academicJournals

Vol. 4(3) pp. 38-44, June 2013
DOI: 10.5897/IJBMBR11.057
ISSN 2141-2154 ©2013 Academic Journals
http:// www.academicjournals.org/IJBMBR

International Journal for Biotechnology and Molecular Biology Research

Full Length Research Paper

Use of SSR markers for genetic diversity studies in mulberry accessions grown in Kenya

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Accepted 11 May, 2013

The knowledge and understanding of the extent of genetic variation of mulberry germplasm is important for conservation and improvement. The objective of this study was to analyze the genetic diversity between and within two mulberry species widely grown in Kenya which include *Morus alba and Morus indica.* Five individuals per species were genotyped with 13 simple sequence repeat (SSR) markers. The SSR markers presented a high level of polymorphism and detected a total of 35 polymorphic bands and 74.47% polymorphic loci. The mean observed heterozygosity per primer was 0.3670 suggesting a high degree of variation. The analysis of molecular variance (AMOVA) revealed that 95% of the variation was found within the species and only 5% between the species. Principal coordinates analyses (PC_OA) clearly distinguished three groups. It was evident from this study that the mulberry accessions did not cluster on the basis of their geographical origin, and neither according to the group of species they fall into. The study showed a close relationship between the two species and therefore mulberry improvement should target sampling more individuals within species rather than among species.

Key words: Mulberry, simple sequence repeat markers, genetic diversity.

INTRODUCTION

Genetic diversity assessment has potential uses in evolution, breeding and conservation of genetic resources (Wu et al., 1999). The extent of diversity in the accessions is paramount for improvement and utilization of genetic resources. Genetic diversity is therefore the backbone of conservation of plant genetic resources for both present and future use (Quedraogo, 2001). Genetic diversity of species also helps formulate appropriate sampling strategies for both *in situ* and *ex situ* conservations, with the aim to protect the variability of taxa so as to preserve ecological processes, rate of establishment, survival and fecundity (Millar et al., 2000).

Information on the levels and distribution of genetic variation in the natural populations of mulberry is of great importance. Mulberry bears different sex types, is monoecious or dioecious, with sex expression varying among species and varieties due to their cross fertilization (Das et al., 1994; Dandin, 1998). Such a high degree of cross species reproductive success is not encountered often in nature, thereby creating variation within and among mulberry populations (Awasthi et al., 2004).

Advancement of molecular biology techniques in the last two decades has aided in the concise classification of individual plants and species. Molecular markers have been used in screening of germplasm, genetic diversity, identifying redundancies in collections, testing accession stability and integrity as well as resolving taxonomic relationships (Kameswara, 2004). SSRs are advantageous over many other markers as they are highly polymorphic, robust, can be automated; only very small DNA is required, highly abundant, analytically simple, readily transferable and have a co-dominant inheritance (Matsuoka et al., 2002).

The genetic structure of mulberry species has been

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studied extensively (Tikader et al., 2009). These studies have revealed a high genetic divergence among mulberry accessions. For instance, Awasthi et al. (2004) and Vijayan et al. (2004, 2005, 2006) examined the genetic structure of indigenous mulberry accessions in India, and found a great degree of polymorphism amongst them. Similarly, Bhattacharya and Ranade (2001) and Sharma et al. (2000) also found a wide variation in genetic distance among different *Morus* genotypes in India and Japan, respectively.

Genetic diversity provides plant breeders with options to develop productive crops that are resistant to virulent pests and diseases and adapted to changing environments. However, a significant proportion of mulberry accessions in Kenya are from other countries such as India, China, Thailand, etc, that have entirely different geographical and ecological features, therefore, when these accessions are subjected to an environment totally different from their natural habitat, it is expectable that they may undergo genetic changes for adaptation (Tikader et al., 2009).

