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# Genetic diversity of drought-responsive genes in populations of the desert forage *Dactylis glomerata*

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**Abstract**

Restriction fragment length polymorphisms were used to estimate genetic diversity in six populations of *Dactlylis glomerata* collected in two different geographical locations in Israel. The objective of this study was to test the hypothesis that drought-responsive genes would have a higher genetic diversity in populations adapted to arid environments. Drought-responsive and non-responsive genes were isolated as cDNAs from leaves of *D. glomerata*. These cDNAs were used as probes on genomic Southern blots. Populations of *D. glomerata* collected in the southern arid region of Israel had greater genetic diversity for drought-responsive genes (0.388, repressed; 0.340, induced) than populations of plants collected in the northern Mediterranean region of Israel (0.308, repressed; 0.314, induced). The populations collected in the Mediterranean region had greater genetic diversity for the genes that were non-responsive to drought (0.185 versus 0.086). The gene flow between populations was relatively high, 2.47 for induced, 1.96 for repressed and 3.5 for non-responsive genes. These results suggest that plant conservation analyses should also consider stress responsive genes in estimates of genetic diversity, to select populations for conservation.


*Keywords:* Central and peripheral populations; Orchard grass; Desertification; RFLP

## 1. Introduction

*Dactylis glomerata* (orchard grass) is a perennial, cross-pollinated species native to Mediterranean areas of Europe. This species comprises a natural polyploidy complex distributed throughout Eurasia. The complex is composed of three ploidy levels that include 15 diploid ($2n = 2x = 14$), three widespread tetraploid ($2n = 4x = 28$), a few localized tetraploid and one hexaploid ($2n = 6x = 42$) subspecies. This complex has been introduced to most other temperate regions of the world because of its high agronomic value [15]. It can now be found widely distributed around the world in different habitats. Some forms of this species are cultivated, while other wild forms are used for livestock feeding in Mediterranean and semi-arid lands.

Drought or reduced water availability is the main factor limiting crop production [1]. Droughts are now more frequent and severe in large regions of the world because of desertification processes [2,3]. Global desertification constitutes a significant risk for the persistence of native populations of plants [4]. Some plant species have morphological, physiological and/or biochemical mechanisms that allow the organisms to grow and reproduce in drought-prone areas. Understanding the genetic bases for the persistence of certain populations in the face of environmental change or stress has agronomic as well ecological implications [5,6].

Genes involved in the responses of plants to short-term and season-long water deficit have been studied in great detail in many agronomic plants but in much less detail in native plants. Genes induced during drought stress are involved in the movement of water across membranes, turnover of proteins, detoxification, synthesis of osmolytes,

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signal transduction and regulation of DNA transcription [7]. Thus, there are genes induced by drought, which can be modeled to be involved in morphological, physiological and molecular adaptations to support plant growth during water deficit [5,8]. Drought-repressed genes also have important functions for the survival and for the eventual development of the plant during water stress [9].

Gene families of drought-responsive genes may differ in structure and complexity in native populations of a species established in different ecosystems. The central–marginal model in evolutionary ecology proposes that populations near the center of the range (central) of a species are dense and show high levels of phenotypic and genetic variation, while the populations on the edge of the range (peripheral) are isolated, bare and chromosomally monomorphic [10]. The center of the range of a species will coincide with the geographic region that is the most ecologically favorable for it in terms of its physiological responses to the physical environment and its interaction with other species. The periphery of a range will usually correspond to an area of extreme marginality for the species [11]. The differences between central and marginal populations will depend on the differences of the environments and the gene flow ($N_m$) among populations. A weak gene flow will allow greater differentiation [12].

Peripheral areas with marginal environments and exposed to stressful and unpredictable conditions favor high phenotypic variation [13]. Those marginal environments also favor recombination of genes involved in dealing with unpredictable conditions [11]. Therefore, peripheral populations are expected to have higher resistance and to show more variation in polygenic traits as compared to central populations [10]. Studies carried out in Israel have shown that peripheral populations of *Hordeum spontaneoum* had a higher diversity than central populations [14]. Gene diversity of *H. spontaneoum* was related to the rate of environmental change and average heterozygosity and gene diversity were positively correlated with rainfall variations. Greater heterozygosity of autopolyploids is proposed to give greater stability of phenotypic expression as compared with related diploids and hence a greater ability to survive in marginal environments [15]. Peripheral populations may constitute a source of genetic variability for breeding cultivated plants for tolerance to abiotic stresses and also may be the subject for core collections in germplasm preservation programs [16,17].

In this study, cloned forms of drought-induced and drought-repressed genes were used to perform restriction fragment length polymorphism (RFLP) analysis on *D. glomerata* populations. Populations of this species were collected in Israel at locations in the northern and southern areas of the country; these two regions have very different climates and may reflect central and peripheral populations sites, respectively. DNA isolated from individuals in these populations were then characterized to test the hypothesis that genetic diversity for drought-responsive genes would be greater in peripheral populations than in central populations.

