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*Full Length Research Paper*

# Genetic diversity in pigeonpea [*Cajanus cajan* (L.) Millsp.] Landraces as revealed by simple sequence repeat markers

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**Genetic relationships among 88 pigeonpea accessions from a presumed centre of origin and diversity, India and a presumed secondary centre of diversity in East Africa were evaluated using six microsatellite markers. Forty-seven (47) alleles were detected in the populations studied, with a mean of eight alleles per locus. Populations were defined by region (India and East Africa) and sub-populations by country in the case of East Africa and State in the case of India. Substantial differentiation among regions was evident from Roger's modified distance and Wright's *F* statistic. Greatest genetic diversity in terms of number of alleles, number of rare alleles and Nei's unbiased estimate of gene diversity (*H*) was found in India as opposed to East Africa. This supports the hypothesis that India is the centre of diversity and East Africa is a secondary centre of diversity. Within East Africa, germplasm from Tanzania had the highest diversity according to Nei's unbiased estimate of gene diversity, followed by Kenya and Uganda. Germplasm from Kenya and Tanzania were more closely related than that of Uganda according to Roger's modified distance. Within India, results did not indicate a clear centre of diversity. Values of genetic distance indicated that genetic relationships followed geographical proximity.**

**Key words:** Pigeonpea, genetic diversity, simple sequence repeat, India, East Africa.

## INTRODUCTION

Pigeonpea (*Cajanus cajan* (L.) Millsp.) is a member of the tribe Phaseoleae which comprises other genera including Phaseolus, Vigna and Lablab which contain important grain legumes (van der Maesen, 1990). Pigeonpea is widely grown in the semi-arid tropics, particularly in the Indian subcontinent where it accounts for over 70% of the world's production and coverage (FAO, 2007). Southern and eastern Africa, particularly Kenya, Malawi, Mozambique, Tanzania and Uganda, constitute the second largest pigeonpea growing areas (Singh, 1991). Other growing regions include Southeast Asia, Central and West Africa and America (Nene and Sheila, 1990). In many countries, pigeonpea is grown in small areas and as a backyard crop (Nene and Sheila, 1990).

It is likely that pigeonpea evolved by interspecific hybridization of *C. cajanifolia* and *C. scarabaeoides* (Nadimplli et al., 1992) somewhere in the Indian sub-continent (van der Maesen, 1980). It is likely that India was also the centre of domestication sometime before 2000BC as evidenced by the presence of several wild

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**Abbreviations: RFLP,** Restriction fragment length polymorphism; **RAPD,** randomly amplified polymorphic DNA; **SSRs,** simple sequence repeats; **dNTP,** deoxynucleoside 5'-triphosphates; **PIC,** polymorphic information content; **UPGMA,** unweighted paired group method using arithmetic averages; **TFPGA,** tools for population genetic analysis; **HW,** Hardy-Weinberg; **S.E,** standard error.

species of pigeonpea including the progenitor species, high morphological diversity among varieties, ample linguistic evidence and variety of use in the daily cuisine (van der Maesen, 1990). Sixteen wild species of pigeonpea exist in India, while only one close relative, C. *kerstingii (Harmes)* is endemic to Africa and another wild relative, *C. scarabaeoides* (L.) Thouars, is thought to have arrived in Africa relatively recently due to its restricted distribution in coastal areas (van der Maesen, 1979). East Africa is considered a secondary centre of diversity of pigeonpea (Smartt, 1990; van der Maesen, 1990). A further centre of diversity occurs in Australia with 15 wild species, 13 of which are endemic (Nene and Sheila, 1990).

After domestication, pigeonpea is believed to have been taken from its origin in India to Malaysia, then to East Africa and then to Egypt through the Nile valley. Thereafter pigeonpea is believed to have been taken to West Africa where it appeared in 2000 BC (van der Maesen, 1990). It is believed that prior to the main slave trade, the crop was taken from the Democratic Republic of Congo and Angola to the new world (van der Maesen, 1986). The main slave trade took it to West Indies where it was named pigeonpea in 1962 (van der Maesen, 1986).

Pigeonpea is largely a self-pollinated crop but some out-crossing occurs through insect pollination. The flower type, the abundance of insect pollinators and weather conditions during flowering can influence the degree of cross-pollination (Bramel et al., 2004a). Out-crossing rates of 40% have been reported (Sen and Sur, 1964). Besides being high in protein (21%) and drought-tolerant, pigeonpea provides many benefits to resource poor farmers including fuel, fodder, fencing material, improved soil fertility and control of soil erosion (Siambi et al., 1992). Despite this, pigeonpea is a neglected crop in terms of research (Minja, 2001). The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) maintains a large *ex-situ* collection of over 13,000 accessions of Cajanus species from around 75 countries (http://singer.cgiar.org).

