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Assessment of genetic diversity within and between pearl millet landraces

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Abstract A minimum core subset of pearl millet [*Pennisetum glaucum* (L.) R. Br.], which comprised 504 landrace accessions, was recently established from the global pearl millet germplasm collection of ICRISAT. The accessions for this core were selected by a random proportional sampling strategy following stratification of the entire landrace collection (about 16,000 accessions) according to their geographic origin and morpho-agronomic traits. In this study RFLP probes were used to quantify the genetic diversity within and between landrace accessions of this minimum core using a subset comprising ten accessions of Indian origin. Twenty five plants per accession were assayed with *Eco*RI, *Eco*RV, *Hin*dIII and *Dra*I restriction enzymes, and 16 highly polymorphic RFLP probes, nine associated with a quantitative trait loci (QTLs) for downy mildew resistance, and five associated with a QTL for drought tolerance. A total of 51 alleles were detected using 16 different probe-enzyme combinations. The partitioning of variance components based on the analysis of molecular variance (AMOVA) for diversity analysis revealed high within-accession variability (30.9%), but the variability between accessions was significantly higher (69.1%) than that within the accessions. A dendrogram based on the dissimilarity matrix obtained using Ward's algorithm further delineated the 250 plants into ten major clusters, each comprised of plants from a single accession (with the exception of two single plants). A similar result was found in an earlier study using morpho-agronomic traits and geographic origin. This study demonstrated the utility of RFLP markers in detecting polymorphism and estimating genetic diversity in a highly cross-pollinated species such as pearl millet. When less-tedious marker systems are available, this method could be further extended to assess the genetic diversity between and within the remaining accessions in the pearl millet core subset.

Keywords AMOVA · Core collection · Genetic diversity · India · Pearl millet · RFLP

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Introduction

Genetic resources of pearl millet [*Pennisetum glaucum* (L.) R. Br.] held at ICRISAT constitute over 21,000 accessions of wild and cultivated pearl millet germplasm from 50 countries. Pearl millet is a highly cross-pollinated species and the accessions held in the ICRISAT gene bank are maintained as populations. The large size of the germplasm collection consisting of individual populations is a major constraint in maintenance, characterization, evaluation and effective utilization. Establishment of a core subset that represents the genetic diversity of the entire collection has been suggested as a successful option for the maintenance and evaluation of large germplasm collections of self-pollinated and clonally-propagated crops (Brown 1989; Grenier et al. 2000). A similar strategy was used at ICRISAT to establish the pearl millet minimum core collection, which is composed of 504 accessions (Bhattacharjee 2000). The accessions for this core subset were identified using the random proportional sampling procedure after stratifying the entire cultivated pearl millet landrace collection (16,063 accessions) based on geographic origin and morpho-agronomic traits.

The evaluation of accessions in this pearl millet core subset based on morphological characters has confirmed that there is a high amount of variation within and between the landrace accessions. However, the between-accession variation was much greater than that observed within-accessions, which indicated that the accessions

were distinct (Bhattacharjee 2000). Genetic diversity within a population depends on the number and frequency of all alleles across all loci, and the genetic constitution of the population (Crossa et al. 1993). For a cross-pollinated species, such as pearl millet, variability between accessions and the proportion of heterozygous individuals within accessions are almost equivalent, which makes it difficult to differentiate the variability between and within accessions. Additionally, the maintenance of accessions in the gene banks through regeneration by selfing or sibbing a few accessions in small plots, could contribute to the loss of alleles and a population shift or loss of vigor that could narrow the original phenotypic variability (Burton 1976).

Previous studies on isozyme diversity in pearl millet landraces from Africa and India found an intra-population diversity of 70–90% of the total diversity, but this was dependant on their regions of origin (Tostain et al. 1987, 1989). Isozymes have failed in the differentiation and classification of landraces because of the small number of marker loci available, which provide poor coverage of the genome, and a low level of allelic polymorphism. The availability of DNA-based markers like RFLPs and RAPDs have provided more-powerful tools for the detailed assessment of genetic diversity in cultivated and wild plant species (Melchinger et al. 1994). Further, these DNA-based markers are environmentally neutral and can provide estimates of the degree and distribution of variation between and within accessions. This has been demonstrated to aid in the reduction of redundant accessions in ex-situ conservation of germplasm (Westman and Kresovich 1997). Information provided by DNA-based markers could establish the base assessment of the degree and distribution of genetic diversity needed to design optimal sampling procedures, regeneration schemes and identification of core subsets.