## 2. Methods

### 2.1. Plant material D. glomerata (diploid subspecies) seeds

Were collected from different locations of northern (Shimron, Givat–Hamore and Carmel) and southern (Pura, Lalow, Sausana) Israel. The east–west axis in the north ran from Atlit on the coast to Gilboa ridge in the south from Beeri to Har-Amasa. The northern region has a Mediterranean climate with annual 450–600 mm of winter rain. The southern region is located on the outer edge of the extreme Judean Desert with ∼300 mm of rain. Plants were grown from seed in a greenhouse on the NMSU campus. All plants were cultured in 0.5-kg pots, in Metro-Mix 360 medium and Osmocote 14–14–14 fertilizer. The plants were watered daily until drought treatments. Drought treatments were cyclic; 6–8 weeks after planting, water was suspended until symptoms of water stress were observed; then, the plants were watered daily for a few weeks and then, the drought treatment was repeated. Stress symptoms were monitored daily with visual observations, plants were considered stressed, when leaves were wilted in the early morning.

### 2.2. RNA isolation and blot analysis

Plants from the Lalow population, representative of the nominally drought resistant, southern populations, were used as the sources of RNA for expressions studies and cDNA library construction. Leaf samples were immediately placed into liquid nitrogen prior to RNA isolation. Samples were collected from either control plants, plants that had never experienced a water deficit stress or from plants after their second drought-stress cycle. Equivalent developmental stages of leaves were collected from control or drought-stressed plants, fully expanded mature leaves. Plants were approximately 3 months old at time of leaf harvest. RNA was isolated using chloroform–phenol extraction [18]. Northern blots of total RNA (5 μg) from stressed and control plants were prepared and hybridized as described earlier [19].

### 2.3. Differential display reverse transcription

The Delta™ Differential Display Kit and Protocol PT 1173-1 from Clontech Laboratories Inc. were used to perform the analysis of differentially expressed genes. Confirmation of differential expression was determined by Northern blot analysis and those DNA fragments were cloned in pGEM-T vectors.

### 2.4. cDNA library construction and characterization of clones

The cDNA libraries of transcripts in leaves from drought-stressed plants (*D. glomerata*, Lalow population) were made in UNI-Zap XR (Stratagene). Nylon membrane replica lifts

from plates of the amplified cDNA libraries were hybridized with $^{32}$P-labeled cDNA from stressed or control plants. Differential plaques were identified, picked up from the plates and placed into SM buffer. A sample of the eluted phage was used to perform PCR amplification of the inserted DNA. Northern blots were used to confirm drought-responsiveness. Inserts from the phage were amplified by PCR using M13 reverse and forward primers and subsequently sequenced. Prediction of function of cDNAs were based on alignments generated using the Blastx Algorithm Version 2.0.12 [20] served at the NCBI web site [www.ncbi.nih.gov/BLAST/].

### 2.5. DNA isolation and Southern analysis

DNA was isolated from leaves using the Puregene kit (Puregene). Samples of leaf tissue were taken from at least 20 plants from each of three populations ($n = 120$). Southern blots were prepared following complete digestion of DNA (10 μg) with *Eco*R1 or *Hin*dIII. A total of 20 DNA samples from each population were set in a single gel. Four replica gels were made for each population. Hybridization patterns were visually scored on the X-ray films after probing with the labeled drought-responsive genes.

### 2.6. Statistical analysis

Polymorphisms for each of the probes were identified on the autoradiograms and scored as binary data. The matrices generated were analyzed with Popgene Version 1.31 software [21] to evaluate gene diversity, genetic distance and genetic differentiation [22–24].

Gene diversity was estimated as the expected heterozygosity in the population assuming Hardy–Weinberg equilibrium. The expected heterozygosity was estimated using the matrices of RFLPs. Each fragment or band was considered as a locus. Therefore, each locus had allele 1, when the band was present or allele 0, when the band was absent. The frequencies ($x_i$) of the alleles for each locus were used for the estimation of the expected heterozygosities within populations, among subpopulations and total populations ($h = 1 - \sum x_i^2$). The variances of gene diversity for each probe and differences of gene diversity variance between northern and southern populations were estimated. Differences of variances of pooled drought-induced, drought-repressed or drought-neutral genes were used to perform *t*-tests. These tests allowed global comparisons of gene diversity of the groups of drought-induced, drought-repressed and drought-neutral genes [24].

Analysis of molecular variance (AMOVA) was made taking into account the frequencies of haplotypes generated by each probe. Analysis of molecular variance and estimation of $F$-statistics or fixation indexes were estimated using the software Arlequin Version 2.000. This software estimates the significance of the fixation indexes using a non-parametric permutation approach, consisting in permuting haplotypes, individuals or populations, among individuals, populations or groups of populations. After each permutation round, the statistics are recomputed to get their null distribution. By doing this process, the normality assumption usually required in analysis of variance tests is no longer necessary, nor is it necessary to assume equal variances among populations or groups of populations [25].

