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Analysis of Genetic Diversity of *Persea bombycina* "Som" Using RAPD-Based Molecular Markers

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Abstract The utility of RAPD markers in assessing genetic diversity and phenetic relationships in *Persea bombycina*, a major tree species for golden silk (muga) production, was investigated using 48 genotypes from northeast India. Thirteen RAPD primer combinations generated 93 bands. On average, seven RAPD fragments were amplified per reaction. In a UPGMA phenetic dendrogram based on Jaccard's coefficient, the *P. bombycina* accessions showed a high level of genetic variation, as indicated by genetic similarity. The grouping in the phenogram was highly consistent, as indicated by high values of cophenetic correlation and high bootstrap values at the key nodes. The accessions were scattered on a plot derived from principal correspondence analysis. The study concluded that the high level of genetic diversity in the *P. bombycina* accessions may be attributed to the species' outcrossing nature. This study may be useful in identifying diverse genetic stocks of *P. bombycina*, which may then be conserved on a priority basis.

Keywords *Persea bombycina* · Muga silk · Genetic diversity · Molecular markers · RAPD

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Introduction

Persea bombycina (King ex Hook. f.) Kost, formerly named *Machilus bombycina* King, is one of the most significant principal host plants of *Antheraea assama* Westwood, the silkworm that produces muga, or golden, silk (Gogoi 1980; Choudhury 1981; Yadav and Goswami 1992). It is a member of the Lauraceae family. The genus *Persea* consists of about 100 species of evergreen trees or shrubs distributed mainly in the tropical and subtropical areas of Asia (Liu et al. 1994). The commercial use of *P. bombycina*, or Som, is restricted to northeast India, although the plant grows abundantly in almost all parts of India, Nepal, Myanmar, Cambodia, Malaysia, and Indonesia (Seth 2000). The northeastern region of India (latitude 22°–29° N, longitude 89°–98° E), comprising Assam, Meghalaya, Manipur, Tripura, Mizoram, Arunachal Pradesh, and Nagaland, with its sericulture fauna and corresponding host plants, is known for its seri-biodiversity and the presence of three silk industries (mulberry, muga, and eri silk).

According to Hooker (1885), India has 16 species of *Persea*, whereas Kanjilal et al. (1990) reported 6 species. The species are so similar morphologically and physiologically that they can hardly be distinguished from one another. Tazima and Choudhury (2005) reported five ecotypes of Som based on the shape of the leaves: "Naharpatiya," "Azarpatiya," "Ampatiya," "Jampatiya," and "Bahpatiya." As leaves are the solitary dietary source for the muga silkworm, which converts them directly to silk fibers, high-value silk production requires improved leaf quality of the host plant. Researchers have reported variations among Som genotypes based on morphology (Choudhury 1981; Raja et al. 1993) and taste of leaves (Bharali 1971), but no systematic study has been done for Som at the biochemical, physiological, or molecular level.

Molecular markers successfully developed during the last two decades have largely overcome the problems associated with phenotype-based classification (Awasthi et al. 2004; Orhan et al. 2007). Moreover, for breeding or improving promising cultivars, precise determination and discrimination of the genotype are required. The most desirable varieties of food plants and a suitable silkworm race with disease resistance or high-yield characteristics can be developed using molecular markers in selection and breeding.

Random amplified polymorphic DNA (RAPD) markers were first introduced by Williams et al. (1990). The simplicity, efficiency, and relative ease of performing RAPD techniques, without sequence information (Khanuja et al. 1998), have led to their expanded use for taxonomic and systemic analysis and phylogenetic studies of plants (Rath et al. 1998; Bartish et al. 2000; Meimberg et al. 2001, 2006; Chaveerach et al. 2002; Hug and Roger 2007; Mokkamul et al. 2007), species differentiation (Lihova et al. 2000; Wallace 2002; Zhang et al. 2007; Verma et al. 2007), and phylogeographic variation (Chaveerach et al. 2006; Wu et al. 2006), as well as for studying breeding and genetic relationships. RAPD techniques have recently been used in genetic diversity studies for plants like *Astragalus* (Adiguzel et al. 2006), *Bambusa* spp. (Sun et al. 2005), *Dysosma versipellis* (Qui et al. 2006), and *Calliandra* Benth (Mattagaja Singh et al. 2006).

