Chapter 9

MOLECULAR CHARACTERIZATION OF INTER- AND INTRA-SPECIFIC GENETIC VARIATION IN ABELMOSCHUS SPECIES USING SINGLE PRIMER AMPLIFICATION REACTION (SPAR) METHOD

Understanding the diversity and phylogeny of a plant species or genus is of great significance, primarily because of its relevance to the existence and adaptation to environment, in which it grows. The existence of variations in a population especially, at the molecular level is important in determining the opportunities to improve them through selection of existing variations or creating new mutations (Barrett and Schluter, 2008) in traits related to quality (Fitzgerald et al., 2009) and disease control (Zhu et al., 2000). Moreover, molecular level variations within a plant population are considered to represent its evolutionary potential of the constituent taxa. Genetic variation is detrimental for conservation strategies at species level (Holsinger et al., 1999) and therefore assessment of genetic variability using molecular marker should be the first step in evaluating the long-term conservation aspects of species in natural environmental conditions (González-Astorga and Castillo-Campos, 2004).

The DNA based molecular markers, which screen both nuclear and organellar genomes with precision, have acted as versatile tools for the fast and unambiguous genetic analysis and are in vogue, not only to characterize population genetic structure but also to understand the innate mechanism of speciation and evolution of constituent taxa. PCR based DNA markers like RAPD (Mirali and Nabulsi, 2003; Zhang et al., 2005), ISSR (Mondal, 2002; Wang et al., 2004), AFLP (Rottenberg and
Parker, 2003), SSR (Rossetto et al., 2004) and DAMD (Heath et al., 1993) are some of the most efficient and cost effective markers, that are routinely used by plant breeders (Mohan et al., 1997; Gupta et al., 1999). In recent years, the PCR-based single primer amplification reaction (SPAR) methods are gaining significance as effective tool for genetic diversity analysis in plants and they collectively provide a comprehensive account of the nature and extent of the diversity (Bhattacharya et al., 2005; Ranade et al., 2009; Sharma et al., 2010).

The representative material constituting both the wild and cultivated species can be analyzed by multiple DNA markers in order to draw valid conclusions with regard to the genetic diversity, to identify the gene pool (s) of high and low diversity within each taxon, and lastly to examine to what extent these multilocus DNA markers will be helpful in resolving the taxonomic riddles and (or) be helpful in reconstructing phylogenetic relationships. Molecular markers also help in the assessment of genetic variation and in elucidation of genetic relationships within and among species (Chakravarthi and Naravaneni, 2006). To conserve and use these plant genetic resources effectively, it is essential to develop markers that not only distinguish individuals and accessions, but also reflect the inherent diversity and relationships among collection holdings (Kresovich and McFerson, 1992).

DNA sequence information of okra is limited. Very few reports dealing with analysis of genetic variation in Abelmoschus species, based mostly on RAPD methods (Martinello et al., 2001; Aladele et al., 2008; Saifullah and Rabbani, 2008; Saifullah et al., 2010; Nwangburuka et al., 2011; Prakash et al., 2011), SRAP (Gulsen et al., 2007) and SSR (Sawadogo et al., 2009) are on hand. Most of them dealt with limited use of only one marker system to assess intra-specific genetic
variation and have been studied only on okra i.e. *A. esculentus*. Collecting DNA marker data to determine whether phenotypically similar cultivars are genetically similar too, would be of great interest in okra breeding programme (Duzyaman, 2005).

For the present investigation, three wild relative species of *Abelmoschus* viz., *A. tetraphyllus*, *A. moschatus* ssp. *moschatus* and *A. angulosus* var. *grandiflorus* have been used along with the cultivated species okra, *A. esculentus*. This is the first attempt to analyse these wild species, which are more adaptive and more tolerance to major diseases of okra like YMV, okra leaf curl disease, *Fusarium* wilt etc. (Charrier, 1984; Samarajeewa and Rathnayaka, 2004). Characterizing the germplasm for resistance or tolerance to these diseases through DNA marker based approaches will help in the improvement programme for a better knowledge of the organization of the genus *Abelmoschus*.

Hence the objectives of the present study was to determine the level of genetic diversity and the patterns of genetic variation and differentiation within and among the populations of *Abelmoschus* viz., *A. tetraphyllus*, *A. moschatus* ssp. *moschatus*, *A. angulosus* var. *grandiflorus* and *A. esculentus* based on RAPD, ISSR and DAMD markers and the cumulative approach SPAR. The present study is the first attempt to make use of these markers which has proved to be highly effective in discriminating various genotypes. Thus the study will enable to exploit these genotypes for effective crossing between them so as to obtain desirable segregates for further identification of superior lines, in early stages of crop growth. Genetic distance calculated from molecular marker data will help to identify genotypes for
mapping populations and also to identify molecular markers linked to desirable traits (resistance to YVMV) by marker assisted selection (MAS).

9.1. Observations:

The germplasm used for present studies in analysis of genetic diversity is summarised in Table 17. Datasets have been generated from three SPAR methods as referred above (RAPD, ISSR and DAMD), individually and later cumulatively, for detection of polymorphism and clustering pattern among them. The data has been summarised in Table 18-25 and illustrated in Figs. 637-692.

9.1.1. Analysis of inter-specific natural genetic variation using RAPD

22 RAPD primers yielded a total of 349 amplification products with 15.86 amplicons primer on average. Of these 349, 335 were polymorphic amplicons with an average of 15.23 bands per primer among the 43 accessions belonging to four species consisting of both wild and cultivated *Abelmoschus* species. The percentage polymorphism ranged from 78.95% to 100% with overall 95.93% polymorphism. Primer OPX-15 produced the highest of 20 amplicons while the lowest of 12 amplicons was produced by OPA-4 and OPX-7. The highest polymorphic information content (PIC) value of 0.3972 was produced by OPC-19, while the lowest of 0.2397 by OPX-01 with an average of 0.3271. The highest Resolving power (Rp) value of 0.6295 was produced by OPC-11 while the lowest of 0.3540 by OPX-1 with an average of 0.4793. The effective multiplex ratio (EMR) was recorded the highest of 17 by OPA-18, OPC-13, OPC-19 and OPX-05 while the lowest of 10.0833 was produced by OPA-4 with an average of 14.6876. Likewise, the highest Marker Index (MI) value of 6.7524 was produced by OPC-19 and the lowest of 3.0624 by OPX-03 with an average value of 4.8259. Figs. 637-656 and Table 18 are
illustrative of the extent of polymorphism observed among the 43 genotypes belonging to four species of *Abelmoschus*.