## 3. Results

### 3.1. Isolation and characterization of drought-responsive genes

Two methods were used to identify and isolate molecular markers for drought-responsive genes, differential screening of a cDNA library and DDRT–PCR. A library of transcripts from leaves of drought-stressed *D. glomerata* was constructed in phage vectors, $4.3 \times 10^6$ pfus, with insets in the range of 1–1.5 kb. A differential screening of this cDNA library identified 88 drought-induced plaques, 45 drought-repressed plaques and 40 drought-neutral plaques. A total of 91 transcripts were PCR-amplified and sequenced. The predicted proteins of 31 of these genes had homology with plant proteins annotated as drought-induced or drought-repressed. Another 11 *Dactylis* clones had homology with GenBank entries identified as unknown or hypothetical proteins. A total of 26 pairs of primers were tested for DDRT–PCR and 46 differentially expressed bands were found; of these, 5 were sequenced, and functions were assigned based on BLAST analysis. Of the five fragments, one represented a unique gene, 2s10, which encoded a portion of the gene for leucine aminopeptidase, and 5s1, which encoded a portion of the gene for lysine ketoglutarate. Transcript abundance changes as a result of drought stress were confirmed by Northern blot analysis. Representative blots are presented in Fig. 1. From these data sets, cDNAs were selected for use as probes in RFLP analyses; six cDNAs representing drought-induced genes, four cDNAs representing drought-repressed genes and three cDNAs representing drought-neutral genes (Table 1).

### 3.2. Southern analysis

Six drought-induced and four drought-repressed cDNA clones were used as probes to estimate the drought-responsive gene diversity in three groups of individuals from the northern population and three groups of individuals from the southern populations of *D. glomerata*. In addition, three drought-neutral cDNAs were used to compare gene diversity of drought-responsive and drought-neutral genes. Conditions to achieve complete digest of the genomic DNA were experimentally determined to be 2.4 U of *Eco*R1 or 2.0 U of *Hin*dIII for each microgram of DNA. Under the hybridization wash conditions used in this analysis (low salt and 65 °C), targets with 84% sequence match to the probe would

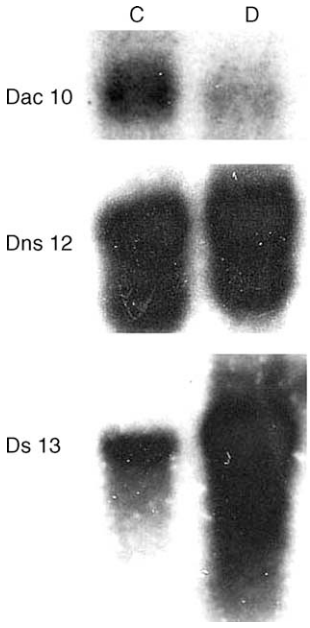


Fig. 1. Characterization of drought-responsive clones. Total RNA (5 μg) from leaves of control (C) or drought-stressed (D) *Dactylis glomerata* plants were electrophoresed, blotted and probed with the indicated cloned genes.

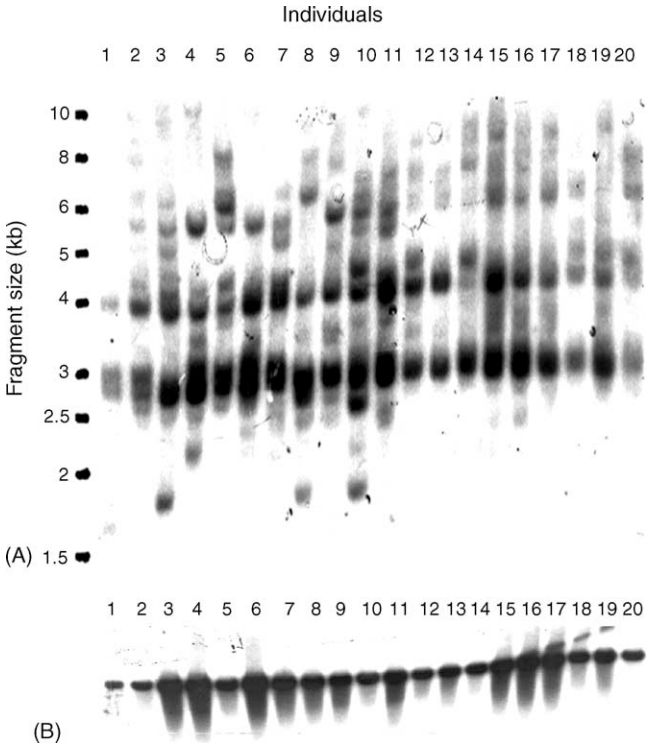


Fig. 2. RFLPs detected among 20 *D. glomerata* individuals from Lalow population. DNA (10 μg) from each individual was restricted with 20 U of *Hin*dIII (A) or 24 U of *Eco*R1 (B), electrophoresed in 0.8% gel at 4 V/cm, blotted onto a nylon membrane and hybridized with either the drought-induced cDNA, 2s10 (A) or drought-neutral cDNA, Dns8 (B).

be detected. These conditions should allow detection of all gene family members for each gene probe.

Drought-induced genes showed great complexity as measured by the number of different fragments in the hybridized Southern blots of *D. glomerata*. The number of polymorphic bands or loci detected by Southern blots ranged from six to nine (Table 1). Moreover, *D. glomerata* populations showed a great diversity of haplotypes, when hybridized with drought-induced cDNA. Drought-repressed genes had similar results, as drought-induced genes regarding the number of fragments detected for each gene. Drought-repressed genes produced a range of 4–10 fragments in *D. glomerata* hybridized genomic DNA Southern blots (Table 1). From the results of the RFLP analysis and given the large number of bands on southerns, it is likely that the drought-induced and drought-repressed genes used as probes against the Southern blots belong to gene families.

Fig. 2A shows a representative autoradiograph of a Southern blot containing genomic DNA probed with a drought-induced gene; DNA from 20 individuals collected at Lalow (southern site) hybridized with a probe for *Lap*. Most of the bands detected in this autoradiograph were polymorphic and different haplotypes could be identified.

Table 1
Clones used as probes for RFLP analysis of *Dactylis glomerata* populations and number of bands detected by hybridization to Southern blots

| [mRNA] | Clone | GenBank # | Functional annotation | # of bands[a] |
|---|---|---|---|---|
| Increase | 2s10 | BG724444 | Leucine aminopeptidase | 9 (H) |
| | Ds29 | CO535097 | Dehydrin | 9 (H) |
| | Ds13 | BG724451 | Group 3 LEA | 8 (E) |
| | Ds30 | BG724459 | Initiation factor 5A | 7 (E) |
| | 5s1 | BG724449 | Lysine ketoglutarate reductase | 6 (E) |
| | Dns1 | BG724452 | Superoxide dismutase | 6 (H) |
| Decrease | Dns9 | CO535093 | Unknown-a | 10 (E) |
| | Ds14 | CO535096 | Nuclease | 7 (H) |
| | Dns2 | BG724453 | Amino oxidase | 4 (H) |
| | Dac10 | CO535094 | LRR-disease resistance protein | 4 (H) |
| Neutral | Ds5 | CO535095 | Unknown-b | 3 (E) |
| | Dns8 | CO535092 | Cysteine protease | 2 (E) |
| | Dns12 | BG724457 | Unknown-c | 2 (E) |

[a] Genomic Southern blots containing digests by either *Eco*R1 (E) or *Hin*dIII (H).

Both drought-induced and drought-repressed cDNA clones produced complex banding patterns, when used as probes on Southern blots. Complex banding patterns were observed in groups from both northern and southern populations. In contrast, the banding pattern observed in Southern blots hybridized with drought-neutral cDNA clones was much less complex (Fig. 2B); drought-neutral genes detected only a few fragments regardless of the site of collection of the individual. Moreover, a high percentage of the loci of drought-neutral genes were monomorphic.

### 3.3. Gene diversity

Gene diversity was estimated for every probe in every population of *D. glomerata*. Six drought-induced, four drought-repressed and three drought-neutral cDNA clones were used to measure gene diversity in three groups from the northern and three groups from the southern populations of *D. glomerata*. Only a single restriction enzyme and probe combination was used for each gene. For example, the Southern blots generated with *Hin*dIII digests probed with clone 2S10 were used for all of the individuals to define the genetic diversity for leucine aminopeptidase. Differences in average gene diversity between northern and southern populations were used to estimate the variance of the differences and test them with $t_{L-1} = d/s(d)$, where $d$ is the average difference of gene diversity between northern and southern populations and $s(d)$ is the square root of the variance of the differences [24].

Gene diversity for all drought-induced genes had a tendency to be larger in groups in the southern population as compared to groups in the northern population (Table 2). The greater gene diversity in four out of six drought-induced genes was found in groups in the southern populations. The larger gene diversity for superoxide dismutase ($H = 0.39$), late embryogenesis abundant ($H = 0.36$), and dehydrin ($H = 0.43$) was found in Sausana, a group from the southern population. Also, the greater gene diversity for the initiation factor 5A ($H = 0.39$), was found in Lalow, a group from the southern population. On the other hand, the higher values of gene diversity for leucine aminopeptidase and lysine ketoglutarate reductase were found in the groups from the northern population of Shimron and Givat–Hamore, respectively. There was variation for gene diversity among the groups within each population.