Characterizing the types and extent of genetic variation is essential to identifying genotypes so that they can be effectively used by breeders, geneticists, and conservationists (Orhan et al. 2007). Earlier classifications and evaluations of *P. bombycina* were based solely on morphological and physiological characteristics, which are easily influenced by the environment. Although reliable and consistent classification can be obtained through genetic information, a literature survey failed to uncover any published work in this area. Moreover, given the lack of knowledge of genetic differentiation among the Som genotypes and the wild nature of the muga silkworm, it is difficult for farmers to retain a uniform quality of silk, a circumstance that directly affects its market value. Hence, we set out to study genetic variation among Som genotypes, using RAPD markers, and any correlation between the genotypes and the areas from which they were collected.

Materials and Methods

Sample Collection

Young leaves were selectively collected from 36 *Persea bombycina* plants from the Som germplasm at the Central Muga Eri Research and Training Institute (CMERTI), Lahdoigarh, Jorhat, and from 12 plants from the germplasm at the North-East Institute of Science and Technology (NEIST), Jorhat, Assam, India. After collection, these specimens were lyophilized, placed in sealed plastic bags, and chilled (−80°C) until genomic DNA was extracted.

DNA Extraction

Lyophilized leaves (about 200 mg) were powdered, using a mortar and pestle, with a small amount of silica gel (60–129 mesh size) and extracted using the modified CTAB method (Doyle and Doyle 1990). The quantity of genomic DNA was determined by electrophoresis on a 0.8% agarose gel against a known quantity of unrestricted lambda DNA.

RAPD Fingerprinting

To optimize PCR amplification, experiments were carried out with varying concentrations of template DNA, primer, *Taq* polymerase, and $MgCl_2$, as well as dNTPs. For PCR-RAPD analysis, 13 primers were used from the OPA, OPAA, OPO, OPBH, and OPB series of Operon Technologies (Alameda, CA). Amplification was performed on an ATC401 Thermal Cycler (4.8 version) with 15 µl reaction mixtures containing 0.05 µg template DNA, 0.4 mM each dNTP (dATP, dTTP, dCTP, and dGTP), 0.25 U Hot Start *Taq* DNA polymerase, 10 pmol each primer, and 2.4 mM $MgCl_2$ (all from Fermentas, MBI, USA).

The amplification regime was as follows: 95°C for 10 min; 94°C for 2 min; then 35 cycles at 94°C for 45 s, 35°C for 45 s, and 72°C for 1.45 min; and a final extension at 72°C for 6 min. Amplified PCR products (15 µl) were separated on an

agarose gel (1.5%; w/v) in 1× TBE buffer at 70 V for 150 min. The gel was visualized by ethidium bromide staining and photographed under UV light by a Gel Doc system (G:Box HR, Syngene, UK).

Data Analysis

Each gel was analyzed by scoring the bands as present (1) or absent (0). The data matrices were entered into the NTsys version 2.02K package, a pairwise comparison of populations was made, and genetic diversity parameters were determined. Genetic similarities based on Jaccard's coefficient were calculated among all possible pairs, using the Simqual option, and ordered in a similarity matrix. A dendrogram was constructed by the unweighted pair group method with arithmetic average (UPGMA; Sokal and Sneath 1963) to group individuals into discrete clusters. The ability of the primers to distinguish between accessions was assessed by calculating their resolving power, R_p (Prevost and Wilkinson 1999), using the formula $R_p = \sum I_b$, where I_b (band informativeness) $= 1 - (2 \times |0.5 - p|)$; p is the proportion of accessions containing band I. This function has been found to correlate strongly with the ability to distinguish between genotypes. The goodness of fit and the robustness of the dendrogram were tested (by comparison with a dendrogram obtained using a different coefficient) by bootstrapping using the Winboot software developed at IRRI. A minimum of 1,000 bootstraps was used in the analysis to compute probabilities as percentages for each node of the tree.