The promotion of pigeonpea as a high value cash crop and the introduction and adoption of improved varieties is slowly reducing the genetic diversity of pigeonpea landraces (Bramel et al., 2004a). Limited pools of pigeonpea germplasm have been characterized previously by protein and isozyme electrophoresis (Ladinzinsky and Hamel, 1980; Singh et al., 1981; Krishna and Reddy, 1982; Kollipara et al., 1994), restriction fragment length polymorphism (RFLP) (Nadimpalli et al., 1992), randomly amplified polymorphic DNA (RAPD) (Ratnarpakhe et al., 1995) and microsatellites (Litt and Luty, 1989) which have also been termed simple sequence repeats (SSRs) (Jacob et al., 1991) and simple tandem repeats (Edwards et al., 1996). Yang et al, (2006) examined diversity of 46 cultivated and 50 wild accessions of pigeonpea using nearly 700 DArT markers. SSRs are multiple tandem repeat sequences whose unit of repetition is between one to five base pairs (Conduit and Hubbell, 1991). The unique value of microsallites for molecular genetic analysis arises from their high level of polymorphism, their abundance in the genome allowing for wide genome coverage, their co-dominance and ease of detection via automated systems (Milbourne et al., 1997; Witsenboer et al., 1997). At the time of this study, nine SSR markers had been developed for pigeonpea and six had been used in an extensive diversity assessment (Buhariwalla and Crouch, 2004; Bramel et al., 2004b). The distribution of genetic diversity between and within pigeonpea landraces from different geographical regions is largely unknown. An understanding of the distribution of genetic diversity is essential for both utilisation and conservation strategies. The objective of this study therefore, was to determine the genetic diversity and relationships within and among pigeonpea landrace accessions from the presumed primary centre of origin and domestication, India and a secondary centre of diversity in East Africa, using SSR markers.

## MATERIALS AND METHODS

### Plant materials

Eighty-eight (88) pigeonpea accessions were selected for this study based on geographical origin (Table 1). Thirty eight (38) accessions were from East Africa (18-Uganda, 10-Kenya and 10-Tanzania) and 50 were from India. Within India, seven were from Andhra Pradesh, eight were from Madhya Pradesh and Tamil Nadu and nine each from Maharashtra, Gujarat and Orissa. Between one and five individuals were sampled from each accession. The total number of individuals from India was 209 and that from East Africa was 168. The seed samples for these accessions were obtained from the genebank at ICRISAT.

### Primer selection and genotyping

Ten plants of each accession were grown in rehydrated Jiffy-Belt® pellet soil placed in 6 cm diameter wells in plastic trays. Three young leaves were harvested from a maximum of five 2 - 3 week-old plants per accession. Total genomic DNA was extracted from the leaves using the DNeasy Plant Minikit (Qiagen) according to the manufacturer's instructions and diluted to 10 ng/ul.

Initially, PCR amplification of all nine available SSR primer pairs were optimized using a modified Taguchi method (Buhariwallah and Crouch, 2004) that is designed to reveal the effects and interactions of specific reaction components simultaneously using a few reactions. In the Taguchi method, reaction components that are likely to affect the PCR process are arranged in an orthogonal array. Each component occurs at one of three predetermined levels (A, B and C), each of which occurs at an equal number of times within the orthogonal array. Here 0.2 pmoles (A), 0.3 pmoles (B) and 0.5 pmoles (C) of each fluorescently labeled primer was used, 5 ng (A), 10 ng (B) and 15 ng (C) of DNA; $Mg^{2+}$ concentrations of 1 mM (A), 1.5 mM (B) and 2 mM (C); dNTP concentrations of 0.1 mM (A), 0.15 mM (B) and 0.2 mM (C) and Taq polymerase of 0.75 U (A), 1 U (B) and 1.2 U (C) was used. This resulted in nine different

**Table 1.** Origin and identity of germplasm used in this study.

| ICRISAT Accession No. | Country | State/District | ICRISAT Accession No. | Country | State/District |
|---|---|---|---|---|---|
| 15510 | Uganda | Mbale | 13672 | India | Tamil Nadu |
| 15511 | Uganda | Mbale | 8524 | India | Tamil Nadu |
| 15512 | Uganda | Kumi | 14015 | India | Orissa |
| 15513 | Uganda | Soroti | 14012 | India | Orissa |
| 15514 | Uganda | Soroti | 14009 | India | Orissa |
| 15515 | Uganda | Soroti | 14005 | India | Orissa |
| 15516 | Uganda | Soroti | 13031 | India | Orissa |
| 15517 | Uganda | Soroti | 13012 | India | Orissa |
| 15518 | Uganda | Lia | 13017 | India | Orissa |
| 15519 | Uganda | Masiroli | 8842 | India | Orissa |
| 15520 | Uganda | Masindi | 8828 | India | Orissa |
| 15522 | Uganda | Masindi | 14055 | India | Maharashtra |
| 15523 | Uganda | Hoima | 14021 | India | Maharashtra |
| 15524 | Uganda | Hoima | 14020 | India | Maharashtra |
| 15525 | Uganda | Kiboga | 12209 | India | Maharashtra |
| 15526 | Uganda | Kiboga | 12211 | India | Maharashtra |
| 15527 | Uganda | Kiboga | 10805 | India | Maharashtra |
| 15528 | Uganda | Shiboga | 10786 | India | Maharashtra |
| 12007 | Tanzania | Kilosa | 10781 | India | Maharashtra |
| 12008 | Tanzania | Iringa | 10772 | India | Maharashtra |
| 12010 | Tanzania | Mbeya | 14044 | India | Madhya Pradesh |
| 12011 | Tanzania | Meya | 12937 | India | Madhya Pradesh |
| 12025 | Tanzania | Masassi | 11882 | India | Madhya Pradesh |
| 12026 | Tanzania | Masassi | 11883 | India | Madhya Pradesh |
| 12053 | Tanzania | Bagamoyo | 10057 | India | Madhya Pradesh |
| 12086 | Tanzania | Kondoa | 10055 | India | Madhya Pradesh |
| 12114 | Tanzania | Dodoma | 10052 | India | Madhya Pradesh |
| 12115 | Tanzania | Dodoma | 10048 | India | Madhya Pradesh |
| 13062 | Kenya | Kitui | 14054 | India | Gujarat |
| 13063 | Kenya | Kitui | 14048 | India | Gujarat |
| 13075 | Kenya | Kitui | 8998 | India | Gujarat |
| 13076 | Kenya | Kitui | 9000 | India | Gujarat |
| 13081 | Kenya | Embu | 9011 | India | Gujarat |
| 13082 | Kenya | Embu | 8915 | India | Gujarat |
| 13094 | Kenya | Kirinyaga | 9005 | India | Gujarat |
| 13095 | Kenya | Kirinyaga | 8946 | India | Gujarat |
| 13102 | Kenya | Machakos | 8966 | India | Gujarat |
| 13103 | Kenya | Machakos | 12887 | India | Andhra Pradesh |
| 13691 | India | Tamil Nadu | 12867 | India | Andhra Pradesh |
| 13688 | India | Tamil Nadu | 12861 | India | Andhra Pradesh |
| 13683 | India | Tamil Nadu | 12206 | India | Andhra Pradesh |
| 13667 | India | Tamil Nadu | 12207 | India | Andhra Pradesh |
| 13658 | India | Tamil Nadu | 11941 | India | Andhra Pradesh |
| 12200 | India | Tamil Nadu | 8072 | India | Andhra Pradesh |

