et al. 1996) indicated that the putative hybrids are genetic intermediates between the two parental species. This did no hold true in our study, where the hybrid *D. excelsior* (*D. purpurea* × *D. grandiflora*) showed a high degree of relatedness to species of the section *Digitalis*, suggesting that the traits characteristic of *D. purpurea* are dominants. Inter-sectional hybridisation of several species of *Digitalis* also demonstrated that the inheritance of phytochemical traits (major types of cardiac glycosides) is section-dependent (Lichius 1991).

This study represents only the first step in using RAPD markers as a tool to implement studies of molecular systematics in *Digitalis*. The inclusion of other species and the use of an increased number of primers might provide a greater resolution of the affinities among these species. Also, direct sequencing of amplified bands may be important for recreating phylogenies.

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