The ability to identify genetic variation is indispensable to effective management and use of genetic resources. This work, though very essential, has however not been undertaken in Kenya. It was therefore prudent to subject the mulberry accessions to genetic characterization using molecular markers to understand the extent of available and to design strategies to eliminate duplicates and nearly similar accessions in the national gene bank.

MATERIALS AND METHODS

Plant materials

Ten mulberry accessions which included Thailand, Thika, Embu, Ithanga, Limuru (*Morus alba* L.), S41, S13, S54, S36 and Kanva-2 (*Morus indica* L.) were obtained from The International Centre of Insect Physiology and Ecology (ICIPE) mulberry germplasm site at Nairobi headquarters' and Kenya Agricultural Research Institute (KARI) at Thika.

DNA extraction

Genomic DNA was extracted from the young fresh leaf samples using cetyl trimethyl ammonium bromide (CTAB) procedure described by FAO/IAEA (2007). The DNA quantity and quality was visually quantified using the agarose gel electrophoresis method as described by Manniatis et al. (1982). DNA samples were stored at -20°C.

PCR amplification reaction for the simple sequence repeat (SSR) primers

Fifteen microsatellite primers used to amplify the genomic DNAs extracted from the ten mulberry accessions were of two sets: SS and Mulstr primers obtained from Invitrogen, United Kingdom. SS primers amplification was performed in a final volume of 15 µl containing 10 mM Tris-HCl (Ph 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.6 µl each primer, 10 ng of genomic DNA, 1 U *Taq* polymerase and molecular biology water. This was run using a touchdown protocol. The amplification cycle included a 3 min denaturation at 95°C, followed by 16 cycles of denaturation at 94°C for 30 s, 30 s primer annealing at 63°C and decreasing the annealing temperature by 0.5°C at each succeeding cycle, and 1 min elongation at 72°C. Then, 24 cycles of 30 s at 94°C, 1 min at 56°C and 1 min at 72°C were conducted, followed by 5 min at 72°C. The Mulstr primer amplification was carried out in 15 µl reactions containing 10 ng template DNA, 2 µl each primer and 1U *Taq* polymerase on a thermocycler programmed as follows: initiatial denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min denaturation, primer specific annealing temperature for 1 min and 72°C for 1.5 min extension and a final extension step at 72°C for 5 min and a cooling step at 4°C. The amplified SSR products were electrophoresed in 2% agarose gel in 0.5 XTBE buffer at 150 V for 2 h, stained in ethidium bromide for 30 min, and photographed on a UV transilluminator at 312 nm.

Data analysis

Scoring of bands was done visually. Fragments were scored as present (1) or absent (0) for each of the SSR markers. Nei's (1973) gene diversity, Shannon information index (Shannon and Weaver, 1949), number of alleles (Na), expected heterozygosity (H_E), observed heterozygosity (Ho), genetic similarity and genetic distance estimated by Nei's coefficient between pairs were analyzed using popgene software version 3.5 (Yeh et al., 1999). The data matrix was then subjected to analysis of molecular variance (AMOVA) to partition the genetic variation into within and among the populations' components using GenAlEx software (Peakall and Smouse, 2006). Principal coordinate analysis (PCoA) and scores for the first and second components were also plotted using GenALEx software.

RESULTS

Genetic diversity among accessions

From a total of fifteen SSR primers used to investigate ten mulberry accessions, only thirteen produced clear amplification products and polymorphisms. The thirteen SSR primers generated a total of 35 polymorphic bands. The highest number of bands was 15 which were generated by SS09 primer while the lowest number was 2 bands generated by SS19, SS20, M2, M6 and M5 primers. A total of 74.47% polymorphic loci were generated by the 13 primers studied. The bands produced by these primers varied in size from 110 to 420 bp. The observed number of alleles per locus in all mulberry accessions ranged from 2 to 15 with a mean of 6.3. The observed heterozygosity per primer ranged from 0.0500 to 0.7000 with an average of 0.3670 suggesting a high degree of variation. Expected heterozygosity per primer ranged from 0.0473 to 0.5053 with an average of 0.3610 (Table 1).

The mean observed heterozygosity per accession varied from 0.15 to 0.4595, hence, suggesting a high degree of variation among the accessions. The Shannon information index showed a high diversity across the accessions ranging from 0.1040 in S36 accession to 0.3185 in Thailand accession, with a mean of 0.4399. In all the accessions, Thailand accession was the most highly diverse according to the observed heterozygosity and Shannon information index.

Table 1. Band sizes, observed number of alleles (Na), observed (Ho) and expected (He) heterozygosity for thirteen microsatellite markers assayed across ten mulberry accessions.

Primer	Repeat motif	Primer sequence (Aggarwal et al., 2004; Zhao et al., 2005)	Band size (bp)	Na	Ho	He
SS05	$(CA)_5CC(CA)_{27}$	F:TCCAGCAAAGATGTGACAAAAGTT R:TTGCCTTCCCGATTATGCTG	320-400	5	0.05	0.0473
SS02	$(CA)_{49}$	F:GCTTCGATCAATCTAGCTTCCC R:GCAAACTACGCCACCCCG	310-420	12	0.2973	0.3318
SS04	$(TG)_{27}$	F:CGAGGGAGGGATGAGGAGC R:CACATTCATCCACCCTCCTATA	190-250	6	0.5444	0.445
SS17	$(CA)_{26}$	F:TACAGGGCTCGGGCAAATG R:TGATCCGAAGCTTGGGGTCT	220-300	9	0.2674	0.3643
SS06	$(TG)_{18}$	F:ACTCAAAATGAAGGAAAAGGAATTATAC R:TTTACTTAAATCCCAGCCACA	180-220	10	0.545	0.4845
SS19	$(TG)_{18}$	F:TTCTGTCGTGTCCTCCGTCAA R:TGAGAACATACACTAATAGGTGAAAAC	300-370	2	0.4000	0.3368
SS09	$(CA)_{56}$	F:AGAACCCTTCCGCCCTATG R:CCTTGGCGTAGGCAAAGTTG	200-290	15	0.2125	0.4992
SS20	$(CA)_{14}ACAA(CA)_{12}$	F:CCCTTTCATCGCCTCCTCC R:CTCTGCCATTCAGTAGCGG	300-350	2	0.500	0.3947
SS18	$(CA)_{27}$	F:TCTTCGCCCGTTGTTCGC R:AGCAATTTTCTTCAACTCACCTTCT	180-210	10	0.1805	0.2916
M2	$(GTT)_{11}$	F:CGTGGGCTTAGGCTGAGTAGAGG R:CACCACCACTACTTCTCTTCTTCCAG	190-210	2	0.4000	0.5053
M6	$(GT)_{15}$	F:TCCTTAGGTTTTTGGGGTCTGTTTACAT R:CCTCATTCTCCTTTCACTTATTGTTG	110-190	2	0.5000	0.3947
M4	$(GAA)_6$	F:GGTCAAGCGCTCCAGAGAAAAG R:GGTGCAGAGGATGAAAGATGAGGT	140-160	5	0.175	0.1197
M5	$(CCA)_8$	F:CCCCCTGCAATGCCCTCTTTC R:TGGGCGAGGCAGGGAAGATTC	160-180	2	0.7000	0.4789

Genetic diversity among species

The number of polymorphic loci generated by the SSR primers per single species varied from 29 in *M. alba* to 21 in *M. indica.* The percentage of polymorphic loci in *M. alba* was 61.7% while that of *M. indica* was 44.68%. The observed heterozygosity varied from 0.2415 to 0.3404 while Shannon information index ranged from 0.3051 to 0.3905 in *M. indica* and *M. alba*, respectively. Therefore these showed little variation between the two species studied.

Genetic similarity among and within species

The genetic similarity among and within species was estimated on the basis of analysis of molecular variance (AMOVA), genetic identity (I) and distance (D), UPGMA dendrogram and Principal coordinates analysis (PCoA).