Four drought-repressed cDNA clones were used to estimate gene diversity for drought-repressed genes in *D. glomerata*. The highest values of gene diversity of drought-repressed genes again were present in groups from the southern population (Table 2). The Pura population had the greater gene diversity for amine oxidase ($H = 0.401$), unknown protein a ($H = 0.351$) and LRR-disease resistance protein ($H = 0.459$), while the greater gene diversity for nuclease ($H = 0.349$) was found in the Sausana group.

The three drought-neutral genes used to estimate gene diversity in *D. glomerata* showed lower complexity than drought-induced or drought-repressed genes, as a consequence, gene diversity was low (Table 2). In some cases, gene diversity was zero, e.g. cysteine protease in the Lalow and Sausana groups in the southern population. The Southern blots of these populations probed with cysteine protease were monomorphic. The highest value of gene diversity among the drought-neutral genes was for Dns12 in the Carmel group (northern population) with $H = 0.327$. Average genetic diversity for the drought-neutral genes was usually greater in the northern population than in the southern population.

A pooled analysis on the drought-induced genes, using *t*-test as proposed by Nei and Kumar [24], showed significant differences ($\alpha = 0.01$) for the group of genes (Fig. 3). In this

Table 2
Genetic diversity ($H \pm$ S.E., $n = 20$) for drought-induced, drought-neutral and drought-repressed genes in *Dactylis glomerata* populations

| Genes | Northern populations | | | Southern populations | | |
|---|---|---|---|---|---|---|
| | Givat–Harmore | Shimron | Carmel | Lalow | Pura | Sausana |
| Induced | | | | | | |
| Ds30 | 0.323 ± 0.062 | 0.351 ± 0.041 | 0.287 ± 0.085 | **0.393 ± 0.043** | 0.322 ± 0.057 | 0.335 ± 0.127 |
| Ds13 | 0.257 ± 0.041 | 0.241 ± 0.039 | 0.289 ± 0.051 | N.A. | 0.354 ± 0.039 | **0.367 ± 0.033** |
| Ds29 | 0.371 ± 0.039 | 0.395 ± 0.043 | 0.343 ± 0.067 | 0.400 ± 0.033 | 0.377 ± 0.039 | **0.437 ± 0.034** |
| Dns1 | 0.280 ± 0.067 | 0.283 ± 0.068 | 0.152 ± 0.068 | 0.290 ± 0.076 | 0.292 ± 0.076 | **0.399 ± 0.051** |
| 2s10 | 0.218 ± 0.046 | **0.340 ± 0.033** | 0.245 ± 0.037 | 0.276 ± 0.041 | 0.286 ± 0.035 | 0.317 ± 0.039 |
| 5s1 | **0.375 ± 0.064** | 0.085 ± 0.050 | 0.188 ± 0.058 | 0.275 ± 0.048 | 0.215 ± 0.085 | 0.161 ± 0.036 |
| Repressed | | | | | | |
| Dns9 | 0.254 ± 0.056 | 0.295 ± 0.051 | 0.278 ± 0.061 | 0.316 ± 0.045 | **0.351 ± 0.028** | 0.343 ± 0.033 |
| Dns2 | 0.109 ± 0.064 | 0.319 ± 0.081 | 0.182 ± 0.098 | 0.165 ± 0.113 | **0.401 ± 0.054** | 0.307 ± 0.104 |
| Dac10 | 0.333 ± 0.089 | 0.419 ± 0.035 | 0.324 ± 0.115 | 0.274 ± 0.097 | **0.459 ± 0.014** | 0.252 ± 0.102 |
| Ds14 | 0.293 ± 0.070 | 0.298 ± 0.051 | 0.281 ± 0.082 | 0.249 ± 0.080 | 0.313 ± 0.48 | **0.350 ± 0.066** |
| Neutral | | | | | | |
| Ds5 | 0.144 ± 0.098 | N.A. | **0.327 ± 0.160** | 0.242 ± 0.081 | 0.177 ± 0.046 | 0.272 ± 0.140 |
| Dns8 | 0.191 ± 0.086 | **0.298 ± 0.186** | 0.137 ± 0.042 | 0 | 0.270 ± 0.137 | 0 |
| Dns12 | 0.047 ± 0.047 | **0.299 ± 0.033** | 0 | 0.095 ± 0.00 | 0.201 ± 0.059 | 0 |

For each gene, the population with the highest genetic diversity is indicated with bold font.