reaction combinations. A final reaction volume of 10 µl was used. Reaction conditions were 94°C for 2 min, 35 cycles of 95°C for 35 s, annealing temperature (between 55 and 60°C) for 1 min, 72°C for 30 s, then a final extension of 20 min at 72°C. Protocol optimization was undertaken at 60°C. Amplification products were co-loaded and visualized on ABI 3730 with an internal size standard. Trace files were analysed using the GeneMapper Software Version 3.0.

A subset of 16 accessions from geographically diverse origins

were screened with nine available pigeonpea primers to determine the level of polymorphism revealed by each primer. Out of the nine primers, six were selected for further analysis (CCB 1, CCB 2, CCB 4, CCB 5, CCB 7 and CCB 9), based on the quality of amplification products and their ability to detected high levels of polymorphism. PCR reactions were carried out in a total volume of 10 µl buffer mix containing $MgCl_2$ (1-2 mM), dNTPs (0.1-0.2 mM), DNA (10 - 15 ng), forward and reverse primers (0.2-0.3 pmol) and Promega® Taq polymerase (0.75 units). The PCR cycling profile and visualization were as above.

#### Statistical analysis

Primers were described according to the number of alleles they detected and their polymorphic information content (PIC). Polymorphic information content is a measure of the informativeness of a genetic marker in any species (Botstein et al., 1980; Liu et al., 2000). The PIC of a genetic marker is estimated by:-

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - 2\left[\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} p_i^2 p_j^2\right]$$

where *p*i is the frequency of the $i^{th}$ allele and *n* is the number of alleles (Botstein et al., 1980).

Populations were defined by region; Indian sub-continent and East Africa. Sub-populations were grouped according to geographical origin, by State in India (Orissa, Tamil Nadu, Andhra Pradesh, Maharashtra, Madhya Pradesh and Gujarat) and country in East Africa (Kenya, Uganda and Tanzania). Sub-sub populations were defined as among individual plants, within an accession. Genetic diversity within and among populations and sub-populations was described by the number of alleles, the number of rare alleles, observed heterozygosity and Nei's unbiased estimate of gene diversity or heterozygosity (*H*) (Nei, 1987) where *p*i equals the frequency of the $i^{th}$ allele and *p*j the frequency of the $(i + 1)^{th}$ allele. Rare alleles were defined here as having a frequency of < 0.05. Allele frequencies on a state/country level as well as a regional level were also examined to give an indication of diversity and relatedness. Within accession the number of polymorphic loci were counted, as were the number of alleles and number of alleles unique to an accession.

Distance matrices were calculated according to Rogers' modified distance (Wright, 1978) and cluster analysis performed using the Unweighted Paired Group Method using Arithmetic averages (UPGMA) to generate a dendrogram. Bootstrap resampling ($n$ = 1000) was performed to test the robustness of the dendrogram topology. All analyses were performed using Tools for Population Genetic Analysis (TFPGA) (Miller, 1997).

To quantify the structure of sub-divided populations F-statistics, $F_{IT}$, $F_{IS}$ and $F_{ST}$ (Wright, 1978) were calculated using TFPGA (Miller, 1997). Genetic differentiation was assayed at three population levels; region, country/state and accession levels. Resampling procedures of Jacknife and bootstrap (1000 replications) over loci were used to obtain the standard error (S.E) and 95% confidence intervals for *F*-statistics.

## RESULTS

### Marker characterization in pigeonpea accessions

All SSRs were found to be highly polymorphic among the populations analyzed generating 47 alleles in total. CCB1, CCB2 and CCB7 showed six alleles with PICs of 0.361, 0.39 and 0.445, respectively. CCB 4 had 11 alleles and the highest PIC of 0.658. CCB 5 revealed nine alleles with a low PIC of 0.362. CCB 9 revealed nine alleles with a PIC of 0.432. Allele size in base pairs is shown in Table 2.

### Gene diversity within and among regions

Forty-two (42) alleles were found in India compared to 31 in East Africa (Table 2). Nei's unbiased estimate of gene diversity (*H*) in India was 0.55 and 0.228 in East Africa (Table 3). The observed heterozygosity was 0.175 in India and 0.103 in East Africa. There was a large difference between the observed heterozygosity and Nei's unbiased estimate of gene diversity, *H*, in both populations.

Within East Africa, accessions from Uganda revealed the largest number of alleles (26), compared to Tanzania (21) and Kenya (17), although the number of individuals sampled followed this order. Accessions from Uganda had the largest number of rare alleles (17), followed by Tanzania (7) and Kenya (4). Nei's diversity index is greatest for Tanzania (0.29) and lowest for Uganda (0.15).