Molecular variance among and within species (AMOVA)

Of the total species diversity, 5% was among the species, while 95% was within the species (Table 2).

Nei's (1978) genetic identity (I) and distance (D) among and within the two species of mulberry.

The genetic similarity coefficient among the accessions estimated on the basis of Nei's (1978) unbiased, varied from 0.8455 between Limuru and Ithanga accessions to 0.4077 between Limuru and Thailand accessions with an average genetic similarity of 0.5362. Where I = 0.0 shows no common alleles while I = 1.0 shows equal gene frequencies (Appendix 3c). Therefore, Limuru and Ithanga accessions shared most of the alleles while Limuru and Thailand were more diverse. The maximum genetic distance (0.8972) observed between any two accessions was that between the Limuru and Thailand accessions while the smallest distance (0.1678) was between Ithanga and Limuru accessions. The mean genetic distance was 0.5916. At species level, the genetic similarity coefficient was 0.7613 while their genetic distance was 0.2727 (Table 3).

Molecular analysis using the SSR data matrix to cluster the ten accessions and show genetic variation using Principal coordinate's analysis (PCoA) is shown in Figure 2. The PCoA coordinates one and two extracted 35.45

Table 2. Analysis of molecular variance (AMOVA) for the two species of mulberry (*Morus* sp.) based on SSR markers.

Source of variation	d.f.	SS	MS	Variance component	Total variation (%)	p-value*
Among species	1	43.200	43.200	1.750	5	<0.001
Within species	8	275.600	34.450	34.450	95	<0.001
Total	9	318.800	77.650	36.200	100	

*After 999 random permutations.

Table 3. Nei's (1978) unbiased measure of genetic identity (I) (above diagonal) and genetic distance (D) (below diagonal) for the two mulberry species studied.

	M. alba	***M. indica***
M. alba	****	0.7613
M. indica	0.2727	****

and 17.49% of the total variation, respectively.

DISCUSSION

The observed number of alleles (*Na*) per locus in the mulberry accessions studied ranged from 2 to 15 with an average of 6.3 (Figure 1). This mean was less than 18.6 reported by Aggarwal et al. (2004) but larger than 5.13 reported by Zhao et al. (2007) using SSR markers. The mean observed heterozygosity (Ho) of 0.3670 in this study was lower than 0.4296 reported in 27 mulberry genotypes by Zhao et al. (2005). Zhao et al. (2007) further reported a higher value of 0.4912 within cultivated species of mulberry for the same. Aggarwal et al. (2004) also reported a higher Ho value of 0.59 across 45 mulberry genotypes from species of diverse origin using SSR markers. The highest mean observed heterozygosity across the accessions was observed in Thailand accession with 0.4595 while the least was 0.1500 in S36 accession. The mean observed heterozygosity among the species varied from 0.3404 to 0.2415 in *M. alba* and *M. indica* species, respectively. These findings showed a high genetic variability among the accession than among species. This implies, there is a high genetic variability among mulberry accession grown in Kenya. In addition, SSR markers gave a mean Shannon information index of 0.4399 for these mulberry accessions, a value higher than 0.3200, 0.3000 and 0.2900 reported by Bhattacharya et al. (2005) using DAMN, ISSR and RAPD markers, respectively in both exotic and indigenous mulberry varieties. However, a higher value of 0.7399 was detected in cultivated species of mulberry using SSR markers (Zhao et al., 2007). Thailand accession with a Shannon index value of 0.3185 was the most varied while S36 with a value of 0.1040 was least varied.

The Shannon information index for both *M. alba* and *M. indica* species was 0.3905 and 0.3051, respectively. Vijayan et al. (2004a) reported smaller values of 0.21 ± 0.27 and 0.25 ± 0.29 in *M. alba* and *M. indica* species using ISSR markers, respectively. In contrast, using RAPD markers, Vijayan (2004b) reported smaller values of 0.19 ± 0.27 and 0.17 ± 0.26 in *M. alba* and *M. indica* species; hence indicating a high diversity in the mulberry accessions and species.

