Caspian J. Env. Sci. 2009, Vol. 7 No.1 pp. 23~30
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CJES
Caspian Journal of Environmental Sciences

[Research]

Diversification of mulberry (*Morus indica* var. S36), a vegetatively propagated tree species

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ABSTRACT
Genetic diversity among plants sprouted from seven year old clones of mulberry (*Morus indica* var. S36) was studied using molecular markers such as ISSR and RAPD. The clones showed significant variability in sex expression and the sprouting behavior. These changes were appeared after seven years of rigorous pruning and training (pruned once in every 3-4months). Genetic diversity analysis revealed significant DNA polymorphism differences among these clones. Through multiple regression analysis, four DNA markers associated with specific traits were identified. Cloning, sequencing and bioinformatics analyses suggested probable involvement of transposable elements along with enzymes and transcriptional regulating factors. The results of this preliminary report call for detailed analyses at biochemical, physiological and molecular levels coupled with inheritance pattern of these markers and their corresponding phenotypic traits. The markers identified for sprouting and sex expression are of much use in early identification of hybrids with the respective traits.

Keywords: Genetic diversity, ISSR, Mulberry, RAPD, Somatic mutation.

INTRODUCTION

Assessment of genetic variations in plant population has many potential uses for evolutionists, breeders and conservation biologists. Because the extent of genetic diversity within a population reflects the intensity of interactions among various evolutionary processes such as mutation, genetic drift, and natural selection (Wright 1978; Wu *et al.,* 1999). Understanding the effect of these evolutionary processes upon a crop species is of much significance to formulate appropriate strategies for conservation and utilization. Mulberry (Morus L.) is a tree belongs to the family of Moraceae, and is of great economic importance in countries where sericulture is widely practiced for producing silk fibers. Mulberry is highly amenable to vegetative propagation through stem cutting, budding, grafting and layering in India and other tropical countries. Thus, mulberry plantations developed from a variety through clonal propagation virtually hold a homozygous population. However, the repeated pruning and training (3-5 times per year) for maintaining the plants at a height convenient for leaf plucking and also to sustain the plant growth during the silkworm crop seasons, subject the plants to various kinds of stresses related to wound injury. Studies in many plants revealed that repeated pruning could result in generating variations (Tikader *et al.,* 1995; Wessler, 1996; Grandbastien, 1998). Further, it has also been reported that significant genetic variability could exist in clonal populations of vegetatively propagated crop species (Kreher *et al.,* 2000; Persson and Gustavsson, 2001). In this study, we have attempted to understand the genomic basis of a few changes appeared in a seven-year-old mulberry orchard developed from clones of a single variety and

maintained as low bush through rigorous pruning of seven years.

MATERIALS AND METHODS

The study was conducted in a population of mulberry variety, namely S36, grown continuously for the seven years by repeated pruning. S-36 was developed from Berhampore local (*Morus indica* L), an indigenous mulberry from West Bengal, India, through chemical mutagenesis (Sastry *et al.*, 1984). Cytologically, it is an aneuploid having the chromosome 2n=30 (Dandin *et al.*, 1983), as normal diploid mulberry has 2n=28 chromosomes. The plants were being maintained under similar conditions with recommended package of practices (Kasiviswanathan *et al.*, 1979). Though initially all the plant in the orchard produced only female catkins, after seven years of repeated pruning and training (the plants were pruned from the base after every three months of growth to maintain them as small bushes), some of the plants started producing two types of catkins (male and female). Similarly, some of the plants took more days to sprout after the pruning. Such striking morphological variations among the clones of a single variety have prompted us to undertake the present investigation. These selected plants were categorized as early sprouting males (SME), late sprouting males (SML), early sprouting females (SFE) and late sprouting females (SFE) for convenience. From each stump, the first three primary branches sprouted were chosen for recording the morphological data as well as for collecting the leaf samples to extract DNA. The DNA from the leaf samples was extracted with 'nucleon' phytopure plant DNA extraction kit (RPN 8510, Amersham Life science, England) following the instructions of the manufacturer. The required dilution of the DNA was achieved by quantification on 0.8% agarose gel electrophoresis followed by staining with ethidium bromide, using fixed concentr ations of uncut λ DNA as standard. The PCR amplifications of the genomic DNA with different primers were carried out on a MJ Research Thermo-Cycler (PTC 200) following different cycles as follows.