Within India, accessions from Maharashtra had the largest number of alleles (30) followed by Tamil Nadu with 29 alleles while those from Andhra Pradesh had the smallest number of alleles (18) (Table 2). Accessions from Maharastra also had the largest number of rare alleles (12). Nei's index of gene diversity *(H)* was largest for accessions from Madhya Pradesh (0.559), followed by those from Tamil Nadu (0.528). Accessions from Andhra Pradesh had the lowest index of 0.42. *H* was greater for pigeonpea in all Indian states than the highest value for that in a country in East Africa (Tanzania with 0.29). Accessions from India harbored 19 rare alleles as opposed to 14 in East Africa (Table 3). The most frequent allele in each region was the same at each locus except for CCB4 and CCB7. Where the most frequent allele was the same, it always occurred at a higher frequency in East Africa than in India (Table 2).

### Genetic relationships among states/countries

Roger's modified distance among states/countries is given in Table 4 and illustrated in the dendrogram in Figure 1. Two main clusters are evident, one consisting of countries in East Africa, the other of States in India. Within the East Africa cluster accessions from Kenya and Tanzania were more closely related to each other than to accessions from Uganda. Table 4 shows that accessions from Uganda are more closely related to those from

**Table 2.** Allele frequencies of pigeonpea in India and East Africa.

| Locus | Allele (bp) | Region | | East African countries | | | Indian States | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | India | East Africa | Ug | Tz | Ken | MP | Mrt | Ors | TN | AP | Grt |
| CCB 1 | 198 | 0.01 | | | | | 0.03 | 0.03 | 0.01 | | | |
| | 200 | 0.23 | 0.07 | 0.11 | 0.02 | | 0.41 | 0.15 | 0.11 | 0.32 | 0.32 | 0.01 |
| | 202 | 0.68 | 0.85 | 0.81 | 0.85 | 0.96 | 0.49 | 0.76 | 0.85 | 0.49 | 0.65 | 0.09 |
| | 204 | 0.07 | 0.08 | 0.08 | 0.12 | 0.04 | 0.08 | 0.03 | 0.02 | 0.17 | 0.03 | 0.01 |
| | 206 | 0.01 | | | | | | 0.03 | | | | |
| | 210 | 0.01 | | | | | | | | 0.03 | | |
| CCB 2 | 154 | 0.01 | | 0.01 | | | | 0.02 | | | | |
| | 156 | | 0. 03 | | 0.10 | | | | | 0.01 | | |
| | 158 | 0.30 | 0.02 | 0.01 | 0.01 | 0.07 | 0.04 | 0.30 | 0.40 | 0.43 | 0.16 | 0.04 |
| | 160 | 0.58 | 0.92 | 0.96 | 0.89 | 0.88 | 0.58 | 0.65 | 0.48 | 0.53 | 0.82 | 0.06 |
| | 162 | 0.10 | 0.03 | 0.03 | | 0.05 | 0.38 | 0.03 | 0.09 | 0.03 | 0.03 | |
| | 165 | 0.01 | | | | | | | 0.04 | | | |
| CCB 4 | 216 | 0.01 | | | | | | 0.06 | | | | |
| | 218 | | 0.03 | 0.01 | 0.06 | 0.05 | | | | 0.01 | | |
| | 220 | 0.05 | 0.88 | 0.94 | 0.80 | 0.87 | 0.03 | 0.03 | 0.06 | 0.08 | 0.05 | |
| | 222 | 0.56 | 0.05 | 0.02 | 0.09 | 0.06 | 0.53 | 0.15 | 0.86 | 0.64 | 0.68 | 0.04 |
| | 224 | 0.03 | | 0.01 | | | | | 0.07 | 0.01 | 0.13 | |
| | 226 | 0.04 | | | | | 0.13 | 0.05 | | | 0.11 | |
| | 228 | 0.13 | 0.02 | 0.01 | 0.03 | 0.02 | 0.03 | 0.42 | | 0.08 | 0.03 | 0.03 |
| | 230 | 0.04 | | 0.01 | | | | 0.15 | 0.01 | 0.05 | | |
| | 232 | 0.04 | 0.01 | | 0.02 | | 0.01 | 0.02 | | 0.12 | | 0.01 |
| | 234 | 0.10 | | | | | 0.28 | 0.11 | | | | 0.02 |
| | 236 | | | | | | | 0.02 | | | | |
| CCB 5 | 185 | | | | | | | | | 0.01 | | |
| | 191 | 0.03 | | | | | 0.12 | | | | | |
| | 193 | 0.23 | 0.03 | 0.03 | 0.05 | | 0.38 | 0.18 | 0.10 | 0.07 | 0.16 | 0.06 |
| | 194 | | 0.01 | 0.01 | | | | | | | | |
| | 195 | 0.62 | 0.94 | 0.94 | 0.90 | 1.00 | 0.50 | 0.74 | 0.55 | 0.78 | 0.84 | 0.04 |
| | 197 | 0.01 | | 0.01 | | | | 0.02 | | 0.03 | | |
| | 201 | | | | 0.01 | | | | | | | |
| | 205 | 0.01 | | | | | | 0.06 | | | | |
| | 207 | 0.10 | 0.02 | 0.01 | 0.05 | | | | 0.35 | 0.12 | | |
| CCB 7 | 152 | 0.01 | 0.03 | | | 0.14 | | | | 0.04 | | |
| | 153 | | | 0.01 | | | | | | | | |
| | 154 | 0.20 | 0.68 | 0.94 | 0.43 | 0.32 | 0.03 | 0.17 | 0.37 | 0.35 | 0.24 | |
| | 156 | 0.73 | 0.29 | 0.06 | 0.57 | 0.54 | 0.79 | 0.81 | 0.57 | 0.60 | 0.74 | 0.09 |
| | 158 | 0.05 | | | | | 0.18 | | 0.02 | | 0.03 | |
| | 160 | 0.01 | | | | | | 0.02 | 0.04 | | | |
| CCB 9 | 148 | 0.01 | | | | | | | 0.02 | | | |
| | 154 | | 0.02 | 0.03 | | 0.05 | | | | | | |
| | 158 | 0.04 | | | | | 0.10 | | 0.02 | 0.08 | | |
| | 160 | 0.31 | 0.04 | 0.02 | 0.08 | 0.02 | 0.37 | 0.21 | 0.18 | 0.14 | 0.55 | 0.06 |
| | 162 | 0.53 | 0.91 | 0.93 | 0.87 | 0.92 | 0.45 | 0.67 | 0.55 | 0.68 | 0.45 | 0.03 |
| | 164 | 0.01 | 0.01 | 0.01 | | 0.02 | | 0.01 | | 0.03 | | |
| | 168 | | | | 0.01 | | | | | | | |
| | 172 | 0.02 | | | | | | 0.09 | | 0.05 | | |
| | 174 | 0.08 | 0.02 | 0.01 | 0.04 | | 0.08 | 0.01 | 0.23 | 0.04 | | 0.01 |