The percent polymorphic loci revealed an average of 74.47% in all the mulberry accessions studied using SSR markers. This value was lower than 85 and 91% shown in RAPD and DAMD markers by Bhattacharya and Ranade (2001). Earlier, Sharma et al. (2000) had reported a higher average value of 81.2%. Additionally, a higher value of 86% was reported by Vijayan et al. (2004a) and Zhao et al. (2007). These values suggest a high level of genetic variability among the accessions studied.

Such high level of genetic diversity can be attributed to the mode of reproduction of these accessions. Vegetatively propagated species such as mulberry often have minute unobserved changes in passage of time and accumulation of these changes can lead to significant changes in plant population. Such variability could come from mutation (Vijayan, 2009) and/or long cultivation periods (Bhattacharya and Ranade, 2001). Mulberry is a highly heterozygous and outcrossing species and therefore it is expectable that its accessions exhibit a high level of polymorphism (Bhattacharya and Ranade, 2001; Awasthi et al., 2004).

Molecular variance between and within the mulberry species was high at 95% within the species (*M. alba* and *M. indica)* and low at 5% between the species. These findings are significant in outcrossing woody crops as mulberry. This is supported by Hamrick and Godt (1996) who reported a high genetic variation within populations than between populations of outcrossing woody plants.
Similar results were reported by Alvarez et al. (2001) on tomato using microsatellite analysis. Steiger et al. (2002) also demonstrated a high genetic diversity within the cultivar catimor of *Coffea arabica* using AFLP markers. Earlier, Erstad (1996) studying vegetative and generative characters in ecotypes of woody spp. of *Ribes rubrum* found greater variation within populations than between populations and Heaton et al. (1999) too found comparable variabilities in a RAPD study of chicozapote tree (*Manilkara zapota*), thus confirming our results.

High level of genetic variability within populations such as those detected in this study, are attributed to mating

