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Assessment of genetic diversity in a *Morus* germplasm collection using fluorescence-based AFLP markers

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Abstract To meet various breeding objectives and to conserve the existing genetic resources of mulberry for future use, the present study was undertaken to investigate the amount of genetic diversity and to establish the relationships between mulberry genotypes using fluorescence-based AFLP markers. Genetic diversity was estimated in 45 mulberry accessions from different eco-geographic regions of Japan and other parts of the world. Five primer combinations amplified an average of 110 AFLP markers per primer combination, ranging in size from 35 to 500 bp. A high degree of polymorphism was revealed by these combinations that ranged from 69.7 to 82.3% across all the genotypes studied. Several rare genotype-specific bands were also identified which could be effectively utilized to distinguish different genotypes. The wide range in genetic similarity coefficients (0.58–0.99) indicated that the mulberry germplasm collection represents a genetically diverse population. The phenetic dendrogram generated by the UPGMA method grouped 45 accessions into four major clusters, which was in agreement with the results from conventional methods. Clustering of some genotypes into strictly separate groups was not readily apparent and no clear interrelationships could be depicted, in spite of their different geographic origin. In addition, AFLP analysis provided sufficient polymorphism for DNA typing and contributed additional insights into the genetic structure of the mulberry germplasm. These results will help in the formulation of appropriate strategies for conservation and variety improvement in mulberry, for which little or no knowledge of genetic diversity is currently available.

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Introduction

The genus *Morus* L. (mulberry) is one of the most interesting taxonomic groups on account of its existing genetic variability and its commercial importance in the sericulture industry. Mulberry leaves are an exclusive food source of *Bombyx mori* L. Mulberry is found in a wide range of areas around the world, ranging from tropical to sub-arctic regions and accounting for a large diversity in its genetic resources. Linguistic, ethnological, taxonomic and geographic evidence suggests that a great diversity of mulberry species are present in Asia, especially in China and Japan, and are scantily represented in Africa, Europe and the Middle East (Fig. 1).

Despite its importance, aspects such as the origin and domestication of mulberry have not been elucidated. Most of the mulberry genotypes are “naturalized” because they have been established, adapted and persisted in areas distant from their initial origin, making their classification very difficult. The different methods used to classify the genus *Morus* are primariliy based on conventional systematic studies involving differences in growth form, leaf morphology, length of the styles in the female flowers, shape of the idioblast, fruit color and other agronomical characters (Katsumata 1972). Koiduzumi (1917), grouped mulberry species into two sections, the Dolichostylae and Macromorus, based on the length of the styles in female flowers, and subdivided each section mainly by leaf morphology. Likewise, Hotta (1954) divided mulberry species into two sections, namely the Dolychocystolithiae and the Brachycystolithiae, according to the shape and position of cystolith cells in the leaf, and which is currently being utilized in classifying mulberry germplasm in Japan.

The characterization and conservation of *Morus* germplasm are both essential for safeguarding the future

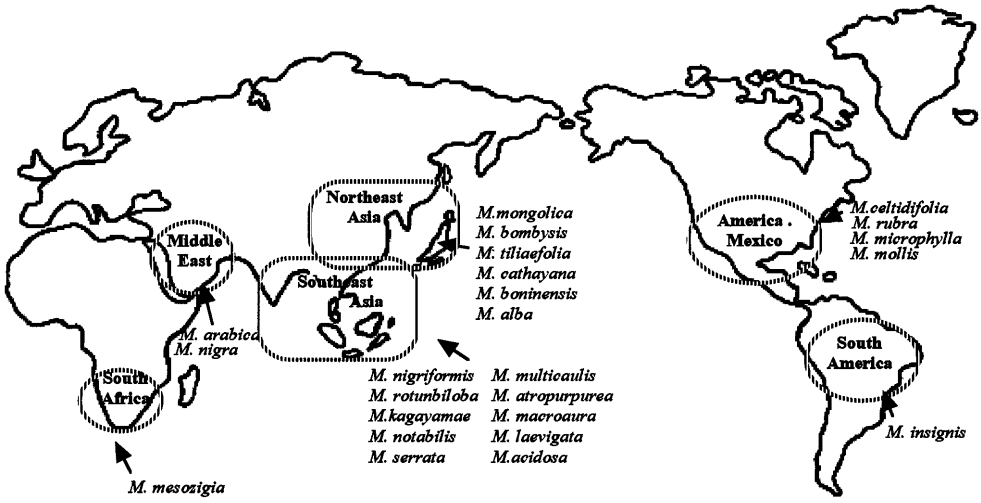

Fig. 1 Geographical distribution of *Morus* sp. showing their great diversity in North and South Asia

of this tree crop. The mulberry germplasm collection at the National Institute of Sericulture and Entomological Science (NISES), Tsukuba, Japan, represents more than 1300 accessions from Japan and other countries which are maintained both in the field and the greenhouse (Machii et al. 1999). A large number of morphological and agronomical characters are being evaluated for utilization in breeding programs (Machii et al. 1997). These efforts are, however, hampered by the limited information available on the genetic diversity and genetic relationships within and among species and varieties in the gene pool. The present classification criteria are not sufficient because of limitations in the identification of useful parents for crossing and the registration of new commercial varieties that are closely related.

Over the past decade, a number of molecular techniques, especially DNA-based markers, have been developed that can provide information on the extent of diversity and genetic relationships. Amplified fragment length polymorphism (AFLP) analysis is a technique through which selected fragments from the digestion of total plant DNA are amplified by the polymerase chain reaction (Vos et al. 1995). The resulting DNA fingerprints provide a large number of genetic markers; and the multiplex ratio, defined as the number of information points analyzed per experiment, is much higher than for other types of markers, such as RFLP (restriction fragment length polymorphism), RAPD (randomly amplified polymorphic DNA) or SSRP (simple sequence repeat polymorphism) (Powell et al. 1996). Compared with RAPD, AFLP analysis detects about 12-times the number of polymorphic loci per assay in soybean varieties (Vogel et al. 1994, cited in Hill et al. 1996; Sanchez et al. 1999). The AFLP technique has been successively used to asseses the genetic diversity in *Lens* (Sharma et al. 1996), soybean (Maughan et al. 1996), *Phaseolus vulgaris* (Tohme et al. 1996), willows (Barker et al. 1999), *Eragrostis tef* (Bai et al. 1999), *Arabidopsis* (Breyne et al. 1999) *Azadirachta* (Singh et al. 1999) and bermudagrass (Zhang et al. 1999).

Attempts have been made in the past to examine the diversity of *Morus* using morphological (Katsumata 1972), cytological (Katsumata 1979) and isozyme (Hirano 1982) markers. To the best of our knowledge, there has been no report regarding the extent of genetic diversity prevalent in mulberry based on molecular markers. Hence, the present study was undertaken to investigate the genetic diversity of, and to establish the relationships between, different genotypes of mulberry using fluorescence dye-labeled AFLP markers. The data presented here are intended to complement the ongoing efforts for the improvement of mulberry germplasm.

Materials and methods

Plant material

The 45 mulberry genotypes (accessions) used in the present study (Table 1) are currently being maintained at NISES, Tsukuba, Japan, under the Gene Bank Project of the Ministry of Agriculture, Forestry and Fisheries (MAFF). Among these, 22 genotypes were collected from different regions of Japan and the remainder were from different parts of the world.

AFLP analysis

For AFLP analysis DNA was extracted, using the modified CTAB method of Murray et al. (1980), from young leaves which were harvested and kept frozen at –80°C.

AFLP electrophenograms were produced for each genotype using the ABI PRISM TM fluorescent dye-labeling and detection technology (Perkin-Elmer), essentially based on the protocols described by Zabeau and Vos (1993) and Vos et al. (1995). A kit supplied by Perkin-Elmer Applied Biosystems (USA), optimized for a genome size of 500 to 6000 Mb, was used according to the manufacturer's instructions except for minor modifications. Genomic DNA (500 ng) from all 45 genotypes was restricted with *Eco*RI and *Mse*I, and the digested DNA fragments were ligated with *Eco*RI and *Mse*I adaptors in a single reaction. The adaptor-ligated DNA was amplified by *Eco*RI and *Mse*I pre-amplification primers using one cycle of 2 min at 72°C, 20 cycles of 20 s at 94°C, 30 s at 56°C and 2 min at 72°C, followed by one cycle for 60 min at 60°C in a Takara thermocycler (Takara Shuzo Co, Japan). The pre-amplified DNA was diluted in a ratio of 1:100 and was used

Table 1 *Morus* germplasm selected for AFLP analysis

S. no	Accession	Genotypes	Polyploidy level	Species	Country of origin
1	58001450	M-32	2*x*	*Morus acidoasa*	Japan
2	58001449	Okinawaguwa	2*x*	*Morus acidoasa*	Japan
3	58000691	Shiwasuguwa	2*x*	*Morus acidoasa*	Japan
4	58001843	M-28	2*x*	*Morus acidoasa*	Japan
5	58000631	Kairyonezumigaeshi	2*x*	*Morus alba*	Japan
6	58000608	Ichinose	2*x*	*Morus alba*	Japan
7	58001987	Gejira-1	2*x*	*Morus alba*	Egypt
8	58000760	Hosoe	2*x*	*Morus alba*	Japan
9	58000647	Kanton II Kou	2*x*	*Morus atropurpurea*	China
10	58001531	Amoi-1	2*x*	*Morus atropurpurea*	China
11	58000609	Ichibei	3*x*	*Morus bombysis*	Japan
12	58000663	Kenmochi	2*x*	*Morus bombysis*	Japan
13	58000683	Shimanouchi	3*x*	*Morus bombysis*	Japan
14	58000778	Yanagida	3*x*	*Morus bombysis*	Japan
15	58001989	Ogasawara-1	4*x*	*Morus boninensis*	Japan
16	58001990	Ogasawara-2	4*x*	*Morus boninensis*	Japan
17	58000836	Cathayana	2*x*	*Morus cathayana*	China
18	58000854	Costarika[a]	2*x*	*Morus celtidifolia*	Costarika
19	58001909	K-2	2*x*	*Morus indica*	India
20	58000811	Local-K	2*x*	*Morus indica*	India
21	58000582	Hachijouguwa	2*x*	*Morus kagayamae*	Japan
22	58001881	Nagamiguwa-1	3*x*	*Morsu laevigata*	India
23	58000589	Rosou	2*x*	*Morus latifolia*	Japan
24	58000649	Kanrasou	2*x*	*Morus latifolia*	Japan
25	58000869	Oshimasou	2*x*	*Morus latifolia*	Japan
26	58000744	Naganuma	2*x*	*Morus latifolia*	Japan
27	58000837	Macroura[a]	2*x*	*Morus macroura*	Malaysia
28	58000801	Enbu[a]	2*x*	*Morus mesozygia*	Africa
29	58000758	Beikoku-13[a]	2*x*	*Morus microphylla*	USA
30	58000838	Multicaulis	2*x*	*Morus multicaulis*	China
31	58000834	Lenbang	2*x*	*Morus multicaulis*	Indonesia
32	58000895	Debabi	2*x*	*Morus nigriformis*	Lebanon
33	58000894	Paraguay-2D	2*x*	*Morus nigriformis*	Paraguay
34	58000803	Iraq-1	2*x*	*Morus notabilis*	Iraq
35	58000887	Pakistan-12	2*x*	*Morus notabilis*	Pakistan
36	58000880	Turkey-2	2*x*	*Morus notabilis*	Turkey
37	58000563	Siam	2*x*	*Morus rotunbiloba*	Thailand
38	58000857	Chiang kam	2*x*	*Morus rotunbiloba*	Thailand
39	58000871	Poo	2*x*	*Morus rotunbiloba*	Thailand
40	58000868	Noi	2*x*	*Morus rotunbiloba*	Thailand
41	58000856	Bai poe	2*x*	*Morus rotunbiloba*	Thailand
42	58000853	Canada-2[a]	2*x*	*Morus rubra*	Canada
43	58001448	Kuromiguwa	22*x*	*Morus nigra*	Lebanon
44	58001884	Tenjikuguwa	6*x*	*Morus serrata*	India
45	58000662	Keguwa	6*x*	*Morus tiliaefolia*	Japan