Results and Discussion

Genomic DNA was extracted from Som leaves by a modified Doyle and Doyle (1990) method. Due to the presence of high concentrations of phenolics, additional chloroform:isoamyl alcohol (24:1) steps were performed. High-purity DNA was selected for RAPD studies. Genomic DNA was extracted using the modified CTAB method, adding polyvinylpyrrolidone (PVP) to overcome the problem of phenolics in the leaf tissue. These secondary compounds generally accumulate in tissues in higher plants, so the problem becomes severe as the material gets older (as observed in this case). Contamination with polysaccharides is particularly problematic (Scott and Playford 1996), as they inhibit the activity of many commonly used molecular biological enzymes, such as *Taq* polymerases, ligase, and restriction endonucleases (Fang et al. 1992). The enzymes form tight complexes with polysaccharides, creating a gelatinous pellet, and the embedded DNA is inaccessible to the enzymes (Sharma et al. 2002). Polyphenol contamination of DNA makes it resistant to restriction enzymes, as has been shown in other taxa where polyphenols copurify with DNA (Katterman and Shattuck 1983) and interact irreversibly with proteins and nucleic acids (Loomis 1974). This phenomenon is mainly due to oxidation of polyphenols to quinones by polyphenol oxidase, followed by covalent coupling or by oxidation of the proteins by the quinones. During homogenization, polyphenols are released from the vacuoles and then react rapidly with cytoplasmic enzymes. DNA isolation protocols generally use CTAB to avoid copurifying polysaccharides

from plant tissues. Keeping this in mind, along with the high amounts of polysaccharides in *P. bombycina* samples, we tried the standard CTAB method (Doyle and Doyle 1990) and also some modifications, including the addition of PVP. A similar approach was used by other workers, adding PVP at different concentrations to the extraction buffer (2% by Dellaporta et al. 1983; 4% by Keb Llanes et al. 2002). These modifications were tried either alone or in combinations, but the DNA yield remained unsatisfactory in terms of quality, restrictability, and amplifiability.

We screened a total of 140 decamer oligonucleotide primers for their ability to generate a consistently amplified band pattern and to assess polymorphism in the tested genotypes. We discarded poorly stained, unique, and very low-frequency bands from the data set used for further analysis. Among the 140 primers tested, 13 selected primers generated 93 bands, of which 48 were polymorphic (Fig. 1). The level of polymorphism revealed by this study was high. Morphologically, the selected genotypes did not show any variation. In contrast, Bhat and Jarret (1995) reported that the use of a small number of primers is sufficient when morphological variation is high. The number of bands per accession ranged from 1 to 11, and the size range of the amplified bands was 250–6000 bp (Table 1). The number of bands per primer ranged from 5 to 11, with a mean of 7.3. The proportion of polymorphic bands across the primers was 12.5–90%, with an average of 59.7%. Estimation of the R_p values exhibited a collective rate of 34, varying from 0.8 for primer OPA-13 to 5.5 for OPO-20, with a mean of 2.6. Relative to variation reported in the South American conifer *Fitzroya cupressoides* (Allnutt et al. 1999), *Populus tremuloides* (Yeh et al. 1995), and the endangered tropical tree *Plathymenia reticuclata* (Lacerda et al. 2001), genetic variation in *P. bombycina* appears to be moderate. Although individual primers used in this study were not able to distinguish all the accessions, a combination of some of the efficient primers with a higher R_p value (OPO-20, OPBH-03, OPAA-16, OPAA-01) can be used to assess genetic diversity (Table 1). Rates and patterns of dispersal and migration between populations will affect the genetic structure of a species: the higher the dispersal ability, the lower the population structuring (Clegg et al. 2002).

According to the genetic distances obtained and the relative position of each accession in the UPGMA tree, using a combined data set for the 13 primers revealed three clusters with 80% similarity. The major group consisted of 24 accessions from CMERTI, Lahdoigarh, whereas the second major group comprised germplasm collected from both CMERTI and NEIST. Two accessions from NEIST (42 and 41) formed a third group. This poor documentation of genotypes is associated with widespread transportation of stock material between the places of collection.

Bands were scored for their presence or absence in 48 accessions of *P. bombycina*. The pairwise similarity values between the genotypes, based on both shared and unique amplification products, were calculated using Jaccard's coefficient. The average value of genetic similarity (GS) shared by 928 accession pairs was found to be 0.78. A similarity matrix (SM) revealed accession pairs 37,38 and 40,45 as the most similar, with similarity values of 0.9876 and 0.9883, respectively. High similarity values were also obtained between accession pairs 28,30 (0.9381) and 27,31 (0.9333). The most diverse genotypes, as revealed by our