9.1.2. Analysis of intra-specific natural genetic variation using RAPD

*Abelmoschus* species *viz.*, *A. tetraphyllus* (var. *tetraphyllus* and var. *pungens*) *A. moschatus* ssp. *moschatus*, *A. angulosus* var. *grandiflorus* and *A. esculentus* were also analysed at intra-specific level (Table 19). For each group, amplification products were also scored and compared individually to determine degree of natural variation in terms of polymorphic loci yielded through 22 RAPD primers.

9.1.2.1. *A. tetraphyllus*

At intra-specific level among the genotypes of *A. tetraphyllus* var. *tetraphyllus* and *A. tetraphyllus* var. *pungens*, 22 RAPD primers generated 306 amplificons with an average of 13.90 bands per primer (Table 19). 269 amplificons were polymorphic with an average of 12.23 bands per primer giving 87.90% polymorphism. The PIC and Rp values were 0.2760 and 0.2634 respectively. The EMR and MI were 10.97 and 3.1728 respectively. Jaccard’s similarity coefficient ranged from 0.29-0.96 with the average similarity of 0.61.

9.1.2.2. *A. moschatus* ssp. *moschatus*

Totally, 281 amplicons were generated in ten accessions of *A. moschatus* ssp. *moschatus* (Table 19). Out of which, 197 were polymorphic with 8.95 polymorphic amplicons per primers. The percentage polymorphism within the accessions was 70.11%. The values of PIC and Rp were 0.2313 and 0.2459 respectively. The EMR and MI was 6.6768 and 1.8273 respectively. Jaccard’s similarity coefficient ranged from 0.48-0.94 with the average similarity of 0.71.
9.1.2.3. *A. angulosus var. grandiflorus*

The total number of amplification products generated with 22 RAPD primers among the different genotypes of *A. angulosus var. grandiflorus* was 207 (Table 19). Of these total amplification products, 82 was polymorphic with only 3.73 polymorphic amplicons per primers. The percentage polymorphism observed was 39.61. The PIC and Rp values were 0.2658 and 0.2608 respectively. The EMR and MI was 2.2120 and 0.6738 respectively. Jaccard’s similarity coefficient ranged from 0.71-0.92 with an average similarity index of 0.82.

9.1.2.4. *A. esculentus*

Of the total 239 amplification products obtained from 22 RAPD primers, 148 amplification products were polymorphic with an average of 6.72 polymorphic bands per primers giving 61.92% polymorphism (Table 19). The PIC and Rp values were 0.9980 and 0.2895 respectively. The EMR and MI was 4.7559 and 4.7292 respectively. Jaccard’s similarity coefficient ranged from 0.62-0.93 with an average similarity index of 0.78.

9.1.3. Analysis of inter-specific natural genetic variation using ISSR

20 ISSR primers produced a total of 209 amplification products, out of which 197 bands were polymorphic with an average of 9.85 polymorphic bands per primer. Percentage of polymorphic bands ranged from 33.33% to 100% giving an average of 93.04% polymorphism among 43 accessions of *Abelmoschus* belonging to four species (Table 20 and Figs. 657-676). Primer 818, 868 and 880 yielded maximum number of 14 polymorphic bands, while a minimum of four polymorphic bands were produced by 841. The lowest PIC value was 0.1347 produced by 842 primer while the highest polymorphic information content value was 0.4238 produced by 862.
primer with an average value of 0.3030. The Rp value was observed lowest of 0.1654 in 842 primer while the highest of 0.5840 by 840 primer with an average value of 0.4244. The EMR was recorded the highest of 15 by 836 while the lowest of 1 was produced by 842 with an average value of 9.4862. Likewise, the highest MI value of 6.6565 was produced by 836 and the lowest of 1.0995 by 841 with an average value of 2.9233.

**9.1.4. Analysis of intra-specific natural genetic variation using ISSR**

*Abelmoschus* species *viz.*, *A. tetraphyllus* var. *tetraphyllus*, *A. tetraphyllus* var. *pungens*, *A. moschatus* ssp. *moschatus*, *A. angulosus* var. *grandiflorus* and *A. esculentus* were also analysed at intra-specific level (Table 21). For each group, amplification products were also scored and compared individually to determine degree of natural variation within the species/taxa in terms of polymorphic loci yielded through 20 ISSR primers.

**9.1.4.1. A. tetraphyllus**

For intra-specific level variation, the genotypes of *A. tetraphyllus* var. *tetraphyllus* and *A. tetraphyllus* var. *pungens* were evaluated. 20 ISSR primers revealed 166 amplicons in 20 genotypes of *A. tetraphyllus* var. *tetraphyllus* and two accessions of *A. tetraphyllus* var. *pungens* of which 134 were polymorphic with an average of 6.70 amplicons per primer, giving 80.72% polymorphism (Table 21). The PIC and Rp value was 0.2761 and 0.2984 respectively. The EMR and MI value was 6.1556 and 1.8442 respectively. Jaccard’s similarity coefficient ranged from 0.50-0.92 with an average similarity index of 0.71.
9.1.4.2. *A. moschatus* ssp. *moschatus*

A total of 154 amplification products were revealed by 20 ISSR primers, of which 113 polymorphic amplification products were produced for ten accessions of *A. moschatus* ssp. *moschatus* with an average value of 5.65 per primer, giving 73.38% polymorphism (Table 21). The PIC and Rp values were evaluated as 0.2408 and 0.3041 respectively. Similarly, the EMR and MI value was recorded as 4.5907 and 1.4117 respectively. Jaccard’s similarity coefficient ranged from 0.53-0.90 with an average similarity index of 0.72.

9.1.4.3. *A. angulosus* var. *grandiflorus*

Of 135 amplification bands revealed in five accessions of *A. angulosus* var. *grandiflorus* by 20 ISSR primers, 52 bands were polymorphic with an average of 2.60 per primer, giving only 38.52% polymorphism. The PIC and Rp values were 0.1316 and 0.1767 respectively (Table 21). While 1.4387 and 0.3319 were the values recorded for EMR and MI respectively. Jaccard’s similarity coefficient ranged from 0.79-0.86 with an average similarity index of 0.83.