AP, Andhra Pradesh; Grt, Gujarat; MP, Madhya Pradesh; Mrt, Maharashtra; Ors, Orissa; TN, Tamil Nadu; TZ, Tanzania; UG, Uganda; and Ke, Kenya.

**Table 3.** Population statistics of Indian States and East Africa countries.

| | Population* | No. of individuals | H | Ho | $A_T$ | No. rare alleles |
|---|---|---|---|---|---|---|
| Region | India | 209 | 0.550 | 0.175 | 42 | 19 |
| | East Africa | 168 | 0.228 | 0.103 | 31 | 14 |
| States | AP | 23 | 0.420 | 0.147 | 18 | 4 |
| | Grt | 29 | 0.452 | 0.102 | 23 | 10 |
| | MP | 40 | 0.559 | 0.131 | 23 | 6 |
| | Mrt | 35 | 0.485 | 0.222 | 30 | 12 |
| | Ors | 42 | 0.479 | 0.155 | 24 | 8 |
| | TN | 40 | 0.528 | 0.273 | 29 | 10 |
| Countries | TZ | 50 | 0.290 | 0.106 | 21 | 7 |
| | UG | 86 | 0.150 | 0.108 | 26 | 17 |
| | Ke | 32 | 0.215 | 0.083 | 17 | 4 |

*AP, Andhra Pradesh; Grt, Gujarat; MP, Madhya Pradesh; Mrt, Maharashtra; Ors, Orissa; TN, Tamil Nadu; TZ, Tanzania; UG, Uganda; and Ke, Kenya.
*H*, Nei,s unbiased estimate of gene diversity; *Ho*, observed heterozygosity; $A_T$, total number of alleles.

**Table 4.** Roger's modified genetic distances among states/countries.

| Subpopulation | Mart | Ors | TaN | AnP | MaP | Grt | Ke | Tan | Uga |
|---|---|---|---|---|---|---|---|---|---|
| Mart | - | | | | | | | | |
| Ors | 0.324 | - | | | | | | | |
| TaN | 0.263 | 0.194 | - | | | | | | |
| AnP | 0.250 | 0.237 | 0.209 | - | | | | | |
| MaP | 0.291 | 0.309 | 0.289 | 0.243 | - | | | | |
| Grt | 0.273 | 0.322 | 0.356 | 0.294 | 0.267 | - | | | |
| Ken | 0.324 | 0.402 | 0.359 | 0.358 | 0.439 | 0.467 | - | | |
| Tan | 0.347 | 0.424 | 0.386 | 0.385 | 0.469 | 0.493 | 0.088 | - | |
| Uga | 0.459 | 0.495 | 0.443 | 0.475 | 0.556 | 0.602 | 0.208 | 0.228 | - |

AP, Andhra Pradesh; Grt, Gujarat; MP, Madhya Pradesh; Mrt, Maharashtra; Ors, Orissa; TN, Tamil Nadu; TZ, Tanzania; UG, Uganda; and Ke, Kenya.

Kenya than those from Tanzania.
The Indian cluster was subdivided into two sub-clusters (Figure 1). The first sub-cluster grouped together accessions from Maharashtra, Andhra Pradesh, Tamil Nadu and Orissa. Within this sub-cluster, accessions from Tamil Nadu and Orissa were found to be most closely related. Germplasm from each of these States were more closely related to accessions from Andhra Pradesh than those from Andhra Pradesh were related to accessions from Maharashtra (Figure 1 and Table 4). Accessions from Maharashtra were the most isolated in the sub-cluster and had the highest number of unique alleles across all States. The second sub-cluster grouped together accessions from Madhya Pradesh and Gujarat.

## Gene diversity within accession

The average number of individuals per accession over all loci was 4.15 and only 17% of accessions did not show any variation within accession. The average percentage of polymorphic loci within an accession ranged from zero to 100 with an average of 47%. Eleven alleles were present in a single accession only (Table 5). Nei's unbiased estimate of gene diversity was 0.226 and average observed heterozygosity was 0.142 across all loci.

