PHYLOGENETIC DIVERSITY AND RELATIONSHIPS AMONG COWPEA (*VIGNA UNGUICULATA* L. WALP.) LANDRACES USING RANDOM AMPLIFIED POLYMORPHIC DNA MARKERS

T. Karuppanapandian[1*], T. Karuppudurai[2], P. B. Sinha[1], A. M. K. Haniya[3] and K. Manoharan[1]

[1]Department of Plant Sciences/[2]Department of Animal Behaviour and Physiology, Centre of Potential in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai-625 021, India

[3]Department of Biotechnology, Srimad Andavan Arts & Science College, Thiruvanaikoil, Tiruchirappalli-620 005, India

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Summary. The objective of the present study was to investigate the genetic diversity and relationships among 24 cowpea (*Vigna unguiculata* L. Walp.) landraces from diverse geographic locations of Tamil Nadu, India, using random amplified polymorphic DNA (RAPD) markers. A total of 155 amplified fragments were generated with an average of 7.8 fragments using 20 random primers and 117 of these fragments were polymorphic with an average of 5.9 fragments per primer for cowpea landraces. The percentage of polymorphism ranged from 25 to 100 %. Gel-obtained binary data (presence (1) and absence (0)) were used for generating a genetic similarity matrix which was utilized in an unweighted pair-group method using arithmetical averages (UPGMA). Genetic distances between all cowpea landraces based on Jaccard's similarity index coefficients ranged from 0.27 to 0.94, but mostly concentrated between 0.80 and 0.94. The UPGMA cluster analysis group of these 24 cowpea landraces into two distinct clusters showed reasonable variability that may be exploited for crop improvement. The present results showed that the cowpea landraces from various locations of Tamil Nadu, India, formed a genetically diverse population and RAPD markers can be

* Corresponding author, e-mail: tkpandian78@yahoo.com

effectively employed to assess genetic diversity and analyze genetic relationships among 24 cowpea landraces.

Key words: cow pea landraces, genetic variability, Jaccard similarity coefficients, polymorphism, RAPD markers

Abbreviations: AFLP – amplified fragment length polymorphism, PCR – polymerase chain reaction, RAPD – random amplified polymorphic DNA, RFLP – restriction fragment length polymorphism, UPGMA – unweighted pair-group method using arithmetical averages

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp.) is an indigenous legume grown mainly by female subsistence farmers. It has been broadly and independently cultivated throughout Southern Indian regions. In addition, it is of relevant socio-economic importance for the family farming system (Parentoni et al., 2001). It is a very essential crop in developing countries of Africa, Asia and Latin America. As it contains high protein content as well as vitamins and minerals, this legume plays an important role in human consumption and animal feeding. It is consumed as dry seeds, fresh green pods and/or leaves. Cowpea is the third most important legume in Asia after ground nut (*Arachis hypogea* L.) and black gram (*Vigna mungo* L.) (Singh et al., 1997; Sivaprakash et al., 2004). Cowpea is widely adapted to semi arid parts of Tamil Nadu, Central, Western, and Southern regions of the country. In many traditional farming systems, cow pea is intercropped mainly with cereals and root crops (Coulibaly et al., 2002; Fall et al., 2003). Cowpea has several agronomic advantages including drought tolerance, high nutritional value and ability to produce some yield in soils that are too poor for cultivation of other more favored species, such as common beans and ground nuts. Cowpea supplies soil nitrogen for other crops by fixing atmospheric nitrogen through symbiosis with *Rhizobium* bacteria and is therefore beneficial in intercropping and crop rotations (Lewin et al., 1987; Parentoni et al., 2001). Despite the importance of cowpea as a food legume in traditional farming systems in Tamil Nadu, India, limited breeding efforts have been made to improve this legume. Little information is available about the extent of genetic diversity among cowpea landraces for long-term conservation and improvement (Fall et al., 2003).

The genetic diversity of landraces was the most immediately useful part of cowpea biodiversity. However, more consistent agronomic and genetic knowledge about these collections is still lacking and it is a serious limitation to utilizing, managing, and conserving landraces of cowpea gene pools (Tosti and Negri, 2002; Fall et al., 2003). Only a few genetic studies on cowpea using molecular techniques (random amplified polymorphic DNA [RAPD], restriction fragment length polymorphism [RFLP], and amplified fragment length polymorphism [AFLP]) have been reported

(Williams et al., 1990; Tosti and Negri, 2002). Coulibaly et al. (2002) verified the existence of polymorphism in different populations of cowpea through AFLP procedure. Subsequently, AFLP markers were utilized to show genetic uniformity in plants derived from somatic embryos (Shenoy and Vasil, 1992). However, these reports fell short of providing quantification of genetic variability within species, especially regarding genetic distances. Evaluations based on RAPD profiles would be suitable for providing such information due to the high level of polymorphism of this technique. In recent years, fingerprinting systems based on RAPD analysis have been increasingly utilized for detecting genetic polymorphism in several plant genera (Powel et al., 1996; Sivaprakash et al., 2004).

To date, few studies have been performed with cowpea using RAPDs and no attempts have been made to evaluate the potential values of using genetic markers of cowpea landraces of Tamil Nadu, India, for genetic improvement and germplasm purposes. In the present work, the phylogenetic diversity as well as relationships among cowpea landraces using RAPD technique were investigated. The objectives of this study were to determine if RAPD markers could be used to estimate genetic diversity among 24 cowpea landraces from diverse geographic locations of Tamil Nadu, India, to investigate relationships among landraces of this species and to determine whether the main groups of landraces could be distinguished using RAPD marker data to describe diversity patterns useful for conservation and crop improvement strategies. Genetic diversity in this study may be used for selecting parents for breeding purposes.

MATERIALS AND METHODS

Plant material

Twenty four cowpea landraces of diverse geographic locations of Tamil Nadu, India, were used in this investigation (Fig. 1). Young, healthy leaves were pooled from 25-day-old field grown cowpeas, washed free of dirt, mopped dry and quickly frozen and powdered using liquid nitrogen. The powders were either used for immediate isolation of DNA or were stored in a deep freezer (-70°C) for a long-term storage.

Isolation of DNA

Total genomic DNA was extracted using the CTAB method of Doyle and Doyle (1987). Fresh leaf samples (500 mg) were ground with liquid nitrogen with a mortar and pestle and then transferred to 1.5 ml centrifuge tubes (preheated in 60°C water) containing 700 мl of urea buffer (8.0 M urea, 0.05 M NaCl, 0.05 M Tris-HCl (pH 7.5), 0.02 M EDTA, 1 % (w/v) sarcosyl), mixed thoroughly and incubated in a water bath at 60°C for 10 min. The tubes were inverted periodically. Then 700 µl of

Fig. 1. Geographical locations of the cowpea landraces listed in Table 2.

phenol:chloroform (1:1, v/v, Tris-HCl (pH 8.0) saturated) were added and the tubes were gently inverted repeatedly. After centrifugation at 10,000 rpm for 10 min at 4°C the supernatant was transferred to a new centrifuge tube, and 0.7 volume of isopropanol and 1/10 volume of 4.4 M NH_4OAc were added and centrifuged at 10,000 rpm for 10 min at 4°C to collect precipitated DNA. The DNA pellet was resuspended with 400 µl of 10 mM TE (Tris-HCl (pH 8.0), 1 mM EDTA) and incubated with 5 µg DNase-free RNase (SigmaAldrich, USA) for 10 min at 65°C. The RNase and the remaining protein were extracted with an equal volume of phenol:chloroform (1:1; v/v, Tris-HCl (pH 8.0) saturated) and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube, and the DNA was precipitated by the addition of a 1/10 volume of 4.4 M NH_4OAc and 3 volumes of 95 % ethanol. Precipi-

tated DNA was collected by centrifugation at 10,000 rpm for 10 min at 4°C, washed with 70 % ice-cold ethanol twice, and dried before re-dissolving in 200 µl of 10 mM TE (Tris-HCl (pH 8.0), 1 mM EDTA). The approximate DNA yields were calculated by using a double beam UV-Vis spectrophotometer (Hitachi U-2000, Japan). The DNA samples were stored at -20°C until further analysis.