PCR amplification with RAPD primers

A total of 12 decamer primers (Table 2) from operon Technologies, Inc, USA were used in this study. The PCR was conducted according to Vijayan and Chatterjee (2003) The PCR product was separated on 1.5% agarose gel in 1x Tris Boric Acid buffer containing 5μg/ml ethidium bromide as stain. Photographs were taken under UV illumination with Nikon (FM2) camera using Kodak 400 ASA film. Scoring of bands was based on presence (1) or absence (0) of a particular band.

PCR amplification with ISSR primers

A total of 8 selected primers (Table 2) UBC (set # 9) were used for the PCR amplification as described earlier (Chatterjee *et al.*, 2004) The PCR product was resolved on 2.0% agarose gel and photographed as described above.

Statistical analysis of the data

The statistical analyses of the data were carried out with PHYLIP 3.5c (Felsenstein 1993) and SPSS/PC+ 10.0 (M.J. Norusis, SPSS Inc., Chicago). Significance of morphological data was estimated by analysis of variance (ANOVA) and means were separated by Duncan multiple range test at $P<0.05$ significant level. The genetic distance among the genotype was calculated following the method of Nei & Li (1979) as the genetic similarity (S) was first calculated as $S= 2Nab/Na+Nb$, where Nab is the number of bands shared by the individuals a and b; Na and Nb are the number of bands in individuals a and b, respectively. The distance index (D) was calculated as 1-S. The average genetic distance (NIED) was also calculated for each clone to identify the most distant ones among the clones. Cluster analysis of the data was carried out using UPGMA method (Sneath and Sokal, 1973), which assumes a constant rate of evolution among the species. Stepwise linear regression analysis was used for identifying markers associated with sex and sprouting. The association of these bands with respective characters was further verified through Beta curve estimation.

Cloning and sequencing of DNA markers

Markers associated with specific traits were excised from the PCR products resolved

on agarose gel (1.5%) and the DNA was eluted using QIAGEN's QIAquick gel extraction kit (QIAGEN, GmbH, D-40724, Hiden). The eluted DNA fragments were cloned to E. coli DH5α using InsT/A cloning kit (MBI, Fermentas, USA) following the manufacture's instruction. Sequencing of the cloned DNA markers was done using ABI PRISM dye terminator cycle sequencer.

RESULTS

Plant height, leaf per branches and number of stomata per unit area showed significant differences among the eight selected individuals (Table 1). The average number of days taken to initiate the sprouting after pruning was varied from 5.0 ± 0.00 in early sprouting clones to 7.07±0.17 late sprouting clones. The average plant height in the former group was 41.72±2.05 cm but the same in late sprouters was 31.38±0.78 cm. Thus, a difference of 10cm in growth was observed between these two groups. The average number of leaves per primary branches also varied from 8.33±0.49 to 10. 00±0.311. The frequency of stomata per unit area was 31.46± 1.74 for early sprouting while it was 37.5±1.32 for late sprouting.

Molecular profiling

The PCR amplifications with the eight ISSR primers yielded 78 markers of which 42 were found polymorphic. (52.85%) (Table 2). The number of markers generated by each primer varied from four by ISSR-864 to 15 by ISSR-835 and ISSR-881. Maximum polymer-phism (73%) was observed in the PCR products of the primer ISSR-881. The RAPD primers also generated an average of 50.0% polymorphism among the clones.

The genetic dissimilarity estimated among the clones according to the formula of Nie and Li (1979) showed considerable genetic differences among the clones. The genetic distance generated by the ISSR markers among the clones (Table 3) showed that the genetic distance between clones of the same group (data in bold letter) was much less than that between two different groups. This clearly points to the fact that considerable genetic diversification has occurred among these groups. This observation was further confirmed through cluster analysis using UPGMA (Fig. 1). In the dendrogram, it could be seen that the clones made 4 primary groups on the basis of their genetic closeness as they are belonging to the same types. For instance, the first primary group comprised of the clone SME-1 and SME-2; both are male plants with early sprouting habits. Similarly, the other groups contain clones of the same types. In the secondary grouping, sprouting has taken predominance than the sex as the clones SME-1, SME-2, SFE-1 and SFE-2 were grouped in one cluster. However, the cluster C and D stood far apart and in the tertiary grouping, the cluster C has joined itself with the cluster E. This clearly shows that the clones SME-1 and SML-1 are genetically more distant from the clones SML-1 and SML-2, though both express the same sex.

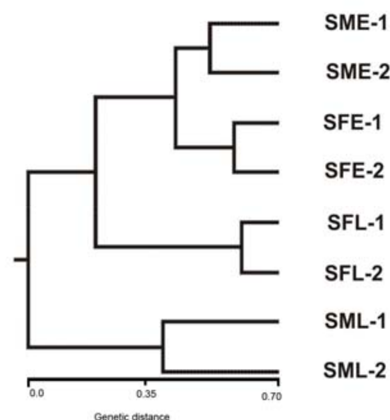

Fig 1. Dendrogram realized from ISSR markers. Abbreviations of clones are as given elsewhere.

The genetic variability estimated from the RAPD markers varied from 0.217 between the clones SME-1 and SML-2 to 0.055 between SFE-1 and SFE-2 (Table 4). However, the intra-group variability was much less compared to the inter-group variability. In this case, as well, the dendrogram realized through UPGMA showed that the clones grouped primarily on the basis of their close genetic affinity as the clone SME-1 and SME-2; both are male plants with early sprouting habits (Fig. 2). Similarly, the other clones also grouped in the same lines. However, in the secondary grouping sex has showed its predominance as all the female clones were grouped into cluster V. Here also it is quite presumable that the cluster IV and I showed maximum genetic distance, though both are belonging to the same sexual group.

The stepwise linear regression analysis has identified four markers closely associated with the changes in the sex expression and sprouting behavior. Among the ISSR markers, ISSR-830.560 (Fig. 3) was found associated with early sprouting while ISSR-825.450 with female sex. Similarly, among the RAPD markers, OPY-5.700 and OPY-15.1200 (Fig. 4) were intimately associated with early sprouting and male sex respectively.

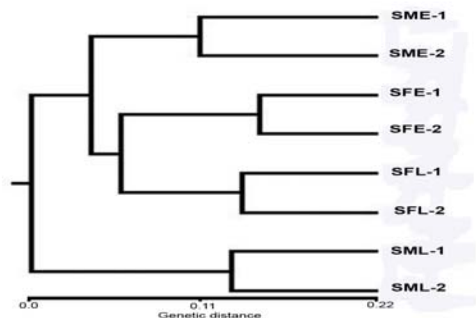

Fig 2. Dendrogram realized from RAPD markers. Abbreviations of clones are as given elsewhere.

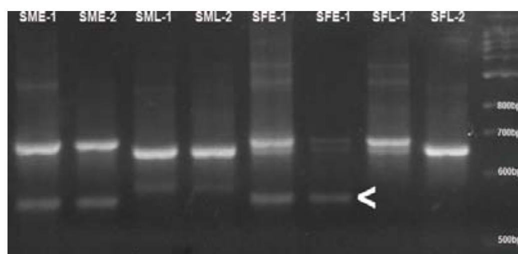

Fig 3. PCR products showing marker ISSR-830.560 associated with early sprouting in mulberry. Abbreviations of clones are as given elsewhere.

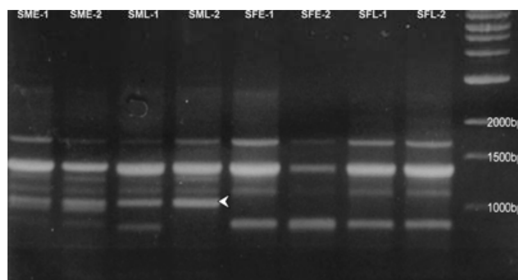

Fig. 4. PCR products showing marker OPY-15.1200 associated with male sex in mulberry. Abbreviations of clones are as given elsewhere.

Table 1. Morphological characters of eight clones of mulberry var. S3-6.

Clone number	Sex	Sprouting	Plant height	Leaves/branch	Leaf size	Stomata/unit area
SME-1	Male	5a	49.90b	14.00ab	155.86b	24.00a
SME-2	Male	5a	37.54b	11.00ab	161.02b	25.00a
SML-1	Male	8b	35.33b	10.67a	135.54a	29.83a
SML-2	Male	8b	30.30a	9.33a	164.44b	36.50a
SFE-1	Female	5a	49.20b	15.00b	169.67b	27.83a
SFE-2	Female	5a	30.27a	10.33a	130.03a	37.67a
SFL-1	Female	7ab	26.93a	8.33a	126.13a	39.17a
SFL-2	Female	9b	32.97ab	11.67ab	127.87a	44.50b

*Values in the same column with different superscript letters are significantly different at P=0.05. Abbreviations of clones are as given in the material and method section.