Studies based on RAPD-, RFLP- and other PCR-based markers in crop species have demonstrated that RFLP markers, because of their co-dominant nature, are polymorphic, reproducible and ideal for the discrimination of genotypes (Karp and Edwards 1995). To-date, most DNA marker-based studies in pearl millet have used RFLP markers (Liu et al. 1994). However, use of these markers has been restricted to studies of recombination rates (Liu et al. 1996) and the development of molecular maps for economically important traits such as downy mildew resistance (Jones et al. 1995) and drought tolerance (Yadav et al. 1999). There has been no reported study which used these markers to partition genetic diversity within and between germplasm accessions. In the present study, we assessed genetic diversity within and between a subset of core landrace accessions with a set of selected RFLP markers to confirm the assessment made with morphological traits. Thus, the specific objectives of this study were to: (1) assess the distribution of the RFLP-based diversity within and between the ICRISAT pearl millet landrace collection, (2) assess the utility of RFLP markers for the classification of accessions into distinct groups, and (3) assess the relationship between RFLP-based genetic similarity and generalized distance, based on morphological characters.

Table 1 List of probe-enzyme combinations tested

Probes	Copy number	Linkage group	Restriction enzyme (s)
Downy mildew resistance			
PgPSM858	2c	1	*Dra*I
PgPSM565	1c	1	*Hin*dIII
PgPSM716	1c	4	*Hin*dII/*Dra*I
PgPSM305	low copy	4	*Hin*dIII/*Dra*I
PgPSM648	1c	4	*Dra*I
PgPSM713	1c	6	*Eco*RI
PgPSM618	1c	7	*Dra*I
PgPSM834	1c	7	*Hin*dIII
PgPSM857	1c	7	*Hin*dIII
Drought tolerance			
PgPSM214	1c	2	*Dra*I
PgPSM025	1c	2	*Eco*RV
PgPSM321	1c	2	*Eco*RI
PgPSM592	1c	2	*Hin*dIII
PgPSM443	1c	2	*Dra*I

Materials and methods

A sample of ten diverse pearl millet landrace accessions of Indian origin was identified from the established minimum core subset of 504 accessions (Bhattacharjee 2000). All the accessions of Indian origin in the minimum core (158 accessions) were stratified by Ward's minimum variance clustering method using six quantitative traits (days to flowering, plant height, spike exsertion, spike length, spike thickness and 1,000-grain weight). From each of the resulting ten distinct clusters, one representative accession was selected randomly. One hundred seeds per accession were sown in the field in two-row plots with 50 seeds per row. These plants were selfed. Samples of about 50 selfed seeds from 25 random plants per accession were sown in low-nutrient peat and a sharp sand compost in one pot (11.5 cm in diameter). The pots were thinned to a stand of 25 seedlings at 15 days after emergence. Leaves from these 25 seedlings were bulk harvested at the 3–5 leaf stage (13–15 days old), frozen in liquid nitrogen and stored at –80 °C until analyzed. Genomic DNA from 5 g of leaf material was extracted as per the method of Sharp et al. (1988). About 20 µg of genomic DNA were digested with four restriction enzymes (*Eco*RI, *Hin*dIII, *Eco*RV and *Dra*I).

From the MilletGenes database (http://jii05.jic.bbsrc.ac.uk/8000/millet.html), 16 highly polymorphic loci mapped by Liu et al. (1994) were selected for analysis. The associated DNA probes had been derived from a pearl millet *Pst*I genomic library and were linked to QTLs that controlled downy mildew resistance and drought tolerance (Table 1). These probes provide coverage over five of the seven pearl millet linkage groups.

Restricted DNA and λ-DNA markers (Amersham) were separated by 0.8% agarose-gel electrophoresis and transferred to nylon membranes (Hybond-N^+, Amersham) by the Southern procedure (Reed and Mann 1985). The probes were isolated by electrophoresis in low melting point agarose and labeled with ^{32}P-α-dATP by the random primer labeling method (Feinberg and Vogelstein 1983). Conditions used for hybridization were similar to those described in Sharp et al. (1988). Depending on the level of ^{32}P incorporation, photographic films (Kodak) were exposed at –70 °C for 5–10 days.