Polymerase chain reaction (PCR) and agarose gel electrophoresis

Twenty random primers (Operon Technologies Inc., USA) were screened by PCR. PCR amplifications were performed in a 25 µl reaction volume containing 10 X PCR buffer (10 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.01 % (w/v) gelatin) with 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 1 units of *Taq* polymerase (Fermentas GmbH, Lithuania), 0.2 µM of random primer and 50 ng of DNA. Amplification was performed using Eppendorf Master Cycle gradient (Eppendorf, Germany), programmed for initial denaturation at 94 ºC for 2 min and 45 cycles of 94 ºC for 1 min, 36 ºC for 1 min, and 72 ºC for 2 min. The amplification was completed with a 5 min final extension at 72 ºC. The amplification products were separated by electrophoresis on 1.5 % agarose (SigmaAldrich, USA) gels with 0.5 X TAE buffer and stained with ethidium bromide (EtBr), (1 µg ml^{-1}). The amplification products were then observed under UV light and photographed and analyzed using Gel documentation system (Biorad, Model 2000, USA).

Gel scoring and data analysis

RAPD-PCR analysis was repeated at least 3 times and only the primers producing strong and reproducible bands were considered for further analysis. Photography of the gels was analyzed using Numerical Taxonomy and Multivariate Analysis System for computer package (NTSYS-pc-version 2.02; Rohlf, 1993). The presence (1) or absence (0) of the RAPD fragment was considered as a single trait and the binary data were used to generate Jaccard's similarity coefficients for RAPD bands showed in Table 2 (Jaccard, 1908). The matrix of similarity coefficients was subjected to unweighted pair-group method using arithmetical averages (UPGMA) to generate a dendrogram using linkage procedure.

RESULTS AND DISCUSSION

The objective of the present study was to assess the extent of genetic diversity and relationships among 24 cowpea landraces being conserved and cultivated by the local communities in various locations of Tamil Nadu, India, possessing entirely different soil and climatic patterns (Fig. 1). The cowpea landraces were analyzed by using 20 random primers. RAPD technique is a simple and quick method for charac-

Table 1. List of primers with their sequence used for the RAPD analysis of cowpea landraces and the number of amplified, polymorphic fragments and percentage of polymorphism yielded by each primer.

Primer code	GC content (%)	Primer sequence (5' to 3')	No. of amplified fragments	No. of polymorphic fragments	% of polymorphism
OPA-01	70	CAGGCCCTTC	6	3	50.0
OPA-02	70	TGCCGAGCTG	4	1	25.0
OPA-03	60	AGTCAGCCAC	8	6	75.0
OPA-04	60	AATCGGGCTG	6	5	83.3
OPA-05	60	AGGGGTCTTG	10	4	40.0
OPA-06	70	GGTCCCTGAC	5	5	100.0
OPA-07	60	GAAACGGGTG	8	7	87.5
OPA-08	60	GTGACGTAGG	9	8	88.9
OPA-09	70	GGGTAACGCC	11	7	63.6
OPA-10	60	GTGATCGCAG	4	4	100.0
OPA-11	60	CAATCGCCGT	7	6	85.7
OPA-12	60	TCGGCGATAG	11	10	90.9
OPA-13	60	CAGCACCCAC	8	5	62.5
OPA-14	70	TCTGTGCTGG	10	8	80.0
OPA-15	60	TTCCGAACCC	7	6	85.7
OPA-16	60	AGCCAGCGAA	8	6	75.0
OPA-17	60	GACCGCTTGT	10	6	60.0
OPA-18	60	AGGTGACCGT	8	7	87.5
OPA-19	60	CAAACGTCGG	6	6	100.0
OPA-20	60	GTTGCGATCC	9	7	77.8
		Total	155	117	1518.4
		Average	7.8	5.9	75.5