Table 2. Polymorphism generated by different primers on eight clones of mulberry (*Morus indica* var. S-36).

Primer code	Sequence	Total bands	Polymorphic bands	Polymorphism (%)
ISSR				
825	ACACACACACACACACT	11	8	72.73
830	TGTGTGTGTGTGTGTGG	8	4	50.00
834	AGAGAGAGAGAGAGAGYT	9	2	22.22
835	AGAGAGAGAGAGAGAGYC	15	9	60.00
861	ACCACCACCACCACCACC	7	3	42.86
862	AGCAGCAGCAGCGACAGC	9	4	44.44
864	ATGATGATGATGATGATG	4	1	25.00
881	GGGTGGGTGGGTGGGTG	15	11	73.33
Total		78	42	53.85
RAPD				
OPY-5	GGCTGCGACA	12	5	41.67
OPY-7	AGAGCCGTCA	12	7	58.33
OPY-9	AGCAGCGCAC	7	7	14.29
OPY11	AGACGATGGG	6	1	16.67
OPY-13	GGGTCTCGGT	8	5	62.50
OPY14	GGTCGATCTG	9	7	77.78
OPY15	AGTCGCCCTT	11	5	45.45
OPY-19	TGAGGGTCCC	4	0	0.00
OPW-6	AGGCCCGATG	15	9	60.00
OPW-7	CTGGACGTCA	8	5	62.50
OPW8	GACTGCCTCT	8	5	62.50
OPW17	GTCCTGGGTT	4	2	50.00
Total		104	52	50.00

Table 3 .Genetic dissimilarity among eight clones of Mulberry (Morus indica var.S-36) revealed by ISSR markers.

Clone	SME-1	SME-2	SML-1	SML-2	SFE-1	SFE-2	SFL-1	SFL-2	NIED
SME-1	0.000								3.096
SME-2	0.182*	0.000							3.287
SML-1	0.750	0.706	0.000						4.332
SML-2	0.667	0.833	0.313	0.000					4.456
SFE-1	0.208	0.333	0.789	0.700	0.000				3.193
SFE-2	0.289	0.282	0.714	0.784	0.116	0.000			3.161
SFL-1	0.489	0.463	0.459	0.538	0.511	0.476	0.000		3.033
SFL-2	0.511	0.487	0.600	0.622	0.535	0.500	0.095	0.000	3.350

* Data in bold letters show the intra group distance. Abbreviations of clones are as given elsewhere.

Table 4 .Genetic dissimilarity among eight clones of Mulberry (*Morus indica* var.S-36) revealed by RAPD markers.

Clone	SME-1	SME-2	SML-1	SML-2	SFE-1	SFE-2	SFL-1	SFL-2	NIED
SME-1	0.000								1.064
SME-2	0.083*	0.000							0.892
SML-1	0.202	0.124	0.000						1.008
SML-2	0.217	0.166	0.068	0.000					1.086
SFE-1	0.120	0.139	0.175	0.165	0.000				0.884
SFE-2	0.131	0.114	0.176	0.179	0.055	0.000			0.908
SFL-1	0.148	0.132	0.130	0.132	0.108	0.132	0.000		0.845
SFL-2	0.162	0.134	0.132	0.160	0.122	0.121	0.063	0.000	0.893

* Data in bold letters show the intra-group distance. Abbreviations of clones are as given elsewhere.