9.1.4.4. *A. esculentus*

Out of 142 amplification bands produced by 20 ISSR primers in six accessions of *A. esculentus*, 70 bands were polymorphic with an average of 3.50 per primer, giving 49.30% polymorphism. The PIC and Rp values were 0.1585 and 0.2325 respectively. Similarly, the EMR and MI value was recorded as 0.3298 and 0.5970 respectively. Jaccard’s similarity coefficient ranged from 0.68-0.86 with an average similarity index of 0.77 (Table 21).
9.1.5. Analysis of inter-specific natural genetic variation using DAMD

18 DAMD primers produced 255 distinct, clearly visible amplicons of which 236 amplicons were polymorphic and were used for the genetic relationship analysis. The lowest number of polymorphic amplicons only six was generated by DAMD 5 primer while the highest of 18 was scored by DAMD 6 primer with an average of 13.11 per primer. The percentage polymorphism ranged from 60% to 100% showing 91.55% polymorphism (Table 22). Primer DAMD 16 revealed the highest PIC value of 0.4125 while DAMD 2 gave the lowest value of 0.2645 with an average value of 0.3575. The highest Rp value of 0.5752 was produced by DAMD 14 while the lowest Rp value of 0.2869 produced by DAMD 20 giving an average of 0.4679. The highest value of EMR was 18 produced by DAMD 1 and the lowest of 3.60 was produced by DAMD 5 with an average value of 12.2750. In case of MI, 6.8408 were recorded as the highest by DAMD 14 and the lowest of 1.2251 was produced by DAMD 5 with an average value of 4.3655 (Figs. 677-692).

9.1.6. Analysis of intra-specific natural genetic variation using DAMD

The intra-specific genetic diversity within four Abelmoschus species viz., A. tetraphyllus (var. tetraphyllus and var. pungens), A. moschatus ssp. moschatus, A. angulosus var. grandiflorus and A. esculentus has been summarised in Table 23.

9.1.6.1. A. tetraphyllus

For intra-specific level variation, the genotypes of A. tetraphyllus var. tetraphyllus and A. tetraphyllus var. pungens were evaluated. A total of 229 amplicons was generated using 18 DAMD primers within 20 genotypes of A. tetraphyllus var. tetraphyllus and two accessions of A. tetraphyllus var. pungens. Of these, 190 were polymorphic with an average of 10.56 bands per primer giving
82.97% polymorphism. The PIC and Rp values were 0.2754 and 0.2902 respectively (Table 23). The EMR and MI value was 12.7222 and 3.5403 respectively. Jaccard’s similarity coefficient ranged from 0.44-0.90 with an average similarity index of 0.67.

9.1.6.2. A. moschatus ssp. moschatus

Within the ten accessions of A. moschatus ssp. moschatus, 18 DAMD primers revealed a total of 217 ampicons of which 151 were polymorphic with an average of 8.39 ampicons per primer giving 69.59% polymorphism. The PIC and Rp values were 0.2427 and 0.2582 respectively. The EMR and MI value was 6.3650 and 1.7972 respectively (Table 23). Jaccard’s similarity coefficient ranged from 0.55-0.93 with an average similarity index of 0.74.

9.1.6.3. A. angulosus var. grandiflorus

Out of 162 amplification bands produced by 18 DAMD primers in five accessions of A. angulosus var. grandiflorus, 46 amplification bands were found to be polymorphic with an average of 2.56 bands per primer giving 28.40% polymorphism. The PIC and Rp values were 0.1212 and 0.1587 respectively. The EMR and MI value was 1.0072 and 0.2069 respectively. Jaccard’s similarity coefficient ranged from 0.83-0.89 with the average similarity of 0.86 (Table 23).

9.1.6.4. A. esculentus

In six accessions of A. esculentus using 18 DAMD primers, a total of 187 ampicons were produced of which 97 were found to be polymorphic with an average of 5.39 ampicons per primer giving 51.87% polymorphism. The PIC and Rp values observed were 0.1839 and 0.2442 respectively. The EMR and MI value
was 3.4671 and 0.9084 respectively. Jaccard’s similarity coefficient ranged from 0.67-0.92 with an average similarity index of 0.80 (Table 23).

9.1.7. Cumulative analysis of polymorphism using RAPD, ISSR and DAMD at inter-specific level

A total of 813 amplification products using 60 primers of viz., RAPD, ISSR and DAMD were scored, of which 768 were polymorphic amplification products exhibiting an overall 94.46% polymorphism and on average 12.80 polymorphic amplicons among the 43 taxa presently studied (Table 24). To determine the efficiency of the three SPAR methods, certain parameters such as EMR, PIC, Rp and MI were separately calculated for each set of primers used. The RAPD revealed the highest EMR (14.6876), Rp (0.4793) and MI (4.8259) while DAMD revealed the highest PIC (0.3575) among the three methods. The cophenetic values were calculated for the same pairs followed by matrix comparison using MxComp programme of NTSYS-pc 2.02k software to obtain the “r” values of RAPD+ISSR, RAPD+DAMD and ISSR+DAMD were recorded as 0.9018, 0.9562 and 0.9180 respectively.

9.1.8. Cumulative analysis of polymorphism using RAPD, ISSR and DAMD at intra-specific level

The extent of polymorphism at intra-specific level as revealed by the cumulative datasets of three SPAR markers within A. tetraphyllus viz., var. tetraphyllus and var. pungens, A. moschatus ssp. moschatus, A. angulosus var. grandiflorus and A. esculentus has been summarized in Table 25 and elaborated species-wise in the following:
9.1.8.1. *A. tetraphyllus*

The three SPAR methods collectively revealed 701 amplicons within the taxa of *A. tetraphyllus* var. *tetraphyllus* and *A. tetraphyllus* var. *pungens* out of which 593 amplicons were polymorphic generating 9.88 amplicons per primer. The overall percentage polymorphisms in both the varieties were 76.89%. The EMR and PIC in these taxa were 8.7824 and 0.2762 respectively. The Rp values were calculated as 0.2801 while MI was 2.5825 (Table 25). Jaccard’s similarity coefficient ranged from 0.39-0.92 with the average similarity of 0.66.

9.1.8.2. *A. moschatus* ssp. *moschatus*

A total of 652 ampicons were generated by the three SPAR methods in the genotype *A. moschatus* ssp. *moschatus*. Of these, which 461 were polymorphic with an average of 7.68 amplicons per primer giving 70.71% polymorphism. The PIC and Rp values were 0.2459 and 0.2748 respectively. The EMR and MI value was 5.8879 and 1.6798 respectively (Table 25). Jaccard’s similarity coefficient ranged from 0.51-0.89 with the average similarity of 0.70.

9.1.8.3. *A. angulosus* var. *grandiflorus*

The total number of amplicons generated through the collective dataset of RAPD, ISSR and DAMD markers among genotypes of *A. angulosus* var. *grandiflorus* were 504, of these, 180 amplicons were polymorphic with an average of 3.0 bands per primer giving 35.71% polymorphism. The PIC and Rp values were 0.1515 and 0.2040 respectively. The EMR and MI values were 1.5928 and 0.4198 respectively. Jaccard’s similarity coefficient ranged from 0.70-0.90 with the average similarity of 0.80 (Table 25).
9.1.8.4. *A. esculentus*

The three SPAR methods collectively revealed 568 amplicons, out of which, 315 were polymorphic with an average of 5.25 amplicons per primer giving 55.45% polymorphism. The PIC and Rp values observed were 0.2038 and 0.2754 respectively. The EMR and MI value was 3.5445 and 2.2008 respectively. Jaccard’s similarity coefficient ranged from 0.67-0.90 with the average similarity of 0.79 (Table 25).

9.1.9. Clustering pattern of different SPAR methods

9.1.9.1. RAPD

Based on pair-wise similarity matrices generated by Jaccard’s coefficient of similarity (Jaccard, 1908) using the SIMQUAL format of NTSYSpc (Rohlf, 1998), a dendrogram was constructed by using UPGMA with the SAHN module of NTSYSpc to show a phenetic representation of genetic relationships as revealed by the similarity coefficient (Sneath and Sokal, 1973). The dendrogram constructed for 43 genotypes belonging to *Abelmoschus* revealed four major clusters as I, II, III, IV and an additional distinct out-group as V belonging to *A. tetraphyllus* var. *tetraphyllus* (AT-2) collected from Nepal (Fig. 693). Cluster I were representatives of *A. tetraphyllus* consisting of 22 accessions i.e. AT-1 to AT-22 with its two varieties viz., *tetraphyllus* and *pungens* respectively, which can be further resolved into two sub-clusters. Sub-cluster IA includes 18 of *A. tetraphyllus* var. *tetraphyllus* accessions of which seven are from Maharashtra (AT-3, AT-4, AT-8, AT-10, AT-18, AT-19, AT-20), four from Rajasthan (AT-9, AT-11, AT-13, AT-14), two each from Uttar Pradesh (AT-6, AT-15) and Madhya Pradesh (AT-12, AT-16), and one each from Goa (AT-7), Gujarat (AT-1) and Tamil Nadu (AT-13). Notably, genotypes AT-7 and
AT-8 showed identical values of genetic distance with highest genetic similarity. While sub-cluster IB comprises of three accessions, of which one accession of *A. tetraphyllus* var. *tetraphyllus* collected from Madhya Pradesh (AT-17) while the other two accessions comprises of *A. tetraphyllus* var. *pungens* collected from Mizoram (AT-21, AT-22). Cluster II includes five accessions which all were representatives of *A. angulosus* var. *grandiflorus*, i.e. AA-33 to AA-37 collected entirely from Kerala. Cluster III comprises of six accessions which are representatives of cultivar variety Pusa Sawani of *A. esculentus* (AE-38 to AE-43). The fourth cluster IV includes 10 accessions which are representative of *A. moschatus* ssp. *moschatus* (AM-23 to AM-32) which consists of three accessions each from Maharashtra (AT-29, AT-30, AT-31) and Madhya Pradesh (AT-26, A-27, AT-28), two from Kerala (AT-24, AT-25), one accession each from Goa (AT-23) and Karnataka (AT-32).

**9.1.9.2. ISSR**

The UPGMA dendrogram was constructed by using the cumulative data of 20 ISSRs to analyze the genetic similarity in all the 43 genotypes of four *Abelmoschus* species using Jaccard’s coefficients. The tree clearly shows well-separated cluster groups according to their respective species. The tree had, at least four major clusters marked I, II, III and IV with large parenthesis (Fig. 694). Cluster I were representatives of *A. tetraphyllus* consisting of 21 accessions i.e. AT-1 to AT-22 with its two varieties *viz*., *tetraphyllus* and *pungens*, which can be further resolved into two sub-clusters. Sub-cluster IA includes 17 accessions of *A. tetraphyllus* var. *tetraphyllus* of which seven are from Maharashtra (AT-3, AT-4, AT-8, AT-10, AT-18, AT-19, AT-20), four from Rajasthan (AT-9, AT-11, AT-13, AT-14), three from
Madhya Pradesh (AT-12, AT-16, AT-17), two from Uttar Pradesh (AT-6, AT-15) and one each from Goa (AT-7), Gujarat (AT-1) and Tamil Nadu (AT-13). Notably, genotypes AT-5 and AT-6 showed identical values of genetic distance with highest genetic similarity. Sub-cluster IB comprises of two accessions of *A. tetraphyllus* var. *pungens* (AT-21 to AT-22) collected from Mizoram. Cluster II includes five accessions which all were representatives of *A. angulosus* var. *grandiflorus*, i.e. AA-33 to AA-37 collected entirely from Kerala. Cluster III comprises of 11 accessions, of which 10 are representatives of *A. moschatus* ssp. *moschatus* (AM-23 to AM-32) which consists of three accessions each from Maharashtra (AT-29, AT-30, AT-31) and Madhya Pradesh (AT-26, A-27, AT-28), two from Kerala (AT-24, AT-25), one accession each from Goa (AT-23) and Karnataka (AT-32), and one accession of *A. tetraphyllus* var. *tetraphyllus* (AT-2) collected from Nepal. The cluster IV includes six cultivar varieties Pusa Sawani of *A. esculentus* (AE-38 to AE-43).