terization and analysis of genetic diversity among the cowpea landraces studied using single oligonucleotide primers in a PCR with low stringency. The technique requires no sequence information prior to analysis and only a minute amount of DNA (Welsh and McClelland, 1990; Williams et al., 1990). Therefore, unlimited markers have been created by RAPD. The initial pilot reactions were carried out to determine the optimum primer, template, and Mg^{2+} concentrations (data not shown). The RAPD profiles were considered consistent if at least two of the three DNA preparations revealed identically sized prominent bands after amplification with a given primer.

The other reason could be that we have used 60 to 70 % GC content of primers, whereas other workers including Yamamoto et al. (1994) have included also primers with less GC content in their studies. Fukuoka et al. (1992) observed an increase in the number of bands with increasing GC content of the primers. They got an average of 0.8 bands per primer with 40 %, 6.1 bands with 50 %, and 8.6 bands with 60 % GC content. The explanation for this correlation between the GC content of the primer and the number of bands is that the stability of base complementation when G is

pairing with C by 3 hydrogen bonds is higher than the complementation of A with T by two hydrogen bonds (Fukuoka et al., 1992). Furthermore, 20 primers used in this study were pre-selected based on their performance with cowpea DNA in earlier studies (Li et al., 2001; Tosti and Negri, 2002; Fall et al., 2003). The RAPD profiles obtained with OPA-12 primer are shown in Fig. 3. The total number of amplified fragments recorded per primer varied from 4 to 11 (Table 1). The percentage of polymorphism across the cowpea landraces ranged from 25 to 100 %. Among these 20 primers, 11 showed more than 80 % polymorphism. The average percentage of polymorphism across 20 primers was 75.5 %.

The ability to resolve genetic variation may be more directly related to the number of polymorphisms detected by the marker techniques (Gepts, 1993; Ehlers and Hall, 1997; Bai et al., 1998; Bisht et al., 1998). The percentage of polymorphic RAPDs, however, does not correlate with the influence of rare and common alleles on the genetic diversity as a fragment of the lowest frequency has the same importance as a fragment with the highest frequency across the genome (Welsh and McClelland, 1990; Gepts, 1993; Ehlers and Hall, 1997). As heterozygotes are not normally detectable, results are not readily usable for computing Hardy-Weinberg equilibrium or Nei's standard genetic distance (Lynch and Milligan, 1994). Hence, Jaccard similarity coefficients were used to assess the genetic variability and relationship among 24 cowpea landraces (Table 2). Analysis of the relationship based on 155 RAPD markers revealed genetic diversity among the landraces which ranged from 0.27 to 0.94, and was mostly concentrated between 0.80 to 0.94. A dendrogram (Fig. 2) was constructed based on Jaccard similarity coefficients taking into account the presence (1) or absence (0) of the bands and ignoring their intensities. These coefficients showed two major clusters. The first cluster (cluster A) comprised of 11 cowpea landraces (TN13, TN14, TN15, TN16, TN17, TN18, TN19, TN20, TN21, TN22, and TN24). The second cluster B was a fairly larger one compared to cluster A. It included 13 (TN1, TN2, TN3, TN4, TN5, TN6, TN7, TN8, TN9, TN10, TN11, TN12, and TN23) of the 24 cowpea landraces. Jaccard similarity coefficients value calculated for the samples collected from various locations of Tamil Nadu, India, showed that cowpea landraces from TN23 had a higher level of genetic diversity among all the landraces. Four samples (TN13, TN15, TN22, and TN24) were of higher interest from the results of the dendrogram (Fig. 2).