## Deviations from Hardy-Weinberg (HW) equilibrium

The Markov Chain Method was used to test for deviation from HW equilibrium using the difference between the observed heterozygosity (Ho) and Nei's unbiased estimate of gene diversity (*H*). Results showed a significant ($P \leq 0.05$) deviation from HW equilibrium at each locus when the entire data set was analyzed. The probability in each case was $P = 0$ with S.E = 0. The same situation was true when data from East African and Indian germplasm were

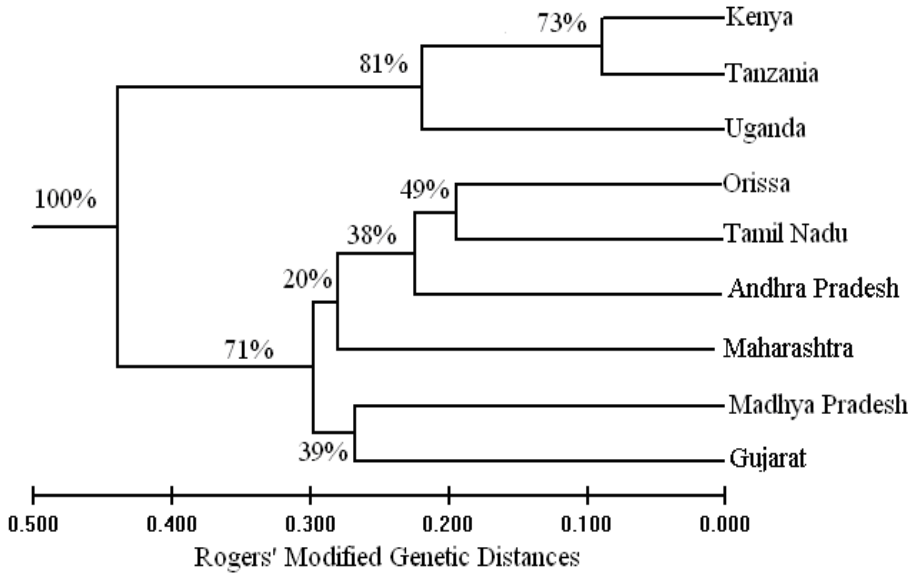


**Figure 1.** Dendrogram of 88 accessions of pigeonpea from India and East Africa according to state/country of origin. The percentage given on each node on the dendrogram represents the percentage of occurrence of the distinction through 1000 bootstrap iterations.

**Table 5.** Alleles present in a single accession only.

| Accession (ICP No.) | Alleles |
|---|---|
| 12007 | CCB2-165 |
| 12207 | CCB1-210 |
| 12209 | CCB4-216 |
| 12887 | CCB5-12887 |
| 12937 | CCB5-194, CCB5-195, CCB7-153 |
| 13683 | CCB1-206 |
| 14021 | CCB4-236 |
| 14009 | CCB9-168, CCB5-201 |

analyzed separately. When the data from various states/ countries were analyzed separately, non-significant differences from HW equilibrium were found in Andhra Pradesh at loci CCB 4 (P = 0.200, S.E ± 0.023), in Gujarat at loci CCB 1 (P = 0.103, S.E ± 0.009), in Kenya at loci CCB 5 (P = 1.00, S.E ± 0.000) and at locus CCB 9 (P = 1.00, S.E ± 0.000) and in Uganda at locus CCB 2 (P = 0.149, S.E. ± 0.032), at locus CCB 4 (P = 0.206, S.E ± 0.043) and at locus CCB 9 (P = 0.440, S.E. ± 0.067) (data not shown).

### Genetic differentiation among populations

Table 6 gives Wright's F statistics according to individual loci and averaged across loci.

Over all loci $\theta_P = 0.270$, $\theta_S = 0.336$, $\theta_{SS} = 0.557$, where P-populations are India and East Africa, S-subpopulations are states/countries and SS-subsubpopulations are within accessions. This shows maximum differentiation among accessions, followed by among states/countries and finally among regions. At all population subdivisions CCB4 made maximum contribution to differentiation, followed by CCB7 (Table 6). *f* values were high across all loci and ranged from 0.527 to 0.389 (Table 6).

## DISCUSSION

Pigeonpea improvement and conservation programs depend on the presence of genetic variability and the accurate characterization of that variability. Microsatellites

**Table 6.** F-statistics between different populations.

| Locus | F | θP or $F_{ST}$ | θS | θSS | *f* |
|---|---|---|---|---|---|
| CCB 1 | 0.636 | 0.049 | 0.090 | 0.380 | 0.413 |
| CCB 2 | 0.743 | 0.180 | 0.219 | 0.562 | 0.413 |
| CCB 4 | 0.823 | 0.507 | 0.565 | 0.710 | 0.389 |
| CCB 5 | 0.681 | 0.144 | 0.238 | 0.458 | 0.411 |
| CCB 7 | 0.7832 | 0.275 | 0.398 | 0.607 | 0.449 |
| CCB 9 | 0.731 | 0.189 | 0.226 | 0.431 | 0.527 |
| Over all loci | 0.750 | 0.270 | 0.336 | 0.557 | 0.437 |

θP or $F_{ST}$ is the degree of differentiation among India and East Africa, θS is the degree of differentiation among states/countries and θSS is the degree of differentiation within accessions. F is the inbreeding co-efficient of an individual relative to the entire population and *f* is the inbreeding coefficient of an individual relative to the sub-population.

provide a useful method of determining genetic variability at molecular level and have been shown to be informative in pigeonpea (Bramel et al., 2004b). There is no 'optimum' number of SSRs to use to elucidate genetic relationships. This will depend on the relatedness of the germplasm being studied and the informativeness of the markers used. In this study, six primers were used, the same number as used in a previous study (Bramel et al., 2004b). Five of them were common among the studies (CCB1, 2, 4, 5 and 9). Here we detected between six and eleven alleles per locus in 88 accessions with a total of 47 alleles, which is less than Buhariwalla and Crouch (2004) who detected between eleven and fifteen alleles per locus, but across a larger number of accessions (940) and a total of 80 alleles. It is worth noting that Yang et al. (2006) found cultivated pigeonpea to have little variation as revealed by DArT markers. From nearly 700 markers, only 64 markers detected variation in 48 accessions and 50% of these had one of the two markers present at low frequency (below 5%).