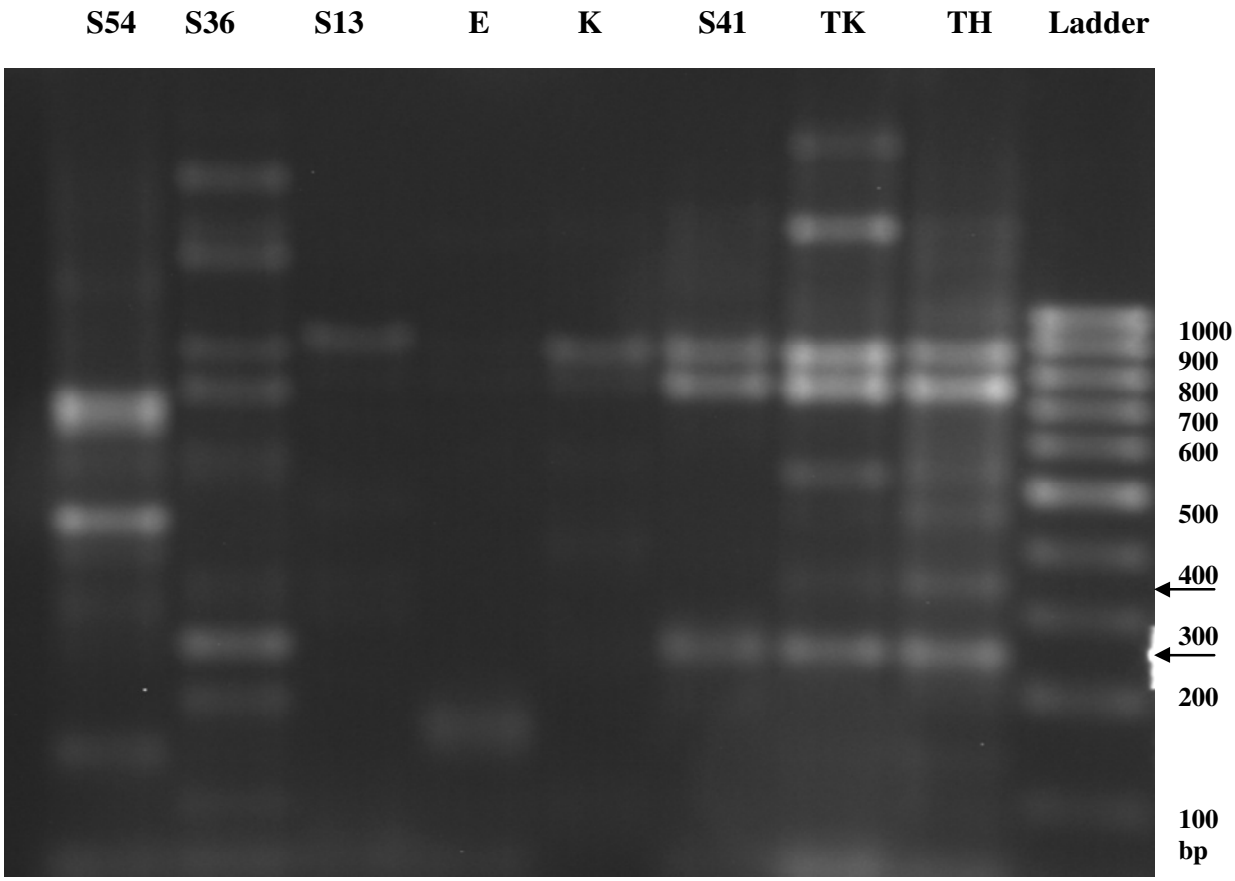

Figure 1. SSR electrophoresis profiles of 8 mulberry accession abbreviated at the top of the lanes, amplified using the primer SS02. The ladder is a DNA marker (100 bp). Arrows indicate some of the polymorphic bands.

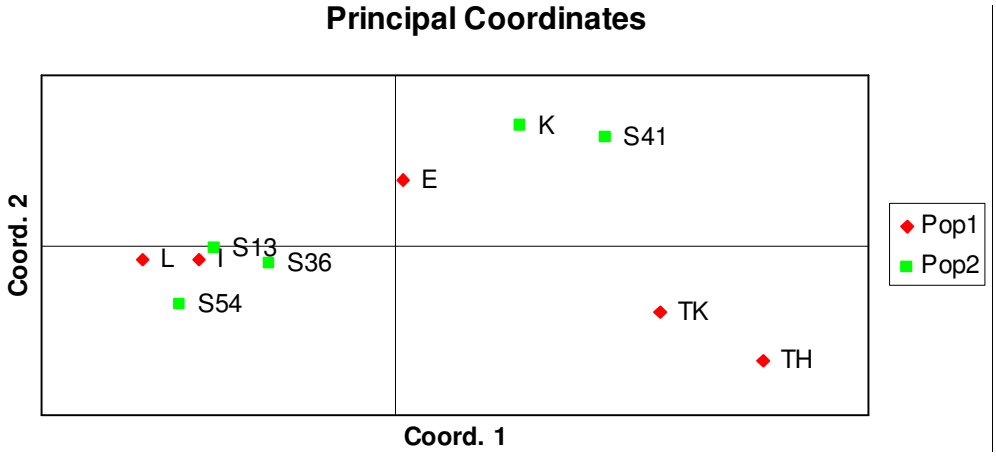

Figure 2. Scatter plot of 10 mulberry accessions based on first and second components of principal coordinate analysis using SSR data. Pop1 = *M. alba*, Pop 2 = *M. indica.*

systems. Cross pollinating species have a higher gene diversity as compared to self-pollinating species (Alvarez et al., 2001). This kind of species exhibit large gene pools, possibly as a result of widespread pollen and seed dispersal (Schierenbeck et al., 1997). Mulberry is believed to be pollinated mainly by wind (Datta, 2000). In addition, fruits are dispersed by birds and other large animals (Martin et al., 2002). This kind of long distance pollination and seed dispersal could explain why there is high genetic variability within species than among species.