The dendrogram and Jaccard similarity coefficient values give an idea about the nature of the individual samples in the whole sample set. TN6, TN8, TN10, and TN11 were found to be the most unique samples in the entire sample set. All cowpea landraces could be distinguished from one another based on these polymorphic bands. Similarities among the test samples calculated with Jaccard coefficient (Jaccard, 1908) indicated that the highest similarity index (0.94) was observed between TN2 and TN3, while the lowest similarity index (0.27) was observed between TN12 and TN24. Sample TN23 from the dendrogram showed that the number of rare alleles was high

Table 2. Jaccard's similarity coefficients among 24 cowpea landraces based on the RAPD data.

Locations	TN1	TN2	TN3	TN4	TN5	TN6	TN7	TN8	TN9	TN10	TN11	TN12	TN13	TN14	TN15	TN16	TN17	TN18	TN19	TN20	TN21	TN22	TN23	TN24
TN1	1.00																							
TN2	0.73	1.00																						
TN3	0.69	0.94	1.00																					
TN4	0.84	0.67	0.63	1.00																				
TN5	0.81	0.62	0.59	0.69	1.00																			
TN6	0.54	0.40	0.41	0.52	0.70	1.00																		
TN7	0.82	0.60	0.57	0.83	0.67	0.64	1.00																	
TN8	0.68	0.54	0.51	0.57	0.82	0.86	0.56	1.00																
TN9	0.34	0.43	0.40	0.30	0.55	0.60	0.33	0.70	1.00															
TN10	0.58	0.66	0.63	0.48	0.74	0.75	0.48	0.85	0.84	1.00														
TN11	0.50	0.57	0.55	0.49	0.65	0.84	0.60	0.78	0.76	0.89	1.00													
TN12	0.71	0.64	0.63	0.66	0.64	0.67	0.59	0.80	0.51	0.71	0.63	1.00												
TN13	0.29	0.35	0.35	0.14	0.33	0.42	0.40	0.31	0.29	0.35	0.31	0.31	1.00											
TN14	0.40	0.29	0.29	0.29	0.43	0.36	0.35	0.38	0.36	0.30	0.34	0.28	0.49	1.00										
TN15	0.29	0.43	0.43	0.31	0.31	0.30	0.37	0.30	0.37	0.43	0.29	0.33	0.91	0.44	1.00									
TN16	0.37	0.28	0.36	0.28	0.41	0.34	0.43	0.37	0.34	0.29	0.32	0.36	0.47	0.91	0.42	1.00								
TN17	0.29	0.33	0.38	0.28	0.30	0.33	0.39	0.28	0.36	0.32	0.34	0.29	0.39	0.69	0.35	0.73	1.00							
TN18	0.32	0.40	0.36	0.33	0.32	0.30	0.32	0.41	0.29	0.32	0.28	0.31	0.32	0.77	0.29	0.75	0.74	1.00						
TN19	0.29	0.29	0.36	0.31	0.36	0.28	0.35	0.33	0.31	0.30	0.38	0.30	0.47	0.79	0.44	0.80	0.69	0.80	1.00					
TN20	0.31	0.39	0.38	0.37	0.28	0.35	0.30	0.43	0.61	0.54	0.46	0.44	0.28	0.59	0.28	0.52	0.39	0.42	0.50	1.00				
TN21	0.32	0.37	0.37	0.28	0.37	0.35	0.41	0.44	0.61	0.53	0.46	0.43	0.30	0.58	0.30	0.52	0.38	0.41	0.49	0.92	1.00			
TN22	0.44	0.39	0.23	0.31	0.28	0.23	0.37	0.28	0.35	0.31	0.31	0.31	0.57	0.41	0.54	0.46	0.39	0.30	0.38	0.41	0.33	1.00		
TN23	0.57	0.49	0.51	0.46	0.54	0.48	0.57	0.48	0.31	0.42	0.44	0.50	0.41	0.28	0.39	0.30	0.29	0.33	0.35	0.31	0.34	0.52	1.00	
TN24	0.36	0.29	0.39	0.41	0.30	0.28	0.29	0.31	0.28	0.35	0.29	0.27	0.45	0.32	0.41	0.36	0.44	0.30	0.33	0.33	0.29	0.83	0.42	1.00

TN1-Kanniyakumari; TN2-Tirunelveli; TN3-Tuticorin; TN4-Ramanathapuram; TN5-Virudhunagar; TN6-Teni; TN7-Madurai; TN8-Sivagangai; TN9-Pudukottai; TN10-Dindigul; TN11-Coimbatore; TN12-Thanjavur; TN13-Tiruchirappalli; TN14-Karur; TN15-Thiruvarur; TN16-Ariyalur; TN17-Perambalur; TN18-Namakkal; TN19-Erode; TN20-Salem; TN21-Cuddalore; TN22-Villupuram; TN23-Dharmapuri; TN24-Tiruvannamalai.

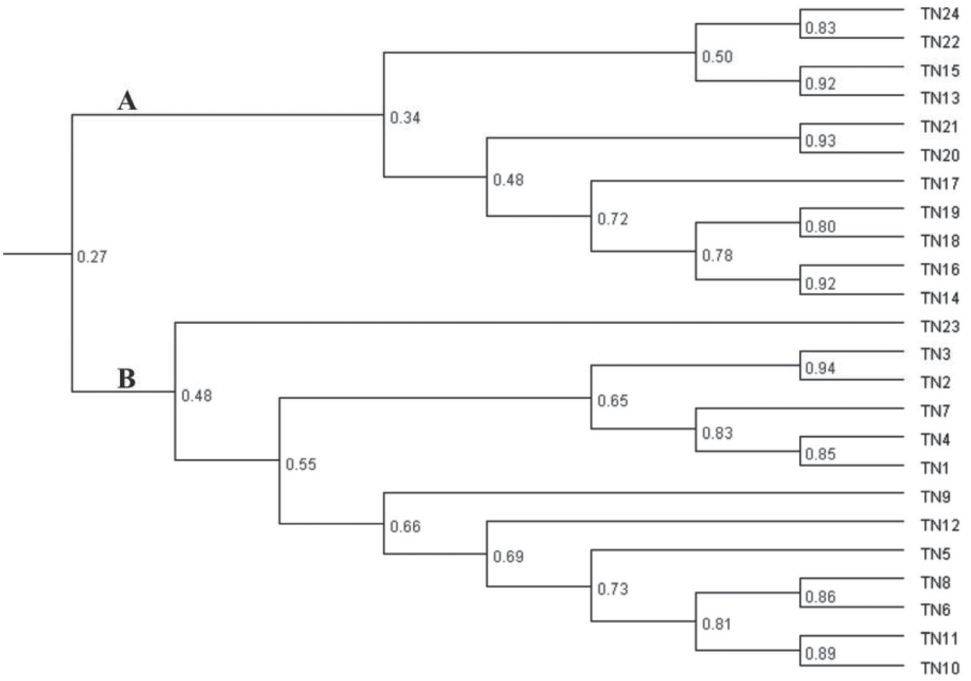

Fig. 2. Dendrogram derived from cluster analysis (UPGMA) showing relationships among the cowpea landraces listed in Table 2. Genetic similarity was obtained by RAPD using the Jaccard's similarity coefficients.

and had contributed significantly to the overall genetic variation in the entire sample set. A similar kind of genetic behaviour has already been reported in our laboratory with green gram (*Vigna radiata* L.) landraces collected from various geographic locations of Southern Tamil Nadu, India (Karuppanapandian et al., 2006).