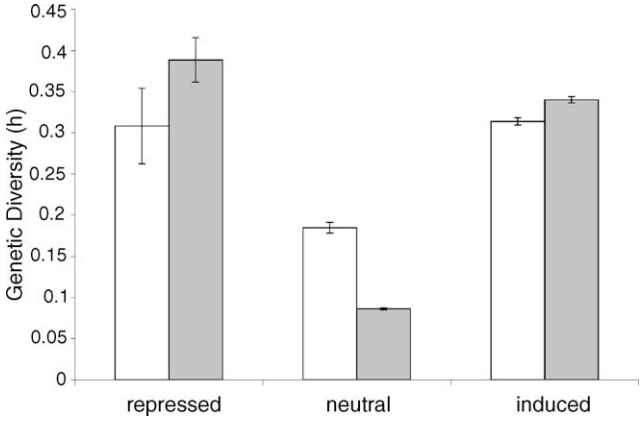


Fig. 3. Average genetic diversity of pooled drought-induced, drought-repressed and drought-neutral genes. The open bars represent northern populations, and the filled bars represent southern populations.

analysis, all the loci of all six drought-induced genes were pooled. Gene diversity for drought-induced genes was larger in southern populations than in northern populations of *D. glomerata*. While there were no significant differences in gene diversity for specific genes, the pooled analysis (*t*-test) performed on the set of four drought-repressed genes showed significant differences ($\alpha = 0.01$) between northern and southern populations. Southern populations had greater gene diversity for the group of drought-repressed genes than northern populations. In contrast, northern populations had higher gene diversity for drought-neutral genes (Fig. 3).

Genetic diversity for drought-neutral genes contrasted with the results obtained for drought-responsive genes. First, drought-neutral genes showed less complex banding pattern as compared to that of drought-responsive genes. Second, the level of gene diversity was lower for drought-neutral genes than for drought-responsive-genes. And finally, drought-neutral gene diversity tended to be greater in the northern population, while drought-responsive gene diversity was greater in the southern population.

### 3.4. Gene flow measurements

Gene flow, defined as the number of migrants exchanged between local populations per generation was estimated using Nei and Kumar's [26] coefficient of differentiation (Gst), as $N_m = 0.5(1–Gst)/Gst$. Values for Gst were obtained from the PopGene treatment of the RFLP data.

Across all genes, gene flow values ranged from a low of 0.9 for the drought-repressed gene, amine oxidase to a high of 5.7 for the drought-neutral gene, Dns12 (Table 3). On an average, the gene flow for the six drought-induced genes was $N_m = 2.47$; for the drought-repressed genes $N_m = 1.96$, and for the drought-neutral genes, $N_m = 3.5$.

The fixation index $F_{ST}$ is a suitable measure of genetic differences among subpopulations. Using these indices, there was a great differentiation among populations of *D. glomerata* for drought-induced and drought-repressed genes, with average $F_{ST}$ of 0.19 and 0.20, respectively. Moreover, this index was significant, as measured by non-parametric permutation approach in the AMOVA, for all drought-responsive genes.

Table 3
Gene flow ($N_m$) and fixation index ($F_{ST}$) estimated for drought-responsive genes in three northern and three southern populations of *D. glomerata*

| Gene (clone ID) | $N_m$ | $F_{ST}$ | *P* |
|---|---|---|---|
| Induced | | | |
| Dehydrin (Ds29) | 4.58 | 0.086 | 0.00001 |
| Group 3 LEA (Ds13) | 3.29 | 0.229 | 0.00001 |
| Leucine aminopeptidase (2s10) | 2.46 | 0.134 | 0.00001 |
| Superoxide dismutase (Dns1) | 2.19 | 0.149 | 0.00001 |
| Initiation factor 5A (Ds30) | 1.30 | 0.231 | 0.00001 |
| Lysine ketoglutarate reductase (5s1) | 1.02 | 0.337 | 0.00001 |
| Average | 2.47 | 0.194 | |
| Repressed | | | |
| LRR-disease resistance protein (Dac10) | 2.42 | 0.147 | 0.00001 |
| Unknown-a (Dns9) | 2.36 | 0.189 | 0.00001 |
| Nuclease (Ds 14) | 2.12 | 0.161 | 0.00001 |
| Amino oxidase (Dns2) | 0.95 | 0.321 | 0.00001 |
| LRR-disease resistance protein (Dac10) | 2.42 | 0.147 | 0.00001 |
| Average | 1.96 | 0.204 | |
| Neutral | | | |
| Unknown-c (Dns12) | 5.7 | 0.037 | 0.047 |
| Cysteine protease (Dns8) | 3.2 | 0.121 | 0.006 |
| Unknown-b (Ds5) | 1.3 | 0.302 | 0.00001 |
| Average | 3.5 | 0.153 | |

*P* indicates the degree of significance of $F_{ST}$ differences among subpopulations.

The $F$-statistics estimated by AMOVA demonstrated a moderate but significant differentiation among populations for the six drought-induced genes tested. The greatest differentiation was for lysine ketoglutarate reductase with $F_{ST} = 0.337$. The lowest differentiation was for dehydrin with $F_{ST} = 0.086$ (Table 3). The average fixation index $F_{ST}$ for all three drought-neutral genes was 0.153.