The genetic similarity coefficients among accessions varied from 0.8455 to 0.4077 with a mean of 0.5362. Similar variation among mulberry genotypes with a genetic similarity ranging from 0.904 to 0.544 with a mean of 0.728 using ISSR markers has been observed (Vijayan et al., 2004a). Sharma et al. (2000) also reported a similar coefficient varying from 0.58 to 0.99 across 45 mulberry genotypes using AFLP markers. Using SSR markers, Zhao et al. (2007) reported a high mean genetic similarity of 0.6131 between wild and cultivated mulberry genotypes. This suggests that the genetic identity across the mulberry accessions studied represents a genetically diverse population.

The genetic distance across the mulberry accessions studied ranged from 0.1678 to 0.8972 with a mean of 0.5916. These results show a high extent of polymorphism across the ten mulberry accessions. Moreover, genetic divergence of 58 mulberry varieties was found to be high as determined by D^2 statistics of multivariate analyses. The genetic distance varied from 0.13 to 0.78 (Fotadar and Dandin, 1998). Other results on genetic distance of both exotic and indigenous mulberry varieties using DAMD, ISSR and RAPD markers showed a distance of 0.30 to 0.68, thereby indicating a wide genetic variability in mulberry. In addition, a minimum genetic distance of 0.049 and a maximum of 0.504 among mulberry genotypes has been reported (Prasanta et al., 2008). In general, the genetic distance reported in this study concurs with results of Prasanta et al. (2008) suggesting that mulberry accessions acquire a considerable amount of genetic diversity.

The genetic identity (I) between *M. alba* and *M. indica* species was 0.7613 suggesting close relationship between the two species. This is further supported by the low genetic distance between the species of 0.2727. Similar findings were reported by Tikader and Kamble (2008) and by Vijayan et al. (2004a).

The kind of genetic variation exhibited in this study can be attributed to the fact that mulberry accessions have been established and adapted in areas distant from their origin thereby considered “naturalized” (Sharma et al., 2000). The mulberry accessions in this study showed a close relationship between Thailand and Thika accessions while S41, Kanva-2 and Embu accessions clustered together. Further, S13, S36, S54, Ithanga and Limuru accession clustered together as evident from PCoA. These form of clustering showed a wide variability among the accessions regardless of their difference in species origin. Limuru, Ithanga, S13, S36 and S54 accessions grouped together revealing the fact that the two species *M. alba* and *M. indica* are closely related. These results are similar to those reported by Prasanta et al. (2008) using ISSR markers. Vijayan et al. (2006) also reported a genetic similarity between *M. alba* and *M. indica* species. Furthermore, Vijayan et al. (2004b) reported genetic closeness of *M. indica* and *M. alba* species using ISSR and RAPD markers.

According to principal coordinates analysis (PCoA), the first and second coordinates extracted 35.45 and 17.49% of the total variation, respectively. Similar results were reported by Zhao et al. (2007) with 30.3 and 25.5% of the total variation in the first and second coordinates using SSR markers in mulberry, respectively. A higher variation was detected by the first and second coordinates with 52.5 and 10.0%, respectively in mulberry using ISSR markers by Zhao et al. (2007). Thus, there is a high genetic variation among the mulberry accessions studied, however the two species, *M. alba* and *M. indica* are closely related.

Conclusion

A high polymorphism exists within than between mulberry species regardless of their geographical origins. The dendrogram and principal coordinates analysis clearly showed that these accessions are genetically diverse despite the two species (*M. alba* and *M. indica)* being genetically related.

ACKNOWLEDGEMENT

We express our hearty thanks to the VLIR AGIBIO Project for financial funding of this research. We also gratefully acknowledge the anonymous referees for corrections and suggestions to the manuscript.

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