9.1.9.3. DAMD

The UPGMA dendrogram constructed by using the cumulative dataset of 18 DAMD primers showed four major clusters marked as I, II, III and IV with large parenthesis and one distinct out-group as V belonging to *A. tetraphyllus* var. *tetraphyllus* collected from Nepal (Fig. 695). Cluster I were representatives of *A. tetraphyllus* consisting of 21 accessions i.e. AT-1 to AT-22 with its two varieties *viz.*, *tetraphyllus* and *pungens* respectively, which can be further resolved into two sub-clusters. Sub-cluster IA includes 20 accessions of *A. tetraphyllus* var. *tetraphyllus* of which seven are from Maharashtra (AT-3, AT-4, AT-8, AT-10, AT-18, AT-19, AT-20), four from Rajasthan (AT-9, AT-11, AT-13, AT-14), two each from Uttar Pradesh (AT-6, AT-15), Madhya Pradesh (AT-12, AT-16) and Mizoram (AT-21,
AT-22), and one each from Goa (AT-7), Gujarat (AT-1) and Tamil Nadu (AT-13). Sub-cluster IB comprises of only one accession of *A. tetraphyllus var. tetraphyllus* collected from Madhya Pradesh (AT-17). Cluster II includes five accessions which all were representatives of *A. angulosus var. grandiflorus*, i.e. AA-33 to AA-37 collected entirely from Kerala. Cluster III comprises of 10 accessions which are representative of *A. moschatus ssp. moschatus* (AM-23 to AM-32) which consists of three accessions each from Maharashtra (AT-29, AT-30, AT-31) and Madhya Pradesh (AT-26, A-27, AT-28), two from Kerala (AT-24, AT-25), one accession each from Goa (AT-23) and Karnataka (AT-32). Cluster IV includes six accessions which are representatives of cultivar variety Pusa Sawani of *A. esculentus* (AE-38 to AE-43). Notably, genotypes AE-39 and AE-41 showed identical values of genetic distance.

9.1.10. Cumulative dataset of RAPD, ISSR and DAMD

The UPGMA dendogram was constructed by using the cumulative data of three SPAR methods to analyze the genetic distance in all the samples using Jaccard's (1901) coefficients of similarity, respectively. Remarkably, the dendrogram generated through both the methods had at least four major clusters marked as I, II, III and IV with large parenthesis one distinct out-group as V belonging to *A. tetraphyllus var. tetraphyllus* collected from Nepal (Fig. 696). Cluster I were representatives of *A. tetraphyllus* consisting of 18 accessions i.e. AT-1 to AT-22 with its two varieties *viz.*, *tetraphyllus* and *pungens* respectively, which can be further resolved into two sub-clusters. Sub-cluster IA includes 18 accessions of *A. tetraphyllus var. tetraphyllus* which seven are from Maharashtra (AT-3, AT-4, AT-8, AT-10, AT-18, AT-19, AT-20), four from Rajasthan (AT-9, AT-11, AT-13, AT-14),
two each from Uttar Pradesh (AT-6, AT-15), and Madhya Pradesh (AT-12, AT-16) and one each from Goa (AT-7), Gujarat (AT-1) and Tamil Nadu (AT-13). Notably, genotypes AT-7 and AT-8 showed identical values of genetic distance with highest genetic similarity. Sub-cluster IB comprises three accessions, of which one accession (AT-17) belongs to *A. tetraphyllus* var. *tetraphyllus* collected from Madhya Pradesh and two accessions (AT-21, AT-22) of *A. tetraphyllus* var. *pungens* collected from Mizoram. Cluster II includes five accessions which all were representatives of *A. angulosus* var. *grandiflorus*, i.e. AA-33 to AA-37 collected entirely from Kerala. Cluster III comprises of six accessions which are representatives of cultivar variety Pusa Sawani of *A. esculentus* (AE-38 to AE-43). Cluster IV consists of ten accessions which are representative of *A. moschatus* ssp. *moschatus* (AM-23 to AM-32) which consists of three accessions each from Maharashtra (AT-29, AT-30, AT-31) and Madhya Pradesh (AT-26, A-27, AT-28), two from Kerala (AT-24, AT-25), one accession each from Goa (AT-23) and Karnataka (AT-32).

9.2. Discussion:

A large number of DNA markers like RAPDs, ISSRs, SSRs, DAMDs and AFLPs have been routinely and successfully used to measure genetic relationships and for estimating genetic variations (Santalla *et al.*, 1998) both at inter- and intraspecific level (Graner *et al.*, 2004). These markers have proved to be useful for identification of cultivars for conservation genetic resources to achieve crop improvement through hybridization programs (Graner *et al.*, 2004). Therefore, the selection of trait specific DNA marker is important and critically depends on the intended use (Gupta *et al.*, 2002).
RAPDs have been described as the generic precursors of SPAR since it is a powerful and extensively used DNA marker and is considered as unbiased and neutral markers for genetic mapping (Michelmore et al., 1991), population genetics (Haig et al., 1994), taxonomy (Abo-Elwafa et al., 1995) and detecting inter- and intra-species variation (Adams and Demeke, 1993; Millan et al., 1996; Goswami and Ranade, 1999; Mailer et al., 1994; Zhang et al., 1998, Rodrigue et al., 1999; Xu et al., 2000) and allows the examination of genomic variation among the target plant population without prior knowledge of DNA sequences (Hadrys et al., 1992). Thus they help in revealing variation in species with low genetic variability (Dawson et al., 1993; Bowditch et al., 1994). The information based on this DNA marker would be of great interest in okra breeding programme. It also helps in generating data to determine whether phenotypically similar cultivars are genetically similar or not (Duzyaman, 2005).

In the present investigation, it is amply clear that 22 RAPD profiles produced 349 amplicons of which 335 were polymorphic giving high percentage of 93.95% polymorphism across the 43 genotypes belonging to four species of Abelmoschus. The cluster analysis plotted using Jaccard’s coefficient could easily distinguish the differences and similarities among 43 genotypes based on their randomness in their genetic make-up as well as in somatic chromosome counts by clustering them into four distinct clusters, each cluster representing the respective species. Only one accession belonging to A. tetraphyllus var. tetraphyllus (AT-2) behaved almost as an out-group, which can be visibly noted, not only from its gel profile but also from its origin of collection site i.e. Nepal. The true basal species A. moschatus ssp. moschatus has been clustered from the rest, while the other A. angulosus var.
*grandiflorus* is showed its close affinity towards *A. tetraphyllus* var. *tetraphyllus*. *A. esculentus*, considered to be derived of cross between *A. tuberculatus* and *A. ficulneus* consisting of six accession of the cultivated variety Pusa Sawani had clustered separately showing its close affinity towards *A. tetraphyllus* and *A. angulosus* var. *grandiflorus*. Such observations have already been recorded in their crossability studies (see chapter 8) which showed their genome compatibility with successful cross between *A. esculentus* (♂) x *A. tetraphyllus* var. *tetraphyllus* (♀) and *A. esculentus* (♂) x *A. angulosus* var. *grandiflorus* (♀). It is interesting to note that such studies are important for transferring the resistance of YVMV from wild, *A. tetraphyllus* var. *tetraphyllus* to the cultivated okra species.

At the intra-specific level, high percentage polymorphism was recorded as 87.90% in *A. tetraphyllus* while the lowest recorded was 39.61% in *A. angulosus* var. *grandiflorus* using 22 primers of RAPD. This high level of polymorphism in *A. tetraphyllus* can also be recorded from its lowest genetic similarity of 0.61 suggesting that there were specific genetic variations amongst the accessions, showing high level of polymorphism. AT-2 showed its distinctiveness by clustering separately as it was collected from a different geographical location i.e. Nepal. Since RAPD primers were able to amplify more than one band per accession, residual heterogenicity within the accessions is apparent. The molecular dendrogram reveals that the accessions with the highest level of relatedness were AT-7 and AT-8 with over 90% level of relatedness. This suggest that both the accessions must have similar ancestral decent and may have similar genes and therefore similar origin. It further also implies that crosses between such accessions may not be recommended, since it may not produce much difference. Information on genetic relatedness among
genetic resources of crop plants is useful not only for breeding purposes but also for the conservation of germplasm (Torkpo *et al*., 2006), which is ratified by the report of Ogunbayo *et al*., (2005). Among the five accessions, one accession (AA-37) of *A. angulosus* var. *grandiflorus* clustered separately from the rest. All these accessions were collected from different places of Kerala and they had the same somatic chromosome number of 2n=66 and also the least genetic diversity (Table 19). While the stable chromosome number of 2n=130 in all of six accessions of *A. esculentus*, also inferred low genetic diversity and highest genetic similarity of 0.78 in all Pusa Sawani cultivar. Among *A. moschatus* ssp. *moschatus*, AM-30 collected from Maharashtra clustered separately from the rest and AM-25 and AM-26, AM-27 and AM-28 had shown the least genetic distance. Amplification of large number of polymorphic bands indicated that the primer sets used in this study could be of significance for the assessment of genetic diversity in *Abelmoschus* genotypes. Dendrograms did not show any significant pattern of clustering according to the locations from where genotypes were collected, indicating little or no location specificity among *Abelmoschus* genotypes. Similar results were reported in Azuki bean (Yee *et al*., 1999), Groundnut (Dwivedi *et al*., 2001) and Castor (Gajera *et al*., 2010).

Inter-simple sequence repeats (ISSR) is a PCR-based method developed by Zietkiewicz *et al*., (1994) which does not require prior knowledge of the genome, since SSRs are abundantly present in the plant genome in many copies of varying repeat units (Langercrantz *et al*., 1993). ISSRs segregate mostly as dominant marker following simple Mendelian inheritance (Reddy *et al*., 2002), successfully used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide
range of crop species which include fingermillet (Salimath et al., 1995), wheat (Nagaoka and Ogihara, 1997), *Plantago major* (Wolff and Morgan-Richards, 1998), *Vigna Savi* (Ajibade et al., 2000), sweet potato (Huang and Sun, 2000), rice (Joshi et al., 2000), apple (Goulao and Oliveira, 2001) and strawberry (Arnau et al., 2003). It has also been used for resolving problems concerning with the phylogeny of wheat (Nagaoka and Ogihara, 1997), Asian cultivated rice *Oryza sativa* L. (Joshi et al., 2000) and *Diplotaxis* (Martin and Sanchez-Yelamo, 2000).

From the present study, 20 ISSR profiles revealed a high percentage polymorphism across the 43 genotypes of Indian representative *Abelmoschus* species. The cluster analysis based on UPGMA cluster analysis demonstrated that all the 43 genotypes were well separated from each other based on their wild or cultivated nature and their somatic chromosome counts forming four major clusters I, II, III and IV. *A. tetraphyllus* var. *tetraphyllus* has been grouped together along with two accessions of *A. tetraphyllus* var. *pungens*. *A. angulosus* var. *grandiflorus* showed the close proximity towards *A. tetraphyllus* by clustering together. However it is interesting to note that direct and reciprocal cross between *A. tetraphyllus* var. *tetraphyllus* x *A. angulosus* var. *grandiflorus* were unsuccessful. However, these observations of genome dissimilarity between the crossing partners could not be related to the close clustering pattern between them. Cluster III comprising of *A. moschatus* ssp. *moschatus* had shown its close genetic distance towards *A. tetraphyllus* and *A. angulosus* var. *grandiflorus*. While one accession belonging to *A. tetraphyllus* var. *tetraphyllus* (AT-2, collected from Nepal) was grouped with cluster III, thereby showing its closeness towards *A. moschatus* ssp. *moschatus*. Cluster IV comprising of the cultivated variety of *A. esculentus*, which in turn resolved
separately and but shows close genetic similarity within the accessions. The most polymorphic and reproducible picture has been obtained with poly (AG) or poly (GA) microsatellites which suggests that these are the most frequent simple sequence repeats in *Abelmoschus* genome. Poly (CA) or poly (GA) microsatellites also gave good profiles. This also suggests that these repeats are also distributed in the *Abelmoschus* genome. Out of the 45 primers screened, 20 gave sharp and distinct bands but poly (AT) microsatellites produced no amplification products. This was surprising since poly (AT) repeats are thought to be the most abundant motifs in plant genomes (Morgante and Olivieri, 1993; Depeiges *et al*., 1995). Similar results were found in wheat (Nagaoka and Ogihara, 1997), grapevine (Moreno *et al*., 1998), rice (Blair *et al*., 1999), chestnut (Casasoli *et al*., 2001), *Nothofagus* (Mattoni *et al*., 2002), lentil (Rubeena and Taylor, 2003), jute (Sultana *et al*., 2006) and *Allium* (Mukherjee *et al*., 2013). A possible explanation is that ISSR primers based on AT motifs are self-annealing, due to sequence complementarity and will form dimers during PCR amplification (Blair *et al*., 1999). ISSR markers generated by primer (AG)8YC was converted into a sequence tagged site (STS) marker to identify the fertility restoration gene in rice (Akagi *et al*., 1996). Similarly, UBC 855500 generated by primer (AG)8YT and UBC 8251200 using primer (AG)8T were linked to the gene conferring resistance to race 4 of *Fusarium* wilt in chickpea (Ratnaparkhe *et al*., 1998). The potential supply of ISSR markers depends on the variety and frequency of microsatellites, which changes with species and the SSR motifs that are targeted (Depeiges *et al*., 1995).