RFLP bands were scored as present (1) or absent (0) for individual plants within each accession. The genetic similarity index

Table 2 Analyses of molecular variance (AMOVA) for the 10 accessions with 250 plants

Source of variation	SSD	d.f.	σ^2	%	ϕ Statistic
Between accessions	142.62	9	0.623	69.11	0.691
Within accessions	66.79	240	0.278	30.89	
Total	209.41	249	0.901	100.00	

SSD: sum of squares deviations
d.f.: degrees of freedom
σ^2: estimates of variance components
%: percent of total variance contributed by each component
ϕ: phi statistic

(*gs*) was calculated following Nei and Li (1979): $gs_{ij} = 2N_{ij}/N_i + N_j$ where N_{ij} is the number of bands present in both individuals i and j, N_i is the number of bands present in individual i, and N_j is the number of bands present in genotype j, with regard to all probe-enzyme combinations. The distance matrix was then calculated, $D = 2 (1 - gs_{ij})$, on the basis of inter-individual pair-wise comparisons. Standard errors of individual estimates were obtained by the jackknife method (Miller 1974) using the Genstat 5 Release 3.2 (1995) program.

The variation within and between accessions was partitioned by the analysis of molecular variance (AMOVA; Excoffier et al. 1992). Within this program the data type was set for 'RFLP' data. This analysis is based on the partition of the distance matrix formulated to the Euclidean metric of Excoffier et al. (1992). The Euclidean metric is similar to the distance metric of Nei and Li (1979). A fixation index (ϕ statistics), which is the molecular equivalent of Weir and Cockerham's (1984) *F*-statistics (Michalakis and Excoffier 1996), was also generated. In this study, ϕ_{ST} refers to the correlation of haplotypes within an accession relative to a random sample from the defined group of accessions as a whole. Thus, $\phi_{ST} = \sigma^2_a/\sigma^2_a + \sigma^2_b$ where σ^2_a and σ^2_b are the variance components (expected squared deviations) of the effects of populations (a) and individuals within populations (b) (Excoffier et al. 1992). The significance of ϕ-statistics was tested using a non-parametric permutation test (500 replicates).

Phenotypic diversity within and between accessions was also estimated for the morphological traits recorded based on 25 plant observations for each accession. The observed data were standardized with mean zero and unit variance per trait, and principal component analysis was performed. The first two principal components explaining about 95% of the variation were used to calculate the generalized distances (morphological distance, *md*) between the plants (Goodman 1972).

Simple (*r*) and rank (r_s) correlation coefficients between genetic similarities (*gs*) and morphological distances (*md*) were calculated. A dendrogram was then constructed based on *gs* estimates using Ward's minimum variance method (Statsoft 1997).

Results

Among the 51 different bands detected using 16 different probe-enzyme combinations, four (approximately 9%) were common between all the accessions, two (approximately 4%) were unique to a single accession (IP 4828 and IP 3890), and the rest were shared by two or more accessions (Fig. 1). Within each pearl millet accession, considerable variation was detected. This is evident from the number of haplotypes and their frequencies derived from RFLP data (Fig. 1). The distribution of rare bands, either within an accession or across all accessions studied, was not uniform and frequency of these rare bands among plants within each accession did not contribute much to the ability to detect variation between the accessions. Variability between accessions was most-readily detected by differences between accessions for bands that are common.

Comparisons of genetic similarity (*gs*) estimates across all the 250 plants (25 plants from ten accessions) were restricted to pairs of unrelated plants to minimize confounding effects due to relatedness. Genetic similarity estimates ranged from 0.40 to 0.98 (data not shown) across these plants. Similarly, the morphological distance (*md*) values ranged from 2.0 to 95.9 with a mean of 27.8 (data not shown). Simple (*r*) and rank (r_s) correlation coefficients between genetic similarity (*gs*) and morphological distance (*md*) were 0.01 and 0.03 within the plants, indicating no significant correlation between *gs* and *md* (data not shown).