[a] Genotypes for which the correct passport data at the species level are not available in the germplasm collection

as a template for selective amplification using the primers *Mse*I (Primer-Cxx) and *Eco*RI [Dye (FAM, JOE and TAMRA)-primer-Axx] (see Table 2). The cycling parameters were same as described by the manufacturer. The amplified product (1.0 µl) was mixed with 1.0 µl of a Gene Scan 500 ROX internal lane standard, and 25 µl of de-ionized formamide in a 0.5-ml Genetic Analyzer sample tube and were denatured at 95°C for 5 min. The denatured samples were analyzed on an automated DNA sequencer (ABI model 310, Perkin-Elmer Applied Biosystems).

Data analysis for genetic diversity and relationships among genotypes

AFLP electrophenograms ranging in size from 35 to 500 bp, analyzed by Gene Scan analysis software (version 3.1, Perkin-Elmer/ABI), were scored manually for the presence (1) or absence (0) across all 45 genotypes of mulberry for each primer-pair combination separately. The data matrix obtained was analyzed by taking the total and the shared number of electrophenograms by pairwise comparison using Excel software (version 5.0, Microsoft).

Genetic similarity was calculated based on the index of Nei and Li (1979) as Sxy=2nxy/(nx+ny), where nx and ny are the numbers of fragments in the individuals X and Y, respectively, and nxy is the number of fragments shared between individuals. Dendrograms were constructed by employing the UPGMA (unweighted pair group mean average) method of Saitou and Nei (1987) in Neighbor PHYLIP software (Phylogeny Inference Package, version 3.57c by J. Felsenstein).

Results and discussion

The assessment of germplasm diversity and the management of genetic resources is pivotal for plant breeding in introgressing exotic genes and characteristics into established cultivars (Tanksley and McCouch 1997). Furthermore, fingerprinting genotypes offers an opportunity for the removal of any duplicates that have been introduced through mislabeling or during multiplication in clonally

propagated crops. When compared with other major crops of economic importance, the collection and conservation of mulberry has not received worldwide attention. Japan is the only sericulturally advanced country where wild mulberry genetic resources are widely exploited in establishing new cultivars. Morphological and physiological characteristics are not sufficient to differentiate some *Morus* genotypes because the differences between them are often subtle. Assessment of the germplasm resources is also made difficult because hybridization occurs commonly in nature and the relatedness of many genotypes is unclear.

With the objective of investigating the genetic diversity within, and to establish the relationships between, different genotypes of the mulberry gene pool, an automated fluorescence dye-labeled AFLP technique was used. Fluorescent labeling and automated fragment-detection technology offer significant improvements over radioactive labeling methods by increasing the scoring accuracy, the typing efficiency and in reducing the cost incurred by the AFLP technique (Mitchell et al. 1997; Zhang et al. 1999).