At intra-specific level, *A. tetraphyllus* has shown the highest polymorphism of 80.72% with the least genetic similarity of only 0.71 among its accessions and *A.*
tetraphyllus var. pungens clustered together though they both had different somatic chromosome numbers of AT-21 (2n=132) and AT-22 (2n=66) and both were collected from Mizoram. This implies that geographical location and chromosome number does not really influence to the clustering of these accessions. The ISSR dendrogram placed AT-5 and AT-6 with 100% similarity, thereby suggesting that they were the same genotypes but collected at different locations and time as observed in okra (Aladele et al., 2008). This suggests that these two must have similar ancestral origin and may have similar genes and therefore have closely related origins. It further implies that crosses between such accessions may not be recommended, since the result of such a cross may not produce much difference. The stable chromosome number of 2n=66 in all of the accessions of A. angulosus var. grandiflorus showed the low genetic diversity and highest genetic similarity of 0.83. Species with low genetic variation would be expected to have reduced ability to cope up with environmental alterations during evolution (Frankham, 1995). However, clustering pattern in present study did not clearly show any geographical isolation and/or ecological differentiations.

DAMD, which uses PCR approach to direct the amplification of regions rich in minisatellite repeats by using the core sequence of minisatellites as single primer (Heath et al., 1993; Zhou et al., 1997). Since minisatellite core sequences are conserved across species and are larger than RAPD primers, DAMD can be effectively carried out at relatively high stringencies, thus yielding highly reproducible results (Heath et al., 1993). DAMD technique has been successfully applied to various plant species including Triticum (Bebeli et al., 1997), Gossypium (Karaca et al., 2002), Oryza (Kang et al., 2002) and Cynodon (Karaca and Ince,
In this present investigation, 18 DAMD profiles produced 255 amplicons products of which 236 were polymorphic giving high percentage of 91.55% polymorphism across the 43 genotypes of the Indian representative *Abelmoschus* belonging to four species. UPGMA cluster analysis depicted that all the 43 genotypes were well separated from each other based on their wild or cultivated nature and their somatic chromosome counts forming four major clusters I, II, III and IV with a high genetic distance of 0.41-0.95. Cluster II of *A. angulosus* var. *grandiflorus* showed the close proximity towards *A. tetraphyllus* by clustering together, while *A. esculentus* was distantly related from *A. tetraphyllus*. *A. moschatus* ssp. *moschatus* was closely related to *A. esculentus*. DAMD has not been applied before to *Abelmoschus*, therefore DAMD has been found to be a powerful technique for identifying species specific DNA markers.

At intra-specific level, *A. tetraphyllus* has shown the highest polymorphism of 82.97% with the least genetic similarity of only 0.67. Two accessions of *A. tetraphyllus* var. *pungens* was clustered together with *A. tetraphyllus* var. *tetraphyllus*, both had different somatic chromosome numbers of AT-21 (2n=132) and AT-22 (2n=66). Both the accessions were collected from Mizoram. Among the accessions of *A. moschatus* ssp. *moschatus*, AM-31 had shown the highest genetic distance which had reflected in diverse chromosome numbers of 2n=68 and 2n=72. *A. angulosus* var. *grandiflorus* had the least genetic diversity as they all had the same somatic chromosome number of 2n=66 and also were collected from the same geographical location. Within *A. esculentus*, the cultivated taxon Pusa Sawani, AE-39 and AE-41 had shown the genetic similarity of 100%.
For SPAR analysis, three independent methods such as RAPD, ISSR and DAMD which are all single primer based amplification strategies have been used and comparisons were made in all possible combinations. RAPD, ISSR and DAMD data revealed high percentage of polymorphism by clearly distinguishing all the four species of *Abelmoschus* included in the present study. The genetic similarity from the data matrix estimated by Jaccard's coefficient among four species of Indian *Abelmoschus* had shown that all the species are genetically distinct. These markers are mostly dominant and detect variations both in coding and non-coding regions of the genome. Such methods have been widely used because they do not depend on the availability of genomic nucleotide sequence data for the plants being analyzed. In plants like neem, mulberry, French bean, rice, papaya, betelvine and *Murraya* species, two or more of SPAR techniques have been used in one study to analyze intra- as well as inter-species genetic diversity (Winberg *et al*., 1993; Zhou *et al*., 1997; Metais *et al*., 2001; Verma *et al*., 2004; Bhattacharya *et al*., 2005; Saxena *et al*., 2005; Ranade *et al*., 2006). The different methods generate discreet PCR profiles from several genomic portions that do not always overlap between them and therefore, together ensure a much wider coverage of the genome being analyzed. Therefore, development of trait specific DNA marker(s) is important and critically depends on the intended use (Gupta *et al*., 1994).