The nucleotide sequences of these markers were deposited in the GenBank. The acession numbers for these sequences are AF502074, EU019836 and EU024694 for ISSR830.560, ISSR-825.450 and OPY-5.700 respectively. The sequencing of the fragment OPY-15.1200 could not be done. The BLAST search of these sequences showed that the marker ISSR-830.560 did not show any significant similarity with any of the known genes. However, the sequences of ISSR-825.450 showed significant similarity with NADH-1 sequences of the mitochondrial genome of Nicotiana tabacum (2e-142), genomic DNA of Vitis vinifera (6e-143), mitochondrial trnC, trnN1, trnY, NAD2 genes of Solanum tuberosum (3e-133) and mitochondrial genomic DNA of Beta vulgaris subsp. vulgaris (2e-79). This indicates that this marker could be from the mitochondrial genome. The sequence of the RAPD marker OPY-5.700 also did not show any significant similarity with any known DNA sequence. Further search for the presence of any transposable elements within these markers revealed that in the sequence of the marker ISSR-830.560, the first 16 bases has 100% homology with transposable elements with an e-value of 0.009. Similarly, 18 base pair homology was observed in the marker 825.450 with that of the transposable elements. In the case of OPY5.700 the first 13 bases showed homology with transposable elements (e=4e-04). Search for open reading frame (ORF) with in these sequences has showed that the sequences of the marker ISSR-830.560 holds three ORF regions. From the base 2-127 (LGKKPPFLADDIFIVCVCV AISFVSLLSF LRFLICCCLVG), 226-345 (MFH FDPWPITLFKFRSFQWLSIIYLIRTEKLEIEIG KKN), and 405-560 (MFPITVSDFVSLIVVF TFSYFLSFVSLVVVFMASVVFNFVVCLIFCL VGVFR). Further search with these amino acid sequences using ExPASY BLAST revealed that none of these putative proteins has homology with any of the known protein. The marker ISSR-825.450 was having four short ORFs in the regions 2-127; (MNSLAPRSIRSEPGSRTFSHRFPS PSHRQK EV LIPWCQRATN) 68-220 (MREG SRTGFR PNRARSQRVQSNSVTSLRAGSSLKGMDGR RLNSERGPPCL) 229-405 (CSR RARDPISR PSGGLFSVFSIQCPHCVRKSSSSISFRNAINK SNSLSRHXSFLLSH) and 266-406 (MLDLFIA LRKEIEDEDFL TQCGHW MEKTENNPPE GRLIGSRARRLQR) respectively. The BLAST search with ExPASY showed that the putative protein from the first ORF has strong homology with an N-terminal protein precursor (A4WQ04_RHOSH) and a hypothetical membrane protein (A2QQ91 _ASPNG). In the case of the second ORF, it has strong similarity with a hypothetical protein of Vitis vinifera (A5BTN0_VIT VI) and an AT-rich interactive domain-containing protein 3A (RI3A_HUMAN). The third one has strong homology with Diguanylate cyclase (Q39S35_GEOMG) and the forth one has similarity with the Zinc finger protein 127 (Q60764). The partial sequence

obtained from the RAPD marker OPY-5.700 contained two ORFS from 1-117 (MVSCI LACEILRINRGDWGECGFCENSNFQMSIVE GRL) and 339-476 (MIYLLR HTGIKTQ YCKRYNKWKLQHRESADLFSSDSTKKKKS QKN) positions respectively. ExPASY search for homology with known proteins reveled that the first putative protein has high similarity with a Glycoprotein M (Q77NJ4 _9GAMA) and the second protein has similarity with Integrase, catalytic region precursor (A0UFP6_9BURK) and a Transposase [istA] (Q5TM11_9BURK). Thus, it is seen that the markers associated with sprouting do not have any known homologous DNA sequ ences.

DISCUSSION

This study has demonstrated that in vegetatively propagated species significant genetic variability can accumulate due to somatic mutation caused by various factors like point mutation, somatic recombination and retrotransposon activity. It is a known fact that changes brought about by these agents may not immediately be visible at morphological or proteins level. Nei (1987) reported that due to codon degeneracy approximately 29% of the mutations occurring at the molecular level could not be detected by amino acid assay. However, accumulation of this kind of mutations would finally come to a level where it has to express biochemically and morphologically. In this context, it is important to note that the variety S-36 was developed through treatment of seeds with a chemical mutagent (Ethyl Methyl Sulphonate). Hence, the chance of occurrence of these kinds of silent mutation was quite high, which in the passage of time might have accumulated and started expressing. Similarly, it is also known that almost all plant retrotransposons so far characterized are inactive during the normal growth and development but would be activated by different types of stress (Wessler, 1996; Grandbastien, 1998). The recent discovery that many wild type plant genes have retrotransposon-derived sequence in their regulatory region (Bennetzen, 2000) underscores the role that retrotransposon insertion may have played an influencing role in the evolution of gene structure and its expression pattern. Given the prevalence of repeated pruning in mulberry and the presence of transposon elements in the genus Morus (AY083674), an active role of the retrotransposons in generating such genetic variations among the clones cannot be ruled out.

