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AFLP mediated genetic diversity of malvaceae species

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AFLP (Amplified fragment length polymorphism) marker system is a reliable method in the evaluation of genetic diversity among different species. It was used to explore phenetic relationships and diversity within and between 13 Malvaceae species belonging to 5 different genera. The primary objective of the study was to evaluate the taxonomic potential, usefulness and applicability of AFLP marker system to reconstruct genetic relationships at interspecific and intergeneric level in Malvaceae. In total, 28 accessions comprising 13 species were included in the study but for assorted technical reasons five profiles remained incomplete or with ambiguous banding pattern. Therefore 23 accessions comprising 12 species were included in the final analysis. Two primer pairs produced a total of 73 bands, of which 70 were polymorphic. Neighbor Joining (NJ) tree showed that all 23 accessions were basically classified in three main clusters and several sub-clusters. The tree had well supported branches especially at the level of accessions and species. However, it also had poor bootsrap support at some intermediate and deeper branches. The informative value of the technique was evaluated by comparing the current results with earlier morphological and molecular investigations. Despite some poorly supported parts of the tree, most of the topologies established were in general congruence with earlier studies revealing that AFLP is a robust and reliable tool for DNA fingerprinting and detecting genetic relationships in Malvaceae at different taxonomic levels.

Key words: AFLP, malvaceae, DNA fingerprinting.

INTRODUCTION

Malvaceae is a worldwide family of herbs, shrubs and small trees with a major concentration of genera in the tropical regions (la Duke and Dobley, 1995). The healing qualities of many species have been used in different therapeutic philosophies throughout history (Shaheen et al., 2009). *Alcea rosea* L., a popular garden flower contains a considerable amount of mucilage and is often added to cough tea mixtures (Weiss, 1988). Flowers and seeds of *A. rosea* are reported to have diuretic properties, and seeds and roots are used as demulcents. A decoction of roots boiled with milk is reportedly applied externally for treatment of dermatitis and goiter, also given to pregnant women to ease delivery (Dar et al., 1984). *Malva neglecta* Wallr is said to be used extensively in medicines due to its high mucilage content in the leaves and roots. A literature survey revealed that, it is frequently used in folk medicines *for* the treatment of an abscess (Tabata et al., 1994), inflammatory eczemas (Weiss, 1988), hemorrhoids (Yesilada et al., 1995), as choleretic and for bruises (Honda et al., 1996). A decoction of leaves and roots is used as a gargle for treatment of respiratory disorders and as fomentations for external treatment of skin inflammations, external cancers and gangrenous wounds (Naqshi et al., 1988; Yesilada et al., 1995). In a research conducted by Gurbuz et al. (2005) to evaluate the anti-ulcerogenic potential of different plants used in folk medicines, a decoction prepared from the aerial parts of the *M. neglecta* showed significant gastric protection against the ethanol-induced gastric ulcer in rats. Flowers of *Malva mohileviensis* Downar, are reported

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to be used as a diuretic in Tibetan medicine (Naqshi et al., 1988). Flowers, roots and buds of *Hibiscus rosa-sinensis* are traditionally attributed to antifertility activity in Ayurvedic literature, whereas the leaves are usually used in traditional system of medicine as emollient, aperients, and in the treatment of burning sensation, skin disease and constipation (Ivan, 1999; Kirtikar and Basu, 1999; Pullaiah, 2006, Shaheen et al., 2009).

The Malvaceae family has been very challenging to the taxonomists. Opinions differ widely as to where to draw the line between species and between tribes, and additionally there is some dispute in the description of the family in the order Malvales (Kearney, 1951). There are two main views on the circumscription of the family. One view accepts Malvaceae (*s. str.*) in its traditional sense (Cronquist 1981, 1988), whereas the APG-II system takes a broader circumscription and some closely related families have been merged into an expanded family Malvaceae (Judd and Manchester, 1997; Bayer et al., 1999; Bayer and Kubitzki, 2003). The expanded concept of Malvaceae *(s.l)* comprises about 4,300 species and 245 genera (Bayer and Kubitzki, 2003). A number of outstanding problems in generic delimitation, generic recognition, and generic subdivision in the Malvaceae are enumerated by Fryxell (1997) to underline their need for resolution.

Malvaceous germplasm has been variously investigated by different molecular marker techniques but the earlier studies either focused on the comparison of the Malvaceae with other families in the order Malvales or to explore the genetic relationships and diversity within and among population and limited number of species in the same genus. Very little attention has been given to the analysis at interspecific and intergeneric levels. La Duke and Dobley (1995) has the only worth mentioning work in this regard. In this study, the genetic relationships and diversity within and between 12 malvaceous species belonging to five genera are investigated by using the Amplified fragment length polymorphism (AFLP). AFLP (Zabeau and Vos, 1993; Vos et al., 1995) is a multilocus DNA fingerprinting technique that approaches the ideal as a marker system for assessing genetic diversity among individuals, populations and species (Mueller and Wolfenbarger, 1999). The advantages of this technique include reproducibility and high levels of polymorphism detection in a single assay without any prior knowledge of the genome studied. The technique has been widely used in plant species with which little or no molecular research has been done (DeHaan et al., 2003). Fluorescent labeling and semi-automated detection of fragments have amplified the pace and accuracy of the AFLP technique (Huang and Sun, 1999).

This study is probably the first application of AFLP to a wide range of species belonging to different genera. This study is also the first to include *Malva* L., *Abutilon* Mill. , *Alcea* L. and *Sida* L. in AFLP analysis. The objectives of our study were:

(1) To explore genetic relationships and diversity in malvaceous species belonging to five different genera.
(2) To evaluate the taxonomic potential of AFLP marker system at interspecific and intergeneric level in Malvaceae.

MATERIALS AND METHODS

Plant material

Young leaves of twenty eight samples belonging to thirteen species and five genera of Malvaceae were collected from different parts of the Pakistan for this study with the exception of three individuals of *M. sylvestris* which were collected from Brighton U.K (Table 1). Vouchers of collection were deposited in the Herbarium of Quaid-i-Azam University (ISL). The leaves were stored in sealed plastic bags with silica gel.

DNA extraction

Genomic DNA was extracted from the silica gel dried and fresh leaf tissues by using a simple protocol described by Echevarría-Machado (2005) for rapid DNA isolation from Malvaceae plant species. Approximately 0.1 g leaf material was ground using a mortar and pestle. Homogenized ground tissues with 1 ml of extraction buffer (10 mM tris-Hcl, 50 mM EDTA, 500 mM sodium chloride, 10 mM β-mercaptoethanol, pH 7.0) were transferred to a 2 ml eppendorf tube, and 100 µl of 20% SDS was added. Samples were incubated at 65°C for 10 min. Afterwards 500 µL of 5 M potassium acetate was added, tubes were shaken vigorously and incubated on ice for 20 min. Samples were centrifuged at 16,000 g for 20 min and supernatant was transferred to a new 1.5 mL tube with 300 µL of silica (Sigma S5631). After centrifugation for 30 s at 16,000 g, the pellet was washed twice with 70% ethanol. Pellet was resuspended in 50 µL of distilled water and incubated at 55°C for 5 min. Tubes were spun at 16,000 g for 2 min and supernatant was transferred to a new 500 µL eppendorf tube. Quality and quantity of DNA was assessed by running on 1% TBE buffered agarose gel and stored at -20°C.