CCB 4 was the most informative marker with the highest PIC value and the largest number of alleles. PIC is influenced by both allele number and frequency, with alleles of more even frequency resulting in a higher PIC. In a previous study of pigeonpea in five districts of Andhra Pradesh, CCB 4 had one allele of high frequency with all other alleles being rare (Bramel et al., 2004b). In this study, in India, the most common allele (222) had a frequency of 0.56 and 10 other alleles had a frequency less than 0.13, with only two rare alleles. CCB7 had the second highest PIC value but amongst the lowest number of alleles. The high PIC resulted from the relatively even frequency of two common alleles in nearly all countries/states studied except Uganda, Madhya Pradesh and Gujurat (Table 2).

CCB1, 2 and 8 have been found to have very similar frequency for all alleles except for a few alleles of very rare frequency (Bramel et al., 2004b). In our study we found a similar situation in India, but a marked difference in East Africa for CCB1, CCB2, CCB5 and CCB9. In East Africa these loci were dominated by one allele of very high frequency, all other alleles having much lower frequency, whereas in India, in almost all cases there were two alleles of relatively high frequency. These alleles were the same in nearly all cases. This contributed towards the higher index of gene diversity in India as opposed to East Africa.

## Gene diversity within and among regions

This study reveals greater diversity in India as opposed to East Africa, according to the number of alleles detected, 42 and 31 respectively, the number of rare alleles, 19 and 14 respectively and Nei's unbiased estimate of gene diversity (*H*), 0.550 and 0.228 respectively. Like PIC, Nei's unbiased estimate of gene diversity is influenced by both allele number and frequency with alleles of more even frequency making a greater contribution to the diversity index than alleles of high or low frequency. This supports previous hypotheses that India is the primary centre of diversity, with East Africa being a secondary centre of diversity (Smartt, 1990). Pigeonpea is believed to have traveled from India to Malaysia then to East Africa (van der Maesen, 1990).

Within East Africa, differences in allele number are derived mainly from the number of rare alleles. Accessions from Uganda harbored the largest number of alleles and rare alleles but the very high and low frequency of most of these alleles resulted in the lowest diversity among East African countries in terms of *H* (Table 2). Tanzania had the highest value of *H* (0.29). The higher diversity in Tanzania than Kenya and Uganda may be due to the early introduction of pigeonpea to Tanzania and the relative importance of the crop in that country. The greater diversity in Tanzania and Kenya as

opposed to Uganda may also reflect the main trade routes between India and East Africa. Much of this trade is via sea, with pigeonpea seed frequently entering the region through coastal ports. The lower diversity of Uganda may on the other hand reflect a more recent introduction from Kenya and/or Tanzania and a further genetic bottleneck. Manyasa et al. (2008) found little variation for qualitative characters in pigeonpea from Tanzania, but substantial variation for agronomic traits and genotype x environment interaction. They identified two diversity clusters in Tanzania; coastal, eastern and southern and a second cluster of germplasm from the northern highlands.

Within India, *H* did not vary widely, but from 0.559 in Madhya Pradesh to 0.42 in Andhra Pradesh. Maharastra had the greatest diversity in terms of number of alleles, number of rare alleles and number of unique alleles. Data from this study indicate that there may be a centre of diversity for pigeonpea around the neighbouring states of Madhya Pradesh and Maharastra. This would have to be validated through a larger study. It seems that Andhra Pradesh may have lower diversity than other States, despite Andhra Pradesh being thought of as a center for pigeonpea domestication and where diversity is maintained in farmers fields (Bramel et al., 2004a), Replacement of pigeonpea landraces with other economic crops and erratic rainfalls have been found to be the probable cause for the loss of genetic diversity from the region (Bramel et al., 2004b).

**Genetic relationships among states/countries**

There is a clear distinction in genotypes from India as opposed to East Africa. This was evident in all iterations during bootstrapping (Figure 1). The differentiation was caused by differences in allele composition as well as frequency. Twenty-three alleles were common to both India and East Africa. Four alleles were found in East Africa, but not in India. All of these were rare alleles. Fifteen alleles were found in India but not in East Africa. Again, all but one of these was a rare allele. CCB4 was a major contributor to the discrimination between India and East Africa according to $F_{st}$ estimates (Table 6). Allele 220 of CCB4 was the most frequent in East Africa (0.88) but occurred at low frequency in all the Indian states studied. Allele 222 had a frequency of 0.56 in India but 0.05 in East Africa. CCB7 also caused some discrimination with the higher frequency of allele 154 in East Africa (0.68) than India (0.2) and a higher frequency of allele 156 in India (0.73) than in East Africa (0.29). The differentiation observed may be due to a reduced effective population size and genetic bottleneck effect when pigeonpea was first introduced to East Africa from India. This is evident from the reduced number of alleles and rare alleles. A smaller effective population size will tend to enhance the effects of random genetic drift, resulting in neutral alleles (that is, those that do not differ in their effects on survival or reproduction) becoming either lost or fixed more rapidly in a population. More rapid genetic drift exacerbates the loss of diversity and can result in increased divergence. The presence of rare alleles found only in Africa, and not in India may be a result of sample size or may reflect the generation of diversity through mutation subsequent to being introduced to Africa. This accumulation of novel variation suggests that germplasm in East Africa has been separated from that in India for a substantial amount of time.