The presence of genetic diversity among the clones of vegetatively propagated plants has also been investigated in many other crops. Breto *et al.,* (2001) studied a large number of varieties of Citrus clementina, a vegetatively propagated crop species developed through selection of spontaneous mutations affecting the agronomic traits, and found that the genetic variability unraveled by molecular markers was minimum compared to the variability expressed by agronomic traits. Kreher et al. (2001) using RAPD technique established the presence of significant genetic variability within the clonally propagated shrubs Vaccinium stamineum, Persson and Gustavsson (2001) also analyzed the genetic variability within and among the clonal populations of lingoberry using RAPD markers. A high rate of genotypic diversity (D: mean 0.84) and evenness (E: mean 0.81) were found for the clones. Analysis of molecular variance revealed that most of the variation (89.2%) resides within populations. Like wise, AFLP fingerprinting revealed significant genetic variability within clonal populations of dwarf bamboo (Suyama et al., 2000). In this context, it is to be admitted that molecular technique was applied more for genetic analysis of annual crops than for perennial trees. All these studies points to the fact that heterogeneity is the order of the Nature, hence the homozygous clonal populations of vegetatively propagated plants gather minute often unobservable changes in the passage of time and accumulation of these changes later brings about visible and ergonomically significant changes in the plant population.

Further, the intra-group genetic variability unraveled in this study could be of much use in identifying duplicates in germless accessions as the minimum genetic distance needs to be considered for separating two closely related accessions often posses great difficulty to the conservationists. In the present study, though all the clones belong to the same variety, it could be seen that considerable genetic diversity present among these clones. The clones belong to the same group, however, possessed minimum

variation. This clearly indicates that the changes in the morphologic traits have genetic bases and the expression of those traits was not merely due to environmental effects. On the line of sex, the clones were grouped into male and females. On the basis of sprouting these clones were further diversified into early and late sprouting groups.

The sex expression of plants appears to be a function of hormonal control; there seems to be evidence that dioeciously plants (male and female reproductive organs on separate plants) are potentially bisexual and that a delicate internal hormonal balance results in one sex "over-riding" the other. A traumatic incident might, therefore, act by temporarily upsetting this balance, which is later restored in a way that leads to the opposite sexual character being manifested. Another pointer in this direction is the fact that a variety of artificially applied chemical substances has been shown to affect the sexual expression of a significant number of different plants. It is also reported that some chemicals known as the steroid hormones might be a major key to the sex expression lock (Genus, 1982). Thus, it is possible that to "create" a new sex in a plant by either a physical shock or a controlled chemical process (Osborne, 1985). Effect of application of hormones and chemicals on the sex expression has also been reported from mulberry. Das and Mukherjee (1992) and Tikader et al. (1995) observed sex reversal upon hormonal application or pruning of branches. Lal and Jaiswal (1988) found qualitative and quantitative changes in the activities of the enzyme phosphatase in male and female flower buds. Application of phthalimides also changed the sex expression in Morus nigraL. Thus is obvious that hormonal balance can tilt the expression of sex in mulberry. In the present study the mutation caused by repeated pruning and training may have changed the hormonal balance in favor of male sex expression.

Thus, from this study it is concluded that repeated pruning and training can bring changes in plants at the genomic levels, which was evident from the present study where seven years rigorous pruning at a regular interval of three months brought remarkable changes in a mulberry variety. However, it is to be mentioned that detailed studies involving inheritance pattern, biochemical and physiological basis of these changes are essential to understand more about these variations. Since mulberry is a highly heterozygous tree species with a juvenile period of 3-4 years, studying the inheritance pattern at least up to F2 generation would take a minimum of 10-15 years. Nevertheless, such a study would be undertaken as a second phase of the present investigation. Further, it is necessary to be mentioned here that, a close vigil on populations of clonally multiplied plants may be helpful on spotting, identifying and isolating beneficial mutants like stress resistant, and high yielding plants in mulberry. Similarly, the markers identified for sprouting and sex would be of much use for breeders as this will help them to identify early and late sprouting hybrids with their respective sex at early stages of selection.

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(Received: Nov. 25- 2007, Accepted: Jan. 6 2008)