AFLP analysis

0.5 µl of genomic DNA was digested with restriction enzymes, EcoR1 (20 u/µl) and *Mse*I (10 u/µl) . Digestion and ligation was performed in a single reaction at 37°C for 4 h. A small aliquot of the digested DNA was run on a 1% (w/v) agarose gel to make sure that the DNA digestions were complete. The pre-amplification was performed with EcoR1 (+A) / *Mse*I (0) primer pair. Final amplification was done using two fluorescent labelled *Eco*RI-*Mse*I primer combination: Eco+ACA/Mse+CAG and EcoRI-AAC/MseI-CAT. EcoR1 primer were end-labeled with [γ-^{33}P].The selective amplification PCR profile was 13 cycles of 30 s at 94°C, 30 s at 65°C followed by lowering the temperature of -0.7°C per cycle and 1 min at 72°C, followed by 27 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 30 s. The reaction product was mixed with an equal volume (10 µl) of sequencing loading dye (98% formamide, 10 mM EDTA pH 8.0 and bromophenol blue) and was denatured at 80°C for 5 min and put immediately on ice to inhibit renaturation of the DNA strands during the gel preparation. The products were analyzed on 6% (w/v) denaturing polyacrylamide gels in 0.5xTBE electrophoresis buffer. Electrophoresis was performed at 80 W constant power for 1h 30 min. The gel was fixed for 30 min in 10% acetic acid and transferred to Whatman's 3 mm chromatography

Table 1. Accessions of the 13 Malvaceous species used in the present study.

Accession code	Species	Locality	Voucher specimen number
	Alcea		
A1	*A. rosea* L.	Islamabad, Pakistan	ISL-Ar1 to ISL-Ar4
A2	*A. rosea* L.	Fateh Jhang, Pakistan	
A3	*A. rosea* L.	Wah Cantt, Pakistan	
A4	*A. rosea* L.	Abbottabad, Pakistan	
	Abutilon		
Ab	*A. bidentatum* A. Rich	Islamabad, Pakistan	ISL-Ab
Ai	*A.indicum*	Fateh Jhang, Pakistan	ISL-Ai
	Hibiscus		
Hr	*H. rosa-sinensis* L.	Wah Cantt, Pakistan	ISL-Hr
Hm1	*H. mutabilis* L.	Islamabad, Pakistan	ISL-Hm1 to ISL-Hm2
Hm2	*H. mutabilis* L.	Wah Cantt, Pakistan	
Hs1	*H. syriacus* L.	Islamabad Pakistan	ISL-Hs1 to ISL-Hs2
Hs2	*H. syriacus* L.	Rawalpindi Pakistan	
Hsh1	*H. schizopetalus* (Mast.) Hook. F.	Islamabad, Pakistan	ISL-Hsh1 to ISL-Hsh3
Hsh2	*H. schizopetalus* (Mast.) Hook. F.	Abbottabad, Pakistan	
Hsh3	*H. schizopetalus* (Mast.) Hook. F.	WahCantt, Pakistan	
Hc1	*H. caesius* Gracke	Pir Sohawa, Pakistan	ISL-Hc1 to ISL-Hc2
Hc2	*H. caesius* Gracke	Pir Sohawa, Pakistan	
	Malva		
Ms1	*M. sylvestris* L.	Brighton, U.K.	ISL-Ms1 to ISL-Ms4
Ms2	*M. sylvestris* L.	Brighton, U.K.	
Ms3	*M. sylvestris* L.	Brighton, U.K.	
Mn1	*M. neglecta* Wallr.	Murree, Pakistan	ISL-Mn1 to ISL-Mn4
Mn2	*M. neglecta* Wallr.	Donga gali, Pakistan	
Mn3	*M. neglecta* Wallr.	Nathia Gali, Pakistan	
Mp1	*M. parviflora* L.	Islamabad, Pakistan	ISL-Mp1 to ISL-Mp3
Mp2	*M. parviflora* L.	Fateh Jhang, Pakistan	
Mp3	*M. parviflora* L.	Wah Cantt, Pakistan	
Mm	*M. microcarpa* Pers.	Islamabad, Pakistan	ISL-Mm
	Sida		
Sc1	*S. cordata* (Burm. F.) Bross.	Islamabad, Pakistan	ISL-Sc1 to ISL-Sc2
Sc2	*S. cordata* (Burm. F.) Bross.	Fateh Jhang, Pakistan	

paper, dried in a vacuum drier for 1 h at 80°C and exposed to X-ray film for 24 h.

Data analysis

AFLP bands showing unambiguous amplification were scored manually as present (1) and absent (0) into a binary data matrix. Unrooted Neighbor Joining tree for 23 genotypes was generated by Power Marker V3.0 (Liu and Muse 2005) by frequency based distance using the algorithm of Nei (Nei and Takezaki, 1983)

Branch support was assessed through the implementation of 1000 bootstrap replicates (Felsenstein, 1985).

RESULTS AND DISCUSSION

Knowledge of genetic relationships of plant taxa belonging to different species and genera of the same family is very important for taxonomist. Discovering new relationships is of course not only relevant to classification but it also provides a direction and sequential scale for plant evolution (Savolainen and Chase, 2003).

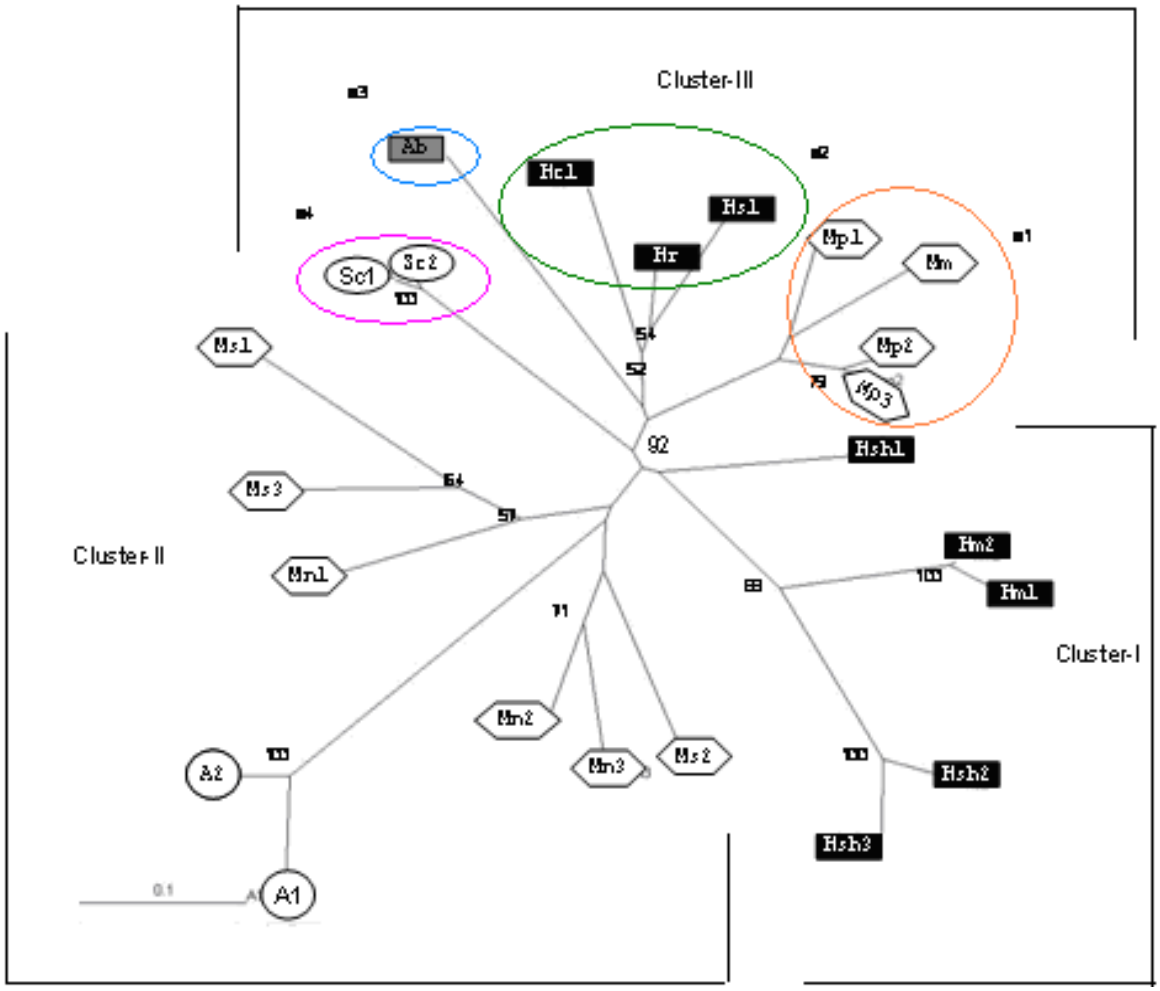

Figure 1. Genetic relationships within and between 23 accessions representing 12 Malvaceous species belonging to five major genera depicted by Neighbour Joining tree based on AFLP marker system. Numbers next to the branches signify the levels of bootstrap support (only levels above 50% are indicated).

The AFLP marker system was selected to estimate genetic relationships among 28 accessions representing 13 species from five major genera of the family Malvaceae because it had previously been successfully used in the family to investigate genetic relationships in a number of studies including cotton (Murtaza, 2006; Pillay and Myers, 1999; Hawkins et al., 2005) and few species of the genus *Hibiscus* (Huylenbroeck et al., 2000; Tang et al., 2003; Cheng et al., 2004; Coetzee et al., 2009). This study is probably the first report of the application of the AFLP methodology to the characterization and genetic relationships between species belonging to five different genera of Malvaceae. 28 accessions were included in this study to clarify their genetic affinities. For various technical reasons, five profiles remained incomplete or with ambiguous banding pattern and these taxa were excluded from analyses.