The analysis of molecular variance of the RFLP for drought-responsive genes showed that the main component of variance is the variance within groups, followed by the variance among groups within populations, and finally, the lowest value of variance was found among populations (Table 4). The variation within groups accounted on average for ~80% of the total variation of drought-induced genes. The variation among groups contributed approximately 20% of the total genetic diversity. The fixation indexes and the variances within groups and among groups were significant ($P < 0.00001$) after a test of 1023 permutations. The variances among populations for all the drought-induced genes were not significant. Therefore, the analysis of molecular variance did not identify significant differences between northern and southern populations for particular drought-induced genes.

The differences in diversity for drought-repressed genes are due mainly to variation within populations. On average for the four drought-repressed genes, the variance within

Table 4
Components of variance (AMOVA) of drought-responsive genes as measured by RFLPs on DNA of individuals from northern and southern populations of D. glomerata (% of total variance)

| | Source of variation | | |
|---|---|---|---|
| | Among groups | Among populations within groups | Within populations |
| Induced | | | |
| Dehydrin (Ds29) | 0.089 (4.44) | 0.083 (4.1) | 1.834 (91.4) |
| Group 3 LEA (Ds13) | 0.039 (1.72) | 0.479 (21.1) | 1.749 (77.1) |
| Leucine aminopeptidase (2s10) | −0.078 (−5.1) | 0.282 (18.5) | 1.321 (86.6) |
| Superoxide dismutase (Dns1) | −0.076 (−7.24) | 0.233 (22.2) | 0.893 (85) |
| Initiation factor 5A (Ds30) | −0.106 (−6.6) | 0.481 (29.7) | 1.245 (76.9) |
| Lysine ketoglutarate reductase (5s1) | −0.003 (−0.3) | 0.349 (34.1) | 0.678 (66.2) |
| Repressed | | | |
| Unknown-a (Dns9) | 0.179 (9.04) | 0.195 (9.9) | 1.606 (81.1) |
| Nuclease (Ds14) | −0.058 (−4.5) | 0.269 (20.6) | 1.094 (83.9) |
| LRR-disease resistance protein (Dac10) | −0.022 (−2.6) | 0.147 (17.3) | 0.724 (85.3) |
| Amino oxidase (Dns2) | −0.041 (−5.5) | 0.279 (37.6) | 0.504 (67.9) |
| Neutral | | | |
| Unknown-c (Dns12) | −0.004 (−3.8) | 0.008 (7.5) | 0.105 (96.3) |
| Cysteine protease (Dns8) | 0.024 (1.4) | 0.018 (10.7) | 0.146 (87.9) |
| Unknown-b (Ds5) | 0.022 (4.1) | 0.138 (26.2) | 0.368 (69.7) |

Values in parentheses are in percent.

populations accounted for 79.5% of the total variation. This variation was highly significant. Variation among populations was smaller than within populations, but it was significant. The biggest variation among populations was for the gene that codes for amine oxidase with ∼37%. The gene coding for a hypothetical protein had the lowest variation among populations, ∼10% to the total variance. The variation among groups was very low and not significant for any of the drought-repressed genes. There were no significant differences between northern and southern populations for gene diversity for particular drought-repressed genes (Table 4).

The molecular analysis of variance showed that approximately 85% of the genetic variation was due to variation within groups for the three drought-neutral genes (Table 4). The higher value of variability within populations was for the clone Dns12 (∼96% of the total variance) and the lower value (∼70% of the total variance) was for the clone Ds5. The variance among populations explained on average ∼15% of the genetic variation of the three drought-neutral genes. The variance among groups (northern and southern) was very low and not significant (Table 4).

## 4. Discussion

Broadly distributed species tend to have greater variation than narrowly distributed species. Moreover, cross-pollinated species have a tendency to maintain more genetic diversity overall and also tend to maintain more variation within their populations than self-pollinated species [27]. The levels of diversity of drought-induced genes ($H = 0.31$–$0.43$) and drought-repressed genes ($H = 0.28$–$0.41$) present in *D. glomerata* populations are higher than those estimated in other outcrossing and widely distributed species. Species that are predominantly cross-pollinated have on average gene diversity of $H = 0.167$. Species with extensive geographic distributions have on average gene diversity in populations of $H = 0.202$ [27]. However, some species have shown higher levels of gene diversity than those estimated for drought-induced and drought-repressed genes in *D. glomerata*. For example, in *Pinus sylvestris* values as high as 0.977 for gene diversity have been described [28].

In marginal environments, free recombination may produce resistant genotypes that will be selected for such adverse environments. Therefore, peripheral populations are expected to have higher resistance and to show more variation in polygenic traits as compared to central populations [10]. Studies in bacteria have shown that genetic diversity within populations reaches its highest levels, when adaptive evolution has slowed significantly [29]. However, the adverse environment is not necessary to restructure the genome or to maintain a high level of genetic diversity.