The statistics of genetic diversity within and between accessions are given in Table 2 for the overall sample of ten accessions. The results confirm that the variance fraction explained by between-accession variation is two-fold greater than that due to differentiation within accessions. The results were significant with a *P* value of 0.001. Since the variance component of the effects of groups is not present in this analysis, ϕ_{ST} is a simple proportion and equivalent to the percentage of total variance attributed to differences between accessions (Table 2). A dendrogram was constructed based on the Nei and Li (1979) similarity indices using Ward's minimum variance method (Fig. 2). Each of these accessions formed distinct clusters that were further subdivided into sub-clusters, depicting within-accession variation. The cluster analysis revealed ten groups at the 10% dissimilarity level. High within-accession variation was observed in each cluster, however, between-cluster variation separated each of these ten landrace accessions into a distinct group. Only two plants, one from each of two different accessions (IP 3098 and IP 8074) grouped

Fig. 1 Polymorphism observed in ten pearl millet core accessions with different probe-enzyme combinations from RFLP data obtained on 25 plants per accession. Different colors used to represent polymorphism are: *Light gray* Presence of bands (scored as 1). *Dark gray* Absence of bands (scored as 0). *White* Polymorphic region (presence of both 1's and 0's among the 25 plants scored). *Grayish black* Polymorphism scored due to presence of bands (presence of few 1's among many 0's for the 25 plants scored). *Black* Polymorphism scored due to absence of bands (presence of few 0's among many 1's for the 25 plants scored) ▶

IPNO
Plant
PSM838
PSM365
PSM25
PSM231
PSM592
PSM214
PSM443
PSM716
PSM716
PSM305
PSM505
PSM648
PSM715
PSM857
PSM618
PSM834

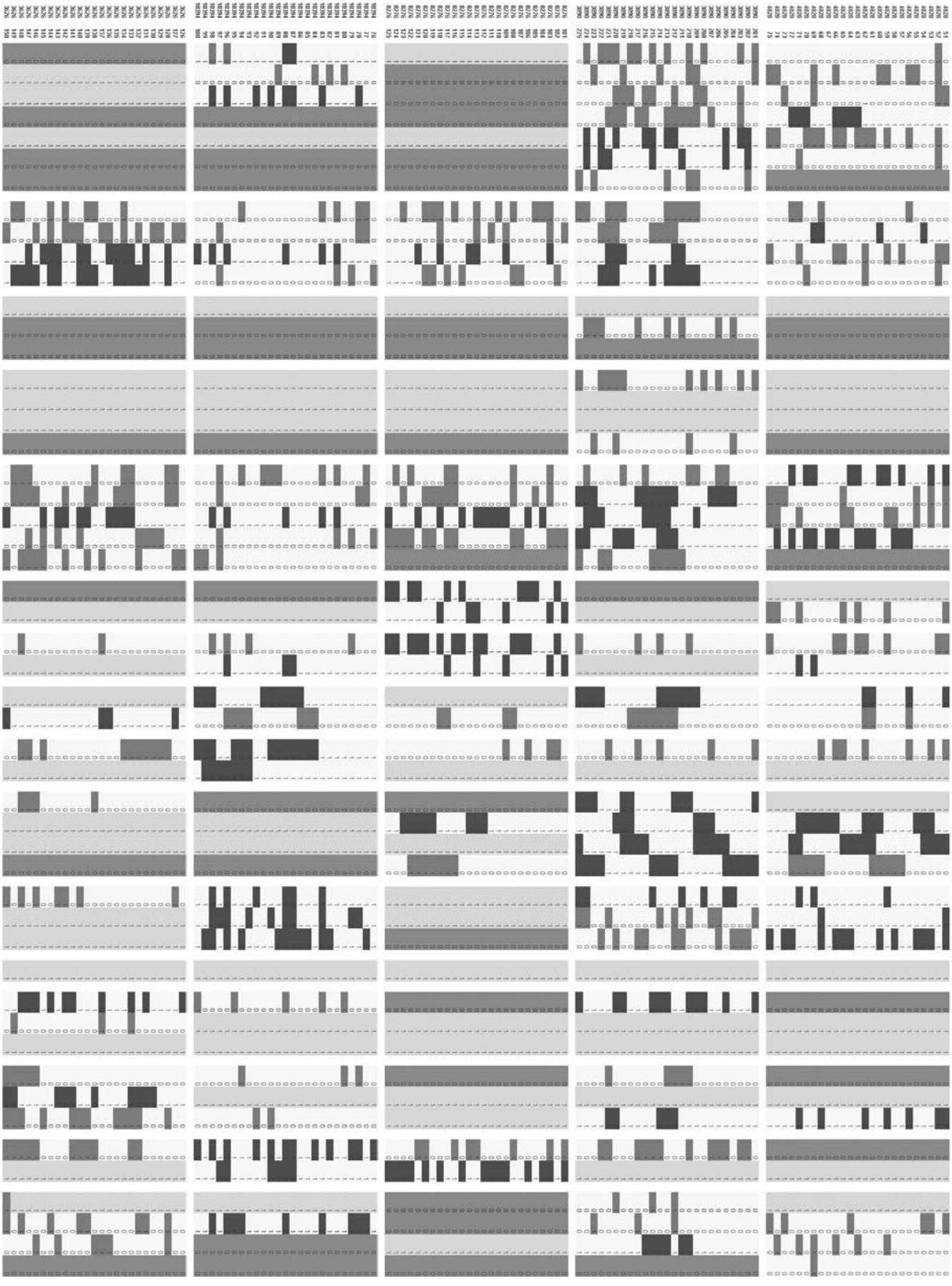

Fig. 1 Legend see page 668

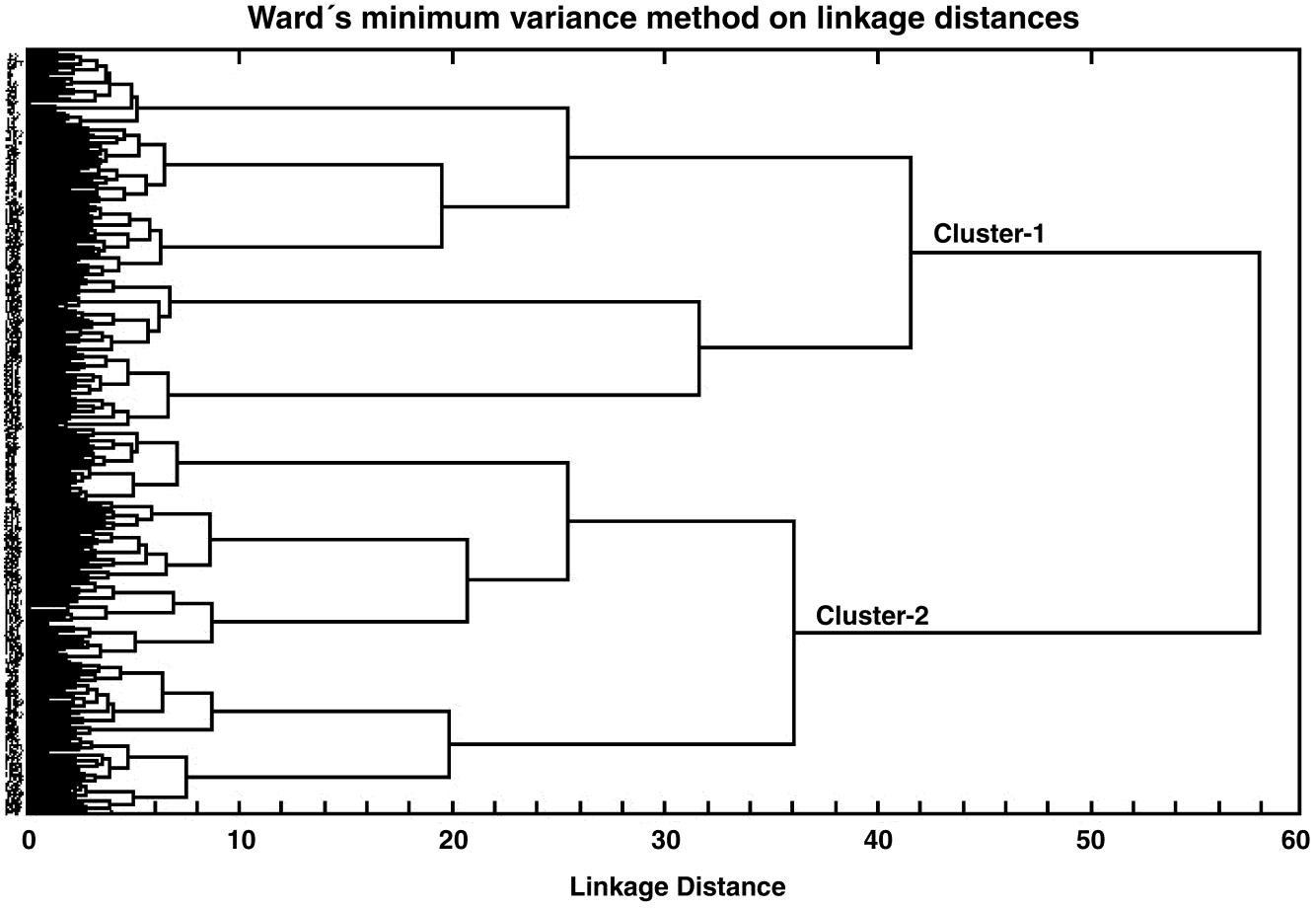

Fig. 2 Dendrogram based on Ward's minimum variance method and linkage distances for molecular data

with other accessions; that is, one plant from accession IP 3098 clustered with the plants of accession IP 8074 and vice versa.