The selected primer pairs amplified a total of 73 unambiguous informative bands. In total 70 (95.8%) of the fragments detected were polymorphic. To visualize relationships among 23 accessions representing 12 species, unrooted neighbor joining tree (NJ) generated by "powermarker" V3.0 (Liu and Muse, 2005) using frequency based distances (Nei and Takezaki, 1983) is depicted in Figure.1. The bootstrap value higher than 50% are indicated, all the other branches have values less than or equal to 49.

All 23 accessions were classified into 3 main clusters and several sub-clusters. Cluster-III has a bootstrap value of 92, whereas cluster-I & II are poorly supported. NJ tree has many well supported branches especially basal topologies and some species groups. However, it also has some weakly supported intermediate and deeper branches. Despite some poorly supported parts, the tree shows a general congruence with the earlier studies based on morphological and molecular data. The individuals belonging to one particular genus are grouped closer to form a cluster or sub-cluster, even in cluster-III,

which is composed of ten accessions belonging to 4 genera, the accessions belonging to the same genus are grouped together to form a sub-cluster within the main cluster, indicating that AFLP can be a valuable tool to reconstruct genetic relationships in Malvaceae at higher taxonomic levels.

The tree separates the eight accessions of *Hibiscus* L. into two clusters. Five accessions are present in cluster-I and the remaining accessions of *Hibiscus* are clustered in cluster- III. The bootstrap support for splitting the taxa in two different clusters is poor but the AFLP results generated here back up the earlier morphological descriptions of the genus. *Hibiscus*, a diverse assemblage of morphologically distinct species groups, has been problematic to the reversionary taxonomist (Pfeil et al., 2002). The genus has been described so heterogeneous for most of its history that despite being subdivided into many sections by different workers, still many sections have undecided or not yet understood boundaries, and a general agreement on the infrageneric classification of *Hibiscus* has not yet emerged (Fryxell, 1997).

Cluster-I is purely composed of *Hibiscus* species, two accessions *of Hibiscus mutabilis* and three accessions of *Hibiscus schizopetalus*. Where multiple accessions were sampled per taxon, the accessions either formed monophyletic pairs (Hm) or polymorphism was detected in one accession of the taxa (Hsh). The initial divergence is between *H. schizopetalus* and *H. mutabilis*. Two accessions of *H. mutabilis* (Hm1 and Hm2) and two accessions *of H. schizopetalus* (Hsh2 and Hsh3) form strongly supported monophyletic pairs and two accessions of each species are connected to each other with a branch having 88 bootstrap support. The results indicate a close relationship between the two species. Species that are closely related based on AFLP analysis may be easily crossable (Chen et al., 2004). This may increase the genetic diversity and ornamental value of these new species. The third accession of *H. schizopetalus* (Hsh1) was placed apart. However, morphological characters of Hsh1 are related to *H. schizopetalus*. Although the underlying reason for this separation is unclear.

The genus *Alcea* represented by two accessions (A1 and A2) is placed in cluster- I, along with three accessions of *M. sylvestris* and three accessions of *M. neglecta*. Although there is no bootstrap support for this clustering, the association of these taxa is consistent with some of the relationships reflected by “alliances”. Bates and Blanchard (1970) placed *Alcea* (as *Althaea*), *Lavatera* and *Malva* in the Malva alliance (La Duke and Dobley, 1995). The monophyly of these taxa was also confirmed by cpDNA analysis (La Duke and Dobley, 1995). Both *M. sylvestris* and *M. neglecta* were represented by 3 accessions each. Interestingly, Two accessions of *M. sylvestris* are clustered with one accession of *M. neglecta* and two accessions of *M. neglecta* are clustered with one accession of *M. sylvestris* in two separate groups (a, b).In group “a”, two accessions of *M. neglecta* (Mn2 and Mn3) forming a monophyletic pair are present with one accession of *M. sylvestris* (Ms2), whereas in group “b”, one accession of *M. neglecta* (Mn1) is present with two accessions of *M. sylvestris* (Ms1and Ms3). In both groups, each species is well differentiated with higher values of inter-species genetic distances than between two accessions of the same species (Table 2). Two accessions of *Alcea* due to longer branch length occupy a distinct position in the cluster.

Cluster-III, the only well supported cluster in the tree is composed of 10 accessions from four genera. The interesting feature of this cluster is the distinctive position of each genus. There is no mismatch pairing of species at generic level. All the accessions belonging to one particular genus are grouped together, so four sub-clusters can be easily differentiated within the main cluster. Sub-cluster “a1”, contains three accessions of *Malva parviflora* and single sampled accession of *Malva microcarpa*. *M. parviflora* (Mp1) and *M. microcarpa (Mm)* form a monophyletic species pair without any bootstrap support. The close relationship between *M. parviflora* and *M. microcarpa* is not unexpected given the strong morphological resemblances as it has been cited as synonymous to *M. parviflora* in earlier literature. Presence of *Sida* L. and *Abutilon* Mill. next to each other in the same cluster is consistent with the findings of La Duke and Doebley’s (1995) cpDNA based phylogeny of the Malvaceae. Whereas the presence of *Hibiscus* along with the members of *Malva*, *Abutilon* and *Sida* in the same cluster is in agreement with the statements by Fryxell (1975), Bates and Blanchard (1970) , but this is in clear contrast with cpDNA based analysis (La Duke and Doebley, 1995). Two accessions of *Sida cordata* (Sc1 and Sc2) in “a4”, sub-cluster forming a monophyletic pair with a bootstrap support of hundred, showed the minimum percentage of polymorphism. The short branch length between two accessions of *S. cordata* indicates that the divergence between them is recent.

Conclusion

Phenetic analysis of the AFLP data revealed three major clusters. Unfortunately we are not in a position to make any conclusive statement about relationships at higher taxonomic level due to lack of bootstrap support at the deeper and intermediate branches of the NJ tree but the phenetic analysis has shed much light on relationships at inter and intraspecific level, has also yielded some conclusions on species relationships.

1. *H. mutabilis* is closely related *to H. schizopetalus* than *H. rosa-sinensis.*
2. *M. parviflora* shows close association with *M. neglecta* whereas *M. microcarpa* has strong genetic affinity with *M. parviflora*

Table 2. Frequency based genetic distances among all pair of taxa.

	A1	A2	Ab	Hr	Hm1	Hm2	Hs1	Hsh1	Hsh2	Hsh3	Hc	Ms1	Ms2	Ms3	Mn1	Mn2	Mn3	Mp1	Mp2	Mp3	Mm	SC
A1																						
A2	0.083																					
Ab	0.403	0.431																				
Hr	0.431	0.458																				
Hm1	0.333	0.389	0.236	0.181																		
Hm2	0.431	0.486	0.333	0.306	0.319																	
Hs	0.431	0.458	0.361	0.333	0.319	0.028																
Hsh1	0.375	0.403	0.222	0.111	0.097	0.306	0.306															
Hsh2	0.361	0.444	0.458	0.347	0.361	0.208	0.236	0.347														
Hsh3	0.403	0.458	0.417	0.361	0.375	0.222	0.250	0.333	0.069													
Hc	0.403	0.403	0.278	0.250	0.208	0.278	0.306	0.194	0.319	0.278												
Mf1	0.472	0.472	0.403	0.319	0.361	0.403	0.431	0.292	0.417	0.375	0.319											
Mf2	0.333	0.333	0.403	0.264	0.333	0.347	0.375	0.264	0.389	0.403	0.264	0.333										
Mf3	0.444	0.472	0.347	0.264	0.333	0.375	0.403	0.292	0.444	0.458	0.347	0.194	0.222									
Mn1	0.486	0.486	0.306	0.306	0.319	0.333	0.361	0.250	0.431	0.444	0.333	0.347	0.292	0.292								
Mn2	0.361	0.361	0.319	0.292	0.306	0.375	0.375	0.236	0.444	0.403	0.319	0.361	0.222	0.250	0.292							
Mn3	0.403	0.403	0.361	0.278	0.319	0.306	0.333	0.278	0.403	0.389	0.222	0.292	0.153	0.208	0.278	0.125						
Mp1	0.333	0.389	0.403	0.264	0.278	0.319	0.347	0.292	0.333	0.375	0.236	0.222	0.250	0.222	0.319	0.306	0.236					
Mp2	0.417	0.472	0.292	0.264	0.250	0.292	0.319	0.208	0.333	0.375	0.292	0.361	0.306	0.306	0.125	0.278	0.292	0.278				
Mp3	0.458	0.486	0.306	0.194	0.236	0.333	0.361	0.139	0.403	0.389	0.278	0.347	0.292	0.319	0.111	0.264	0.278	0.292	0.125			
Mm	0.431	0.458	0.278	0.194	0.208	0.333	0.361	0.111	0.375	0.361	0.250	0.319	0.292	0.319	0.139	0.264	0.278	0.292	0.097	0.028		
SC	0.389	0.417	0.319	0.319	0.278	0.347	0.347	0.236	0.417	0.375	0.264	0.389	0.306	0.333	0.319	0.278	0.236	0.361	0.333	0.264	0.264	
sc	0.417	0.444	0.292	0.292	0.278	0.319	0.347	0.236	0.389	0.347	0.236	0.361	0.278	0.306	0.292	0.278	0.208	0.333	0.306	0.236	0.236	0.028

3. Within the Malvaceous species studied, *S. cordata* accessions were the least diverse.

However these results should be treated as tentative until corroborated with supplementary data. The general congruence of these preliminary results with previous morphology-based classifications and with the results based on other molecular markers leads to the conclusion that AFLP marker system is robust and reliable for reconstructing relationships in Malvaceous species at different taxonomic level. The AFLP profiles and the topologies established can be used as bases for comparison in future studies.

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