AFLP fingerprinting and the polymorphism level in mulberry genotypes

The present study revealed that there is a large genetic diversity in *Morus.* A total of five AFLP primer combinations (Table 2) were selected based on the number of fragments amplified in each genotype and the polymorphism exhibited among closely related genotypes. Table 2 summarizes the percentage of polymorphism in the mulberry germplasm using five different primer-pair combinations. Each primer combination produced an average of 110 amplification products per individual, ranging in size from 35 to 500 bp. However, the maximum number of bands was found to be 123 with primer combination E-ACT/M-CTC, whereas the least number of bands (92) was obtained with primer combination E-AAC/M-CTG (Table 2), thus confirming the high multiplex ratio produced by AFLP markers. On average, 69.7–82.3% polymorphism was detected with each AFLP fingerprint across all 45 genotypes tested, indicating a high marker index (Table 2). Figure 2 represents a section of the AFLP fingerprint pattern that was derived by using primer combination E-AAG/M-CAC in four genotypes, Ichinose (*Morus alba*), Ogasawara-1 (*Morus boninensis*), Kuromiguwa (*Morus nigra*) and Keguwa (*Morus tiliaefolia*). This primer combination produced an average of 109 amplification products over all the mulberry genotypes with a polymorphism of 81.2% (Table 2). The monomorphic peaks (Fig 2, shown by the shaded area) produced with this primer combination were around 20% in all 45 genotypes. In contrast, peaks that could distinguish clearly between different genotypes (Fig. 2; shown by closed arrows) or were polymorphic (Fig. 2; shown by open arrows) among 45 genotypes were also obtained. The matrices of genetic similarity estimates, based on AFLP patterns from two separate primer combinations, were highly correlated (r=0.88–0.91). Moreover, the genotypes used in the present study did not share a similar DNA profile, thereby permitting the unique identification of each individual analyzed. This analysis also indicates that the collection does not contain genetic duplicates and facilitated an analysis of the distribution of genetic diversity among the mulberry genotypes.

Table 2 Average number of peaks (fragments) obtained from five selective primer combinations to detect AFLPs among 45 mulberry genotypes

Primer pair	Average number of peaks	% Polymorphism
E-AAC/M-CTA	112	69.7
E-AAG/M-CAC	109	81.2
E-AAC/M-CTG	92	82.3
E-ACG/M-CAA	118	71.7
E-ACT/M-CTC	123	74.2

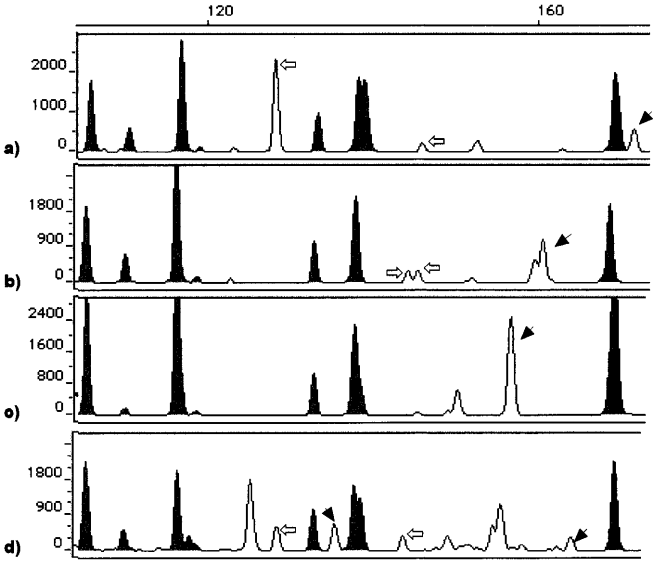

Fig. 2 Comparison of a section of electrophenograms obtained from the ABI 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems). Primer combination E-AAC/M-CTG was used to amplify genomic fragments of genotypes *a)* Ichinose *b)* Kuromiguwa *c)* Tenjikuguwa and *d)* Ogasawara-1 as described in Table 1. Monomorphic, genotype-specific and polymorphic peaks are indicated by *shaded areas*, *closed arrows* and *open arrows*, respectively

Genetic diversity among mulberry genotypes

Little is known about the genetic diversity within and between *Morus* species that has been based only on morphological characteristics. In the present study, the genetic similarity coefficient based on AFLP data ranged from 0.58 to 0.99 across all 45 mulberry genotypes. Such a wide range in similarity coefficients suggests that the

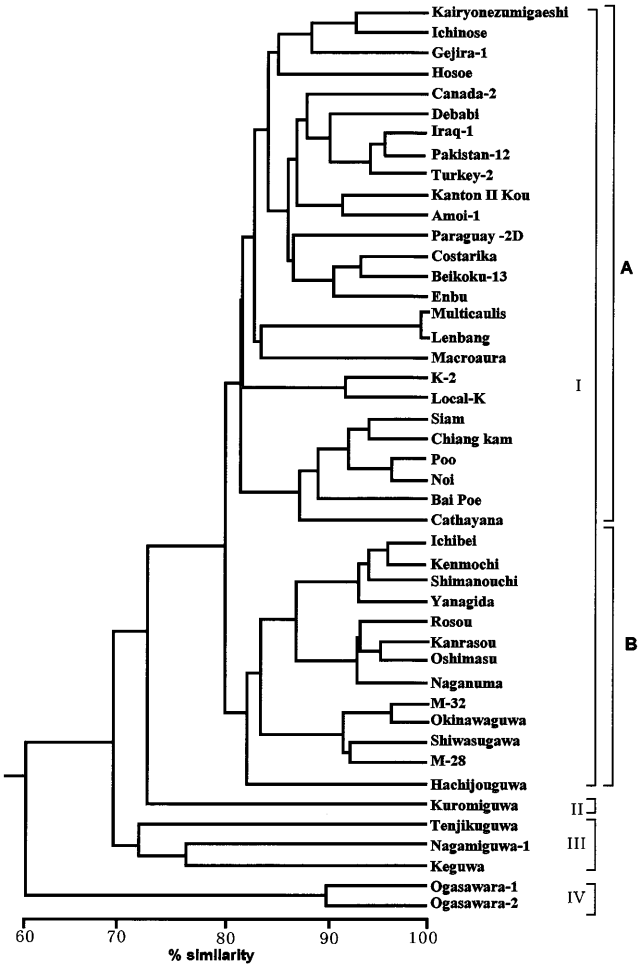

Fig. 3 Dendrogram, drawn from the UPGMA cluster analysis, of Nei and Li (1979) estimates of genetic similarity based on AFLP analysis with five selective primer combinations. A total of 45 mulberry genotypes used in this study (Table 1) were grouped into four major groups as discussed in the text

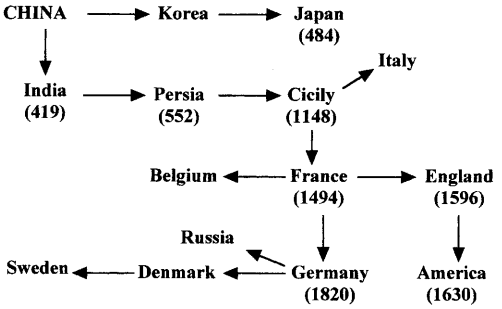

Fig. 4 A line diagram showing the domestication of the most-widely grown mulberry species, *M. alba*, a native of China, to areas as far apart as India, Europe and America

mulberry germplasm collection represents a genetically diverse population. The highest value of the similarity coefficient (0.99) was detected between two genotypes of *Morus multicaulis* collected from China and Indonesia. Among the Japanese genotypes (Table 1), Ogasawara-1 and Ogasawara-2 of *M. boninensis* showed the lowest similarity coefficients, ranging from 0.58 to 0.67.