Discussion

For pearl millet, heterogeneity and heterozygosity at many loci within a landrace accession is high, due to the cross-pollinated nature of the crop and the breeding system used for maintenance in the ICRISAT gene bank. To our knowledge this is the first study to report estimates of genetic diversity within and between landrace accessions of an outcrossing species based on RFLP markers. Previous studies of genetic diversity within the germplasm of pearl millet from Africa and India were performed based on isozyme markers (Tostain et al. 1987, 1989). They reported that accessions from Niger showed very high intra-population variation (70–90%). However, the present study based on RFLP has shown substantially higher inter-population genetic variation than that obtained previously using isozymes. This discrepancy could be due to the different evolutionary properties of RFLP and isozyme loci.

The observed proportion of variation attributable to between-accession differences was inconsistent with the expected population structure for an outcrossing species. The self-pollinated species such as *Hordeum spontaneum* has 43% of within-population variation (Dawson et al. 1993) and the leguminous tree *Gliricidia sepium* has 40% within-population variation. In contrast, cross-pollinated species have been reported to have 72–100% of their total variation attributable to within-population variation (Huff et al. 1993; Nesbitt et al. 1995). In the RFLP analysis of this study on pearl millet, 69.11% of the total variation was attributable to between-accession differences, a level comparable or slightly lower than that reported for self-pollinated species. Clustering of plants based on the dissimilarity matrix following Ward's minimum variance method further differentiated the accessions into ten distinct groups that also represented the between-accession variation. The clusters formed by RFLP analysis revealed a similar pattern to that known for the morphological variability and all the accessions were separated from each other with a minimum of 10% dissimilarity.

The high between-accession differentiation recorded in the study could be a result of the erosion of the original genetic diversity of accessions that is expected in gene banks where genetic drift has occurred in the regeneration of accessions, which would lead to increased between-accession variation. Moreover, within each acces-

sion there might have been substantial inbreeding, which is expected as a consequence of selfing in the cluster-bagging mating system that has historically been used to maintain this collection. This would account for an increase in genetic differentiation between accessions (Parzies et al. 2000). Therefore, to make the best use of landrace accessions, in which genetic variation may be predominately between accessions rather than within accessions, it appears advisable to advance generations in a working sample while minimizing the number of regenerations in the sample used for long-term conservation.

In this study, correlations between the RFLP-based *gs* estimates and morphological distance were very weak ($r = 0.01$ and $r_s = 0.03$). Morphological distance and *gs* estimates are fundamentally different approaches to determine the similarity between genotypes. They rely on different kinds of information and, as a consequence, are subject to different sources of error. In this study, the absence of significant correlations may be a result of the inadequate representation of genetic relationships by the observed morphological traits. This could be due to the limited number of traits observed, the limited variation for these traits, the limited number of underlying genes for these traits and/or possible epistatic interactions between these genes (Schut et al. 1997). Further, the accuracy of *gs* estimates depends on the number of markers, their distribution over the genome and the independent information provided by each DNA marker (Messmer et al. 1993). In this study, *gs* estimates might have had large sampling errors due to the small number of probe-enzyme combinations (16) used to reveal polymorphism. According to Messmer et al. (1993), a minimum of 100 independent polymorphic probe-enzyme combinations are required to keep the standard error of *gs* estimates below 0.05; and this condition has certainly not been met in the present study.

In conclusion, it appears that RFLP data pertaining to diversity within and between accessions of a crop species can be useful in devising strategies for managing germplasm as previously reported for crops such as maize (Dubreuil and Charcosset 1998, 1999), rice (Zhang et al. 1992), barley (Casas et al. 1998), oats (Moser and Lee 1994) and *Paspalum* species (Jarret et al. 1998). Therefore, their use in genetic conservation should be encouraged, where other lower-cost options are not available. The study can also be further extended to assess genetic diversity within and between other groups that can be identified (based on morphological characters) among core accessions of pearl millet collected from Africa. Availability of less-tedious and expensive DNA marker systems, such as sequence-tagged microsatellite site (STMS) markers, will make these molecular methods of diversity assessment even more attractive (Grenier et al. 2000).

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