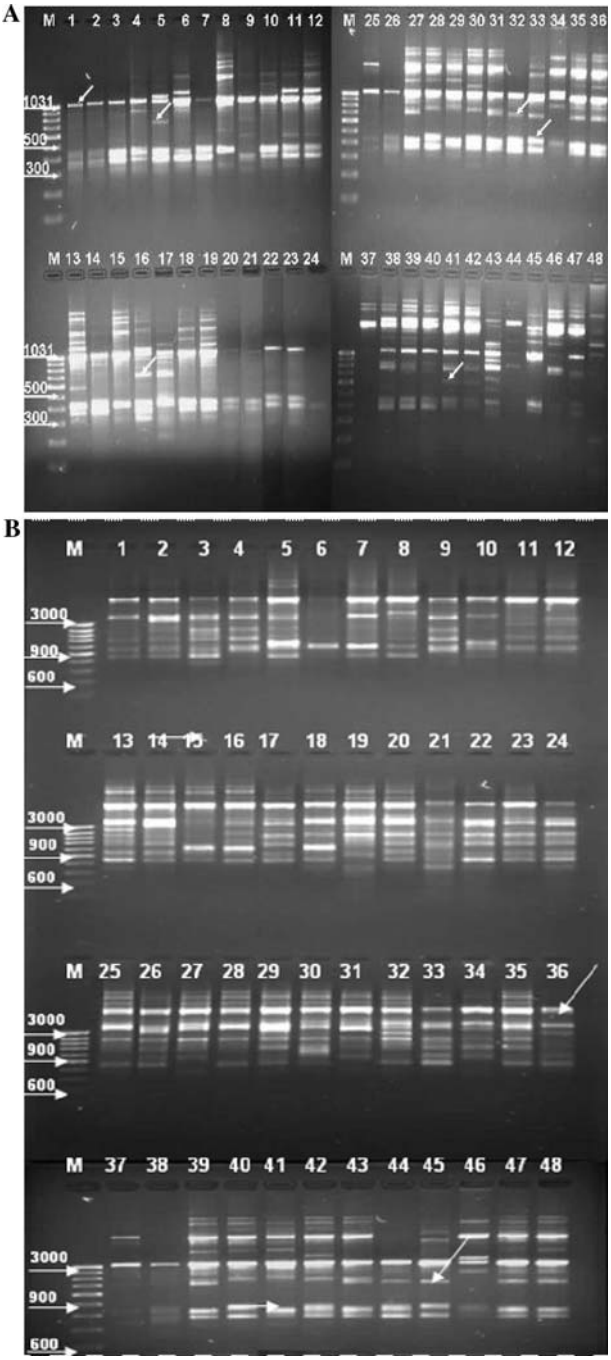

Fig. 1 RAPD profiles of 48 polymorphic accessions generated with primer OPO-20 (**a**) and primer OPAA-01 (**b**). *Arrows* indicate polymorphic and monomorphic bands

Table 1 Banding profile produced by selected RAPD primers

No.	Primer	Sequence 5′→3′	Total bands	Polymorphic bands	PPB	R_p value	Range of band size amplified (bp)
1	OPO-02	ACGTAGCGTC	11	8	72.2	1.9	4000–800
2	OPAA-01	AGACGGCTCC	8	4	50	3.3	6000–750
3	OPA-13	CAGCACCCAC	7	5	71.4	0.8	700–250
4	OPB-17	AGGGAACGAG	6	5	83.3	1.1	900–250
5	OPB-04	GGACTGGAGT	10	3	30	1.8	800–400
6	OPC-05	GATGACCGCC	8	2	25	2.3	1500–200
7	OPO-20	ACACACGCTG	8	1	12.5	5.5	3000–450
8	OPAA-16	GGAACCCACA	7	3	42.9	3.3	900–200
9	OPAA-15	ACGGAAGCCC	7	2	28.6	2.2	1500–400
10	OPAA-06	GTGGGTGCCA	5	4	80	2.7	1200–400
11	OPBH-04	ACCTGCCAAC	5	2	40	2.8	1700–250
12	OPBH-03	GGAGCAGCAA	6	5	83.3	4.7	750–250
13	OPBH-10	GTGTGCCTGG	5	4	80	1.6	7000–950
Total			93	48		30.0	

PPB proportion of polymorphic bands

study, were accessions 16 and 41, which shared the lowest similarity value, 0.7126. The phenetic UPGMA dendrogram based on these genetic similarity values grouped the 48 accessions into two major clusters and one minor cluster (Fig. 2). There is great genetic variation among the accessions belonging to cluster 1. Four accessions grouped in this cluster had a low overall GS value of approximately 0.80. The GS value between accessions 15 and 17 was 0.79. Similar values were obtained between other accessions that were grouped in this cluster. Clustering of different genotypes into distinct groups was not readily apparent in cluster 2. From the data analysis, it is evident that accessions 41 and 42 do not fall into either cluster 1 or cluster 2; they instead form a distinct group of their own. The phenograms generated from RAPD data revealed a highly consistent pattern of grouping, thus strengthening the validity of our data. Evaluation of the degree of support for clusters within the dendrogram through bootstrap analysis revealed high confidence values around the key nodes, such as at cluster 3 (98.9), and at the junction of the three clusters (42.6). The majority of the subclusters were present in the consensus tree; a few minor clusters supported by weak confidence values were absent. Similarly, a matrix based on the SM coefficient was also calculated, and the phenogram generated from this matrix reflected a similar pattern of grouping (not shown).

The coefficient of correlation (r) between the similarity matrices (generated from Jaccard's and SM coefficients), as well as the cophenetic correlation, was determined by a Mantel test (Mantel 1967). The cophenetic correlation was fairly high (Jaccard's: $r = 0.89$, $p = 1$; SM: $r = 0.87$, $p = 1$), indicating that no distortion of the similarity matrices had occurred while constructing the phenograms using UPGMA analysis. Congruency in the similarity matrices generated by

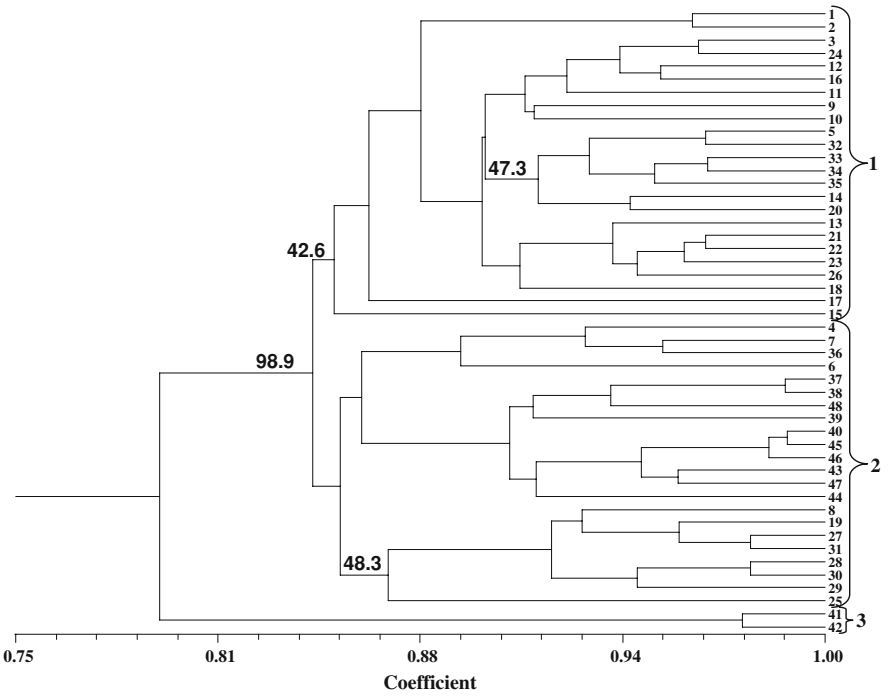

Fig. 2 UPGMA phenetic dendrogram based on Jaccard's coefficient, indicating genetic similarities among 48 genotypes of *Persea bombycina*, derived from RAPD data. The scale represents the similarity coefficient values, and bootstrap values are given at the branch nodes

Jaccard's and SM coefficients was also observed, as indicated by the high coefficient of correlation ($r = 0.99$, $p = 1$).

A principal correspondence analysis (PCA) plot generated from RAPD data of the *P. bombycina* accessions indicated that the accessions were relatively spread out on all three axes (Fig. 3). It is evident from the plot that accessions 5, 41, 42, and 32 are distinct and well separated from the remaining accessions. No major differences were observed between the dendrogram and the PCA plot, except for accession 5. This genotype grouped within cluster 1 using Jaccard's coefficient, but appeared on the far edge of the PCA plot.

The characterization of material based on morphological traits and common local names often leads to duplicated accessions, many of which are synonymous or homonymous or have a false attribution (Zoghlami et al. 2007). The practical utility of any molecular approach for germplasm management is partly determined by the ability to differentiate between large numbers of accessions (Gilbert et al. 1999). RAPD analysis proved to be an effective technique for measuring the magnitude of diversity and discriminating between genotypes. Som is a highly cross-pollinated plant. The RAPD markers in this study may be attributed to more uniform clusters, which are expected for progenies of wild-grown plants with predominant cross-pollination (Arnholdt-Schmitt 2000).

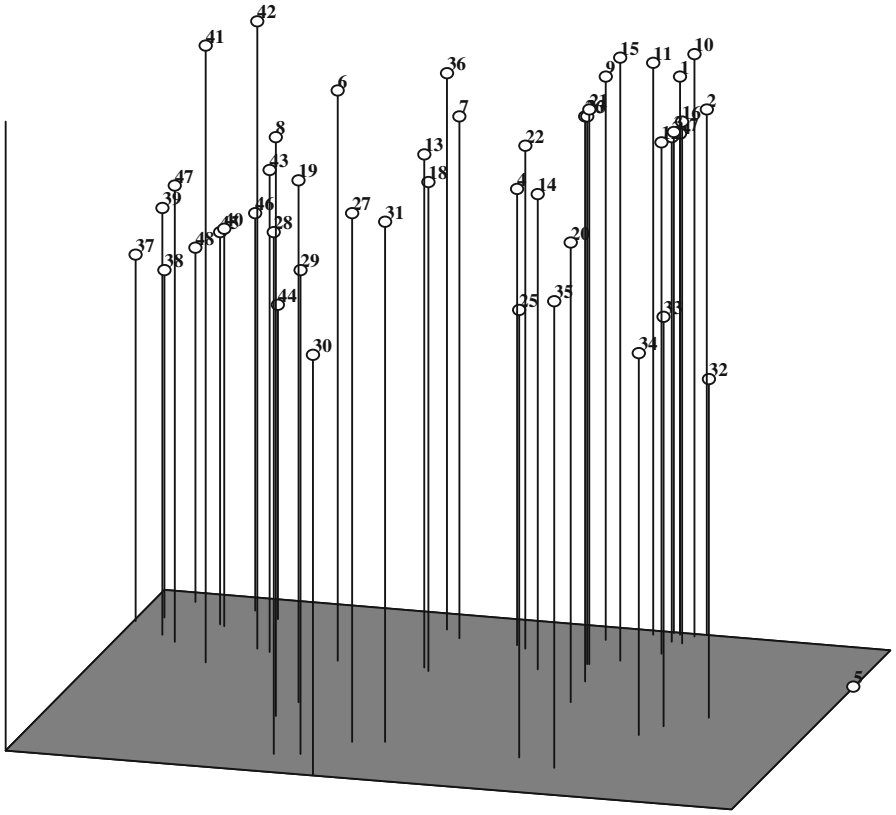

Fig. 3 Three-dimensional plot derived from the principal correspondence analysis of 48 genotypes of *Persea bombycina*

Understanding patterns of genetic variation within tree species is of fundamental importance for successful management in tree conservation programs. Knowledge of possible adaptive variation among areas is essential to the evaluation of biodiversity within and among populations to reveal information on population evolution. This study shows that RAPD is a very effective and useful tool for estimating the degree of genetic diversity as well as for determining the pattern of genetic relationships between genotypes of *P. bombycina*, with polymorphism levels sufficient to establish informative fingerprints with relatively few primer sets. The information obtained from this study could be of practical use for mapping the *P. bombycina* genome as well as for classical breeding. The informative primers identified in our studies will be useful in genetic analysis of mulberry accessions in germplasm holdings. The putative species-specific bands can be used as probes to ascertain whether their numbers of copies are high or low in the mulberry genome, and such specific bands may be used for genotype characterization and grouping germplasm accessions. Further, putative species-specific RAPD markers could be converted to sequence-characterized amplification regions after sequencing and

designing primer pairs to develop robust species-specific markers. The study also provides a basis for *P. bombycina* breeders to make informed selections of parental material based on genetic diversity to help overcome some of the problems usually associated with a tree crop improvement program. The information can then be used in identifying and prioritizing areas with comparatively high genetic diversity for monitoring, management, and protection. Knowledge of population structure is important for ex situ and in situ conservation of natural populations (Williams and Hamrick 1996) by maintaining the total evolutionary potential and minimizing consanguinity.

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