The pair-wise genetic distances of the genotypes based on both shared and unique amplification products were calculated, and UPGMA cluster analysis was used to describe the phenetic relationships among the mulberry genotypes. The resultant dendrogram (Fig. 3) grouped the 45 genotypes into four major groups, I, II, III and IV. Clustering of different genotypes into strictly separate groups was not readily apparent in group I due to a high similarity coefficient between the different genotypes. However, this group was resolved into two distinct subgroups, A and B. Subgroup A comprised 26 genotypes of which Hosoe, Gejira-2, Kairyonezumigaeshi and Ichinose, belonging to the widely grown species, *M. alba*, were closely grouped with other genotypes collected from Canada, USA, Africa, Lebanon, Turkey, Iraq, Paraguay, Pakistan, China, India, Malaysia, Indonesia and Thailand (Table 1, Fig. 3). Furthermore, the genotypes belonging to *M. alba*, *Morus notabilis*, *Morus atropurpurea*, *Morus indica* and *Morus rotunbiloba* were grouped into individual subclusters. No significant morphological differences were found among genotypes of these species in our germplasm collection. Information about the geographic origin of genotypes is often helpful but, as can be seen from the data presented here, in the case of mulberry there is no clear correlation between the estimated relationships and the geographical origins of the genotypes. The rationale of these results is that most of the mulberry genotypes are ”naturalized” because they have been established, adapted and persisted in areas distant from their initial origin. As illustrated in Fig. 4, *M. alba* which is native to China was domesticated in different countries as far apart as India, Europe and America. During this process it is highly probable that, as a result of selection and breeding from germplasm, some genotypes may have been mislabeled or classified as distinct species based on unusual morphological characters. This is also supported by the fact that Gejira-2 collected from Egypt was closely clustered with genotypes of *M. alba* collected from Japan. Similarly, the genotype Cathayana (*Morus cathayana*) from Thailand is closely grouped with genotypes of *M. rotunbiloba* (Bai Poe, Siam, Chiang Kam, Poo and Noi), also collected from Thailand. Moreover, the chromosome number in *Morus cathayana* in Kew Botanic Gardens has been reported to be 4*x*, 6*x* or 8*x* (Katsumata and Ishiguro 1980), whereas the genotype in our germplasm collection is diploid (2*x*). Therefore, it is safe to conclude that Cathayana is a strain of *M. rotunbiloba*. Additionally, the genotypes of *M. multicaulis*, Multicaulis (China) and Lenbang (Indonesia) showed 99% similarity inspite of their different geographic origins (Fig. 3). The genotypes Enbu (Africa), Costarika (Costarika) and Beikoku-13 (USA), for which the correct passport data at the species level are not available in our germplasm collection, showed a high similarity (92%) with other genotypes in subgroup A. These genotypes have most probably been introduced into the collected site from European countries, which do

not represent much diversity in *Morus* species (Fig. 1). This result suggests that the genotypes which look morphologically similar to the most-widely grown species, *M. alba*, are either mislabeled or mistakenly classified as separate species. Cluster analysis, along with morphological observation, indicates that the mulberry species in subgroup A are very close to each other despite their different geographic origins and need detailed study to establish their true identity at the species level.

Subgroup B includes 13 genotypes represented by *Morus kagayamae*, *Morus bombysis*, *Morus latifolia* and *Morus acidosa*, all collected from different regions of Japan. Interestingly, all the genotypes were sub-clustered separately at the species level (Fig. 3), which corroborates the morphological observations. For instance, Hachijouguwa (*M. kagayamae*) and kairyonezumigaeshi (*M. alba*, subgroup A) are distinctly different in their morphological traits. The long-style trait of Hachijouguwa is incompletely dominant to the short-style trait of Kairyonezumigaeshi and was grouped as a different species (Katsumata 1982).

M. nigra, the black mulberry, represented by Kuromiguwa from the Lebanon, was clustered in group II showing a similarity coefficient of approximately 0.71 with the other genotypes. Kuromiguwa is a natural polyploid with a high level of docosaploidy (22-ploid) and is believed to have originated from the polyploid races of *M. alba* and *M. cathayana* (Janaki Amal 1948). Interestingly, we expected Kuromigawa to be the most distinctive genotype in the *Morus* sp., whereas, AFLP results revealed a similarity coefficient of approximately 0.73 with the other genotypes. Expectedly, Tenjikuguwa-1 (*Morus serrata*), Nagamiguwa (*Morus laevigata*), both from India, and Keguwa (*Morus tiliaefolia*) from Japan clustered together in group III showing a similarity coefficient of approximately 0.64 with other genotypes (Fig. 3). *M. boninensis*, represented by Ogasawara-1 and Ogasawara-2 collected from a remote island, Ogasawara, in Japan, were clustered in group IV with the lowest similarity coefficient of 0.58. This is in agreement with the peculiar characteristics observed in *M. boninensis*, which are very different from those of the other mulberry species, and was grouped at the outermost position in the genus *Morus* (Katsumata 1974).

In conclusion, the results obtained by AFLP analysis of mulberry accessions in the present study support to a large extent the genetic relatedness established among them by conventional methods. In addition the observations on genotype-specific amplified bands and the AFLP banding pattern will be helpful in the identification of different genotypes and the molecular tagging of traits of economic importance.

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