Although the differences in diversity between northern and southern populations of *D. glomerata* for particular drought-responsive genes were not significant, the pooled analysis showed that drought-responsive genes have a greater diversity in southern populations than in northern populations. This could be due to a positive selection for alleles for fitness to the marginal environments and the high level of outcrossing that causes a high level of heterozygosity.

A possible cause for the low differentiation among groups for particular genes is a high rate of gene flow between northern and southern populations. The differences between central and marginal populations will depend on the differences in the environments and the gene flow among

populations. A weak gene flow will allow greater differentiation [12,30]. The levels of gene flow detected in these *D. glomerata* populations for drought-responsive genes were high with averages of $N_m = 2.47$ and 1.96 for drought-induced and drought-repressed genes, respectively. Studies in *Centaurea solstitialis* showed a relatively high gene diversity ($H = 0.172$) and low genetic differentiation among populations. These conditions were attributed to the outbreeding system of the species and high gene flow ($N_m = 2.38$), which was associated to seed dispersal [31].

The AMOVA showed that there are no significant differences among groups (northern and southern) for specific drought-responsive genes. However, it was possible to detect significant differences between northern and southern populations by using *t*-test on the pooled drought-induced or drought-repressed data. It is possible that detection of differences is due to the use of a greater number of loci in this pooled analysis. A large number of loci should be examined to estimate genetic variation of a population [26].

The amount of genetic variability of a population is function of the genetic diversity originally available to the species and of the later influence of processes such as selection, gene flow and the mating system [32]. It is expected that variation for neutral genes be equal in northern and southern populations, since the main factor defining the variation of these genes would be the phylogenetic legacy and mutation. Genes that have a neutral influence on the organism would change their presence just by chance. Therefore, variation for specific neutral genes would change the same in northern and southern populations.

A changing environment will keep populations in an infinite state of flux, as different biotypes are favored in turn by changing climatic conditions. These conditions would cause mutations with an adaptive value that have a greater probability to remain in the population by the action of natural selection [33]. According to the minute gene effect hypothesis, although most molecular mutations behave just like neutral genes in populations, they are not strictly neutral and the cumulative effects of a number of them are responsible for the adaptive change of the individual [26]. The bigger diversity of drought-neutral genes in northern populations as compared to that in southern populations of *D. glomerata* may be due to a greater diversity of these genes in the original populations. As the species started to colonize more extreme environments some of these genes may decrease their diversity just by chance or genetic drift. Moreover, central populations can afford the production of individuals with neutral genes that may not have an adaptive value [10]. Favorable environment would allow that neutral genes keep their presence in the population. In contrast, unfavorable environments would allow those genes with an adaptive value to be increased as a consequence of natural selection and neutral genes may remain unchanged. In this study, populations from unfavorable environments (southern) showed bigger diversity for a group of drought-responsive genes as compared to the diversity of this group of genes in populations from favorable environments (northern). However, diversity of the group of drought-neutral genes tended to be greater in northern populations. The biological source of the DNA used to probe for these genes was a southern population. By using moderate stringency conditions in the washes of the Southern blots, we monitored all genes with sequence identity of 84% or higher, eliminating the bias created by using only one population as the source of the probes.

A comparison of plasticity between related diploid and tetraploid *D. glomerata* collected from a sympatric zone in northern Spain did not show significant differences. The results did not support the hypothesis that in general polyploids show greater plasticity than related diploids. Heterozygous individuals may be more able to adjust to variable environments because of their greater biochemical flexibility. Therefore, if multi-locus heterozygosity determined at marker loci reflects genomic heterozygosity, chromosome doubling should have some measurable effects on plasticity. Studies of plasticity associated with polyploidy using genotypes of known levels of heterozygosity could be helpful to understand the genetic basis of plasticity [15]. Another work with populations from two separate altitudinal transects, one in France and the other in Italy, showed that DNA *C*-values of the individuals from the French and Italian transects were negatively correlated with altitude. Phylograms elaborated with AFLP data grouped into different cluster to the individuals from low altitudes and high altitudes for both transects, French and Italian. This was consistent with the variation of DNA *C*-values. They conclude that both DNA *C*-value measures and molecular markers can be used to identify different genotypes and that the polymorphism may be related to tolerance to environmental factors at the genotypic level [34].

Which populations to conserve is an important decision, and the use of genetic analyses to assess diversity is an important component of those decisions [35]. Our results with populations of *D. glomerata* collected in different environments suggest that genetic diversity of genes supporting different functions for the plant differ. Assessing genetic diversity of plant populations using genes or genetic markers expected to be associated with stress resistances or tolerances may be an important consideration to maximize allelic variability in plant collections.

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