The traditional agricultural practices in cowpea cultivation involve cultivation of the seeds obtained from the same field in subsequent generations. The homozygous genetic distribution of these landraces is well maintained as the farmers collect and sow the same seeds for cultivation supported by the fact that cowpea is a self-pollinated crop. It is therefore envisaged that the genetic variation observed in these landraces might have resulted from the long cultivation history of the species as adaptation to the local agroclimatic conditions. Once these adaptive variations have been fixed in the genotypes, they could have subsequently passed on to the next generation. In the long run, these could have resulted in locally adapted genotypes. This agricultural practice probably contributes to the genetic uniqueness by strengthening the specific adaptations obtained by the landraces (Ehlers and Hall, 1997; Li et al., 2001; Badiane et al., 2004). The limited sample size utilized in this study revealed the presence of a unique genetic reservoir in the cowpea landraces of Tamil

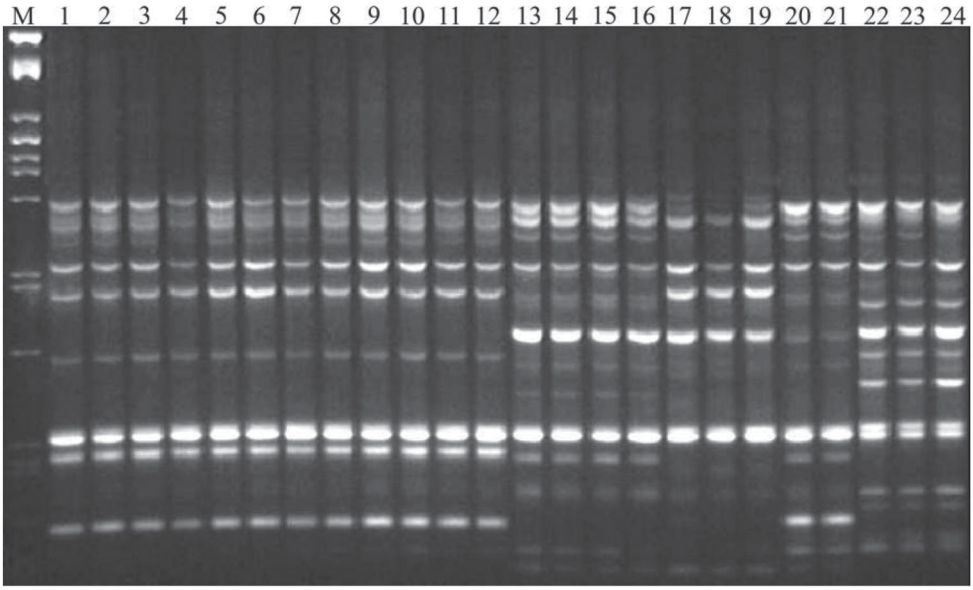

Fig. 3. RAPD profile of 24 cowpea landraces generated with OPA-12 primer. Lane M: molecular weight marker; Lanes 1-24 correspond to the cowpea landraces listed in Table 2.

Nadu, India. The high level of genetic variability suggested that many cowpea landraces had been traded into those regions or migrated with indigenous people who had begun cowpea agriculture in other localities. These cowpea landraces have been cultivated in distinct regions by unrelated small farmers. TN23, which showed the lowest similarity to the other landraces, has an uncertain origin and it seems to derive from a sample collected a few years ago by small land hold farmers.

Examination of the morphological characters of the seed and plant material of these cowpea landraces (data not shown) showed no discrete differences compared with the other samples used in this study. A similar observation was made by other researchers (Coulibaly et al., 2002; Badiane et al., 2004), who studied seed size, colour, habitat, and maturity of seeds. No information was provided as to why TN23 sample showed a distinct variation from the other samples. Therefore, TN23 sample needs to be studied further and analyzed, so that this landrace can be used in breeding programmes. The genetic diversity obtained in this study might be useful in future strategies for evaluation of desired genotypes. Such molecular data would be also useful for detecting DNA patterns unique for a given accession or set of accessions. Finally, our results demonstrate the feasibility of the RAPD technique for quantifying genetic distances among 24 cowpea landraces. The polymorphism among the cowpea landraces can be used in breeding programs to maximize the use of genetic resources. The landraces analyzed in this research have been used by small-scale

farmers. The farmers considered planting of the landraces in small areas less expensive than the commercially improved hybrids. Therefore, genetic improvement of this germplasm is important for traditional agriculture maintenance developed by the small land holder farmers from Tamil Nadu, India.

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