In terms of the data, within East Africa, there are large differences between the results of Uganda from those of Kenya and Tanzania in the distribution of allele frequencies at CCB4 and CCB7, as well as allele composition (Table 2). Uganda is characterized by allele 220 at very high frequency (0.94) in CCB4, whereas both Kenya and Uganda have three alleles of more even distribution, although 220 is still the most frequent in each case. A similar situation occurs in CCB7 where the most frequent allele was different in Uganda than in Tanzania and Kenya (Table 2). Uganda and Tanzania each had two unique alleles. Allele frequencies in CCB4 and CCB7 suggest a more recent introduction of pigeonpea into Uganda, from either Kenya or Tanzania, resulting in a more recent further genetic bottleneck. This hypothesis is supported by the low levels of diversity in Uganda, possibly accentuated by random genetic drift caused by a reduction in effective population size through a relatively recent introduction. The fact that pigeonpea is traditionally introduced through sea ports in East Africa would also support the hypothesis of a more recent introduction to landlocked Uganda.

The relatively close relationship of Orissa, Andhra Pradesh and Tamil Nadu indicates geneflow among these adjoining eastern coastal states, and perhaps adaptation to environmental or cultural practices. The relatively close relationship of germplasm from Madhya Pradesh and neighboring Gujarat also indicated geneflow and/or adaptation. Interestingly, germplasm from Maharastra is more closely related to Andhra Pradesh and Tamil Nadu than to the more northern States of Madhya Pradesh and Gujurat. It is most distantly related to Orissa. The most common allele at each locus was consistent in all states except CCB4 in which the most common allele was 222 except in Maharastra where allele 228 had the highest frequency (0.42). Allele 228 had a relatively high frequency of 0.29 in Gujurat. This may indicate some gene flow between the neighbouring states of Maharastra and Gujarat. In CCB5, allele 193 was most common in Gujarat, while allele 195 was most common in all other States. In CCB9, allele 162 was most common in all states except Andhra Pradesh and Gujarat in which allele 160 was most common. Based on the Roger's Modified distances (Table 4) among the

accessions from India, the greatest genetic distance was observed between accessions from Gujurat and Tamil Nadu.

### Deviation from H-W Equilibrium

Significant departures from HW equilibrium were detected using the Markov-Chain, F and *f* statistics (0.75 and 0.437, respectively, over all loci) (Table 6). Departure from HW equilibrium indicates non-random mating, in this case high levels of inbreeding. This is expected from pigeonpea where significant levels of inbreeding have been observed (Ratnarpakhe et al., 1995). It is possible that levels of inbreeding in the germplasm used in this study are higher than in natural populations. All accessions used in the study were from the ICRISAT gene bank where two plants of the same accession are bagged together in the regeneration process to reduce cross pollination among accessions and increase pollination within accession, either self, or pollination among plants of the same accession, to maintain the integrity of accessions. In recent years, this has been done using cages (iron frames covered with polypropylene net). Non-significant deviation from Hardy Weinberg equilibrium was observed in quite a number of loci in the analysis of the accessions from Uganda. Collection of landraces from Uganda was in 1996 while for the rest of the states and countries; this was done much earlier (1976 - 1986). This further supports the breeding method overestimating inbreeding compared to field conditions.

### Within accession variation

In non-HW equilibrium populations, there is a deficit or excess of heterozygotes, which creates a correlation or non-independence among alleles. This correlation can occur between alleles within individual genotypes, within single populations, and among separate populations. The F statistics allow analysis of structures of sub-divided populations. $F_{ST}$ (or θ) is the degree of gene differentiation among populations in terms of allele frequency. $F_{IS}$ (or *f*) is the deficiency or excess of average heterozygotes in each population and provides an indication of average inbreeding within a sub-population. $F_{IT}$ (or F) is the deficiency or excess of average heterozygotes in a group of populations and provides an indication of average inbreeding within the whole population. Wright (1978) suggested the following qualitative indices for the interpretation of fixation indices; 0 - 0.05 indicates 'little' genetic differentiation, 0.05 - 0.15 indicates 'moderate' genetic differentiation, 0.15 - 0.25 indicates 'large' genetic differentiation while values greater than 0.25 indicate 'very large' genetic differentiation. In this study, values of θss indicate strong population structure at the accession level (Table 6). This is characteristic of a highly inbred population. Evidence of this population structure at the accession level also comes from unique alleles found within accessions.

In conclusion, the markers used were found to be highly informative in the germplasm studied. The most informative marker was found to be CCB 4 as observed by the high number of alleles, PIC value and Nei's unbiased estimate of gene diversity observed at this locus. Higher genetic diversity was observed in India than in East Africa. This is indicated by the higher number of alleles, the number of rare alleles, values of Nei's unbiased estimate of gene diversity and observed heterozygosity in India compared to East Africa. This supports the theory of India as the primary center of diversity of pigeonpea. Among the states and countries, the greatest amount of genetic diversity was observed in Maharashtra and Tamil Nadu and not Andhra Pradesh as earlier suggested by other studies. There is therefore need for further studies to identify the state of highest diversity and possible origin of pigeonpea. The genetic relationships indicate that there is a difference between the accessions from India and East Africa suggesting an evolutionary adaptation of accessions from the two regions. The accessions from Uganda were found to be less closely related than those from Kenya and Tanzania, suggesting an inland adaptation as opposed to a coastal adaptation. The inbreeding nature of pigeonpea and method of regeneration by 'bagging' were found to be the probable cause of heterozygote deficiencies in the populations studied. These would explain the observation of the greatest differentiation within accessions.

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