RAPD marker was found to be more efficient in estimation of molecular diversity of different ecotypes of *Abelmoschus* species than ISSR and DAMD marker as evident from large values of polymorphic loci, average number of polymorphic bands per primer, EMR and MI. The data analysis revealed the highest polymorphism of 95.93% with RAPD, 93.04% with ISSR and 91.55% with DAMD,
while in a cumulative analysis (SPAR), 94.46% polymorphism was observed. The presence of distinct polymorphism in a given population is often due to the existence of genetic variants represented by the number of alleles at a locus and their frequency of distribution in a population. Higher polymorphism is indicative of greater genetic diversity. Heterozygosity is a good measure of detecting polymorphism (Powell et al., 1996). RAPD recorded the highest average number of polymorphic bands per primer of 15.23, followed by DAMD (13.11), RAPD+ISSR+DAMD (12.80) and ISSR (9.85). Likewise, RAPD markers generated more number of bands, its discriminating capacity was also significantly higher than that of ISSR and DAMD. RAPD marker also revealed highest value (0.4793) of resolving power (Rp), which is an efficient tool to assess the capacity of a given primer(s)/marker(s) to distinguish among various genotypes. The MI is a product of “expected heterozygosity” and EMR. As EMR depends on the number of loci per assay unit, RAPD showed much higher values of EMR than ISSR or DAMD. This comparison is also valid for MI, an estimate of marker utility. MI was higher in RAPD as 4.8259 than that of ISSR or DAMD as also recorded in *Allium* (Mukherjee et al., 2013). The high MI is the reflection of efficiency of RAPD marker to simultaneously analyze a large number of bands, rather than level of polymorphism detected. The results suggested that the gene pool quantum utilized in their breeding programmes has been restricted and that introgression of genes from unexploited sources deserves immediate attention. However, comparison of PIC values for three marker systems (a parameter associated with the discriminating power of markers) indicated that the PIC values for DAMD was highest (0.3575), followed by RAPD (0.3271), ISSR (0.3030) and RAPD+ISSR+DAMD was 0.3193 indicating better resolving power of DAMD.
marker over RAPD and ISSR. Polymorphism in a given population is often due to the existence of genetic diversity represented by the number of alleles at a locus and their frequency of distribution in a population. Thus the differences found among the dendrogram generated by RAPDs, ISSRs and DAMDs could be partially explained by the different number of PCR products analyzed (349 for RAPDs, 209 for ISSRs and 255 for DAMDs), reinforcing the importance of the number of loci again and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among *Abelmoschus* species. Similar results have been observed in barley (Loarce *et al*., 1996) and *Cymbopogon* (Bhattacharya *et al*., 2010). Another explanation could be the relative low reproducibility of RAPDs as compared to other markers studied. The observed high proportion of polymorphic loci obtained by RAPD, notwithstanding low reproducibility suggests that there is profound genetic heterogeneity which may be due to high degree of heterozygosity in *Abelmoschus* taxa due to out crossing behaviour. Therefore, authors are of the opinion that RAPD and DAMD are the ideal marker system to analyze natural genetic variation in plants more authentically compared to other SPARs *viz.*, ISSR in *Abelmoschus*.

Parson *et al*., (1997) suggested that differences in the chromosomal location of the three types of markers could influence the diversity assessment since the resolution of these markers are attributed to their specificity and efficiency, which in turn are governed by nucleotide sequence, genomic DNA sequence and target different portions of the genome. Such observations were reported in wheat, where RAPDs were more representative of chromosomal regions enriched in repeated sequences (Kojima *et al*., 1998). It has been clearly observed from our studies that the cophenetic correlation coefficient r-value were statistically significant for all
three marker systems revealing the efficacy of these methods as observed in mulberry, neem and pomegranate (Bhattacharya and Ranade, 2001; Ranade and Farooqui, 2002; Ranade et al., 2009). The highest correlations were found in RAPD+DAMD when compared to RAPD+ISSR and ISSR+DAMD. Similar observations were made by different workers while analyzing genetic variation in plants viz., pomegranate (Ranade et al., 2009), mango (Srivastava et al., 2005, 2007) and papaya (Saxena et al., 2005) to describe variation. This result also supports that the data sets generated by RAPD and DAMD markers revealed the general genetic structuring of the plants. This has importance in breeding programs, germplasm conservation and genetic resource management where relationship and distance estimates give insight into genetic makeup of related accessions.

In intra-specific analysis, A. tetraphyllus showed the lowest similarity of 0.66 and the highest genetic distance of 0.39-0.92. In further details, similarity coefficient value varied from 0.39-0.92 among A. tetraphyllus accessions, 0.51-0.89 in A. moschatus ssp. moschatus, 0.67-0.90 in A. esculentus and 0.70-0.90 in A. angulosus var. grandiflorus accessions. The average genetic similarity from the data matrix estimated by Jaccard's coefficient among four species of Abelmoschus exhibited that all species are genetically distinct and the highest degree of genetic similarity was noticed in A. angulosus var. grandiflorus followed by A. esculentus, A. moschatus ssp. moschatus and A. tetraphyllus. This proves the narrow genetic make-up of A. angulosus var. grandiflorus. Dendrograms did not show any clear pattern of clustering according to the locations from where genotypes were collected, indicating little or no location specificity among Abelmoschus genotypes. However, further study with more varieties and accessions of this species should be performed for
better understanding of the intra-specific genetic diversity of this species. The magnitude and pattern of genetic variation detected in this study can be useful for more systematic germplasm management and utilisation in breeding programmes. The exploitation of crosses between genetically distant parents and those from diverse local sources may produce higher heterosis, better genetic recombination and segregation in their progenies and result in varieties with a broad genetic base which are important in hybrid/cross-breeding programmes (Chahal and Gosal, 2002).

It is quite intriguing to note that constitutive heterochromatin content of AT is found to be relatively high in *Abelmoschus* from our study on flourochrome binding pattern in heterochromatic region (see chapter 6). It has been reported that heavy heterochromatin content plays a very important role in producing genetic diversity in *Allium cepa* and *A. sativum* (Talukder and Sen, 1999). However, almost comparable level of polymorphism in both RAPD and ISSR (95.93% and 93.04% respectively) were observed in all the genotypes of the four *Abelmoschus* species. Such observations were reported earlier in *A. porrum* (Talukder and Sen, 1999).

With this study we can conclude that the molecular analysis at inter- and intra-specific level of *Abelmoschus* through RAPD, ISSR and DAMD provides a powerful tool for the generation of potential diagnostic markers for identification of genetic variants. All three assays differed not only in underlying principle but also amount of polymorphism detected and all of them have different properties thus combination of these three resulted more genome coverage. SPAR methods generate markers unaffected by the environment, with a high throughput for analysis and high degree of reliability and rapidity for identification and discrimination of genotypes and cultivars. The current work elucidates the usefulness of molecular markers in
establishing distinct relationships between accessions where phenotypic expression using morphological methods alone may not be able to determine differences between accessions, especially since phenotypes are influenced by the environment.