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DNA fingerprinting sheds light on the origin of introduced mulberry (*Morus* spp.) accessions in Italy

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Abstract

Mulberries are members of the genus *Morus* L., a taxonomic group showing a great genetic variability and adaptability to different environmental conditions. This study deals with the use of AFLP-based fingerprints as a tool for estimating genetic variability within as well as among three different mulberry species (i.e., *M. alba* L., *M. latifolia* Poir. and *M. bombycis* Koidz.). A high level of polymorphism (72.2%) was found over all the 48 accessions analyzed. Genetic similarity (GS) within single *Morus* species ranged from 0.845 (*M. bombycis*) to 0.884 (*M. alba*) being intermediate in *M. latifolia* (0.869). The between-species mean genetic similarity estimates based on pair-wise AFLP marker fingerprint comparison were very similar ranging from 0.861 to 0.874. The partition of the genetic variation over the three *Morus* species was unexpected: a proportion of the among-species genetic diversity as low as $G_{ST} = 0.084$ pointed out that about 92% of the total genetic diversity found among *Morus* accessions is due to DNA polymorphisms within a species, while only 8% of the total variation was highlighted among species. Our data indicate that some of the introduced accessions showing distinctive phenotypes, clearly differentiated from those revealed in the original habitat where they have been selected and adapted, hide an identical genotype.

Introduction

Mulberries are members of the genus *Morus* L., a taxonomic group showing a great genetic variability and adaptability to different environmental conditions. Mulberries are used commercially as a food source for silkworms as well as for their edible fruits and for amenity plantings. Despite its importance, information on the origin and domestication of mulberry genotypes is scanty.

Mulberry domestication began several thousand years ago, and the origins of most cultivated varieties are believed to be in the areas of China–Japan and the Himalayan foothills (Sànchez 2000). Nowadays, *Morus* is distributed in a wide area of tropical, sub-tropical, temperate and sub-arctic zones (Sharma et al. 2000).

Although mulberry includes mainly diploid cultivars with 28 chromosomes, natural polyploids are also known to be cultivated (Machii et al. 2000). In addition, mulberry breeding programs have induced artificial polyploids, which exhibit increased vigor and adaptability (Machii et al. 2000). Mulberry species have been subjected to intensive selection starting from open pollinated populations or single individuals produced by controlled

hybridization and mutation, resulting in over a thousand varieties, including triploid, tetraploid, and also hexaploid genotypes (Sànchez 2000).

Because of spontaneous as well as artificial hybridization and due to the wide movement of genotypes to areas far from their origin, the taxonomy of the genus *Morus* is not well defined. In addition to a confused and often misleading nomenclature, the lack of a modern monograph makes an univocal classification far from easy. In fact, conventional systematic studies based on several methods, including morphological and agronomical characteristics (Sharma et al. 2000), grouped mulberry species differently. This may, in part, be due to the influence of environmental variation on these characteristics.

Molecular markers can shed light on the origin, on variation within and on relationships among *Morus* accessions. It is known that molecular markers detect polymorphism by assaying subsets of the total amount of the DNA sequence variation in a genome. Polymorphisms detected by AFLP (Zabeau and Vos 1993; Vos et al. 1995) markers reflect the variation of restriction fragment sites and result from DNA sequence variation at primer binding sites and from DNA length differences between primer binding sites. The key of the success of AFLP markers has to be found in their high multiplex ratio, which is much higher than those of other multilocus PCR-based markers such as RAPD or ISSR markers. In fact, owing to their own genetic nature, AFLP markers detect simultaneously many loci (more than 100) which are usually randomly distributed in the genome. Moreover, compared to co-dominant markers with multiple alleles per locus (e.g., SSR), this marker system allows a more precise estimate of marker allele frequencies at single loci and a faster estimate of polymorphisms over several loci, because it requires a lower number of sampled plants per accession to assess presence vs. absence of marker alleles and a much lower number of experiments to investigate a given number of marker loci.

This study deals with genetic variability within as well as among three different mulberry species (i.e. *M. alba*, *M. latifolia* and *M. bombycis*) by using of AFLP-based fingerprinting. Results concerning genetic diversity and similarity statistics among the accessions are reported and inferences on the origin and domestication of introduced mulberry clonal populations are discussed.

Materials and methods

Plant material

A collection of 48 mulberry accessions was provided by the Sericulture Section of Padova, Livestock Experimental Institute of the Ministry for Agriculture and Forestry Policies, Via dei Colli in Italy. It represents a prominent part of the largest germplasm collection of the genus *Morus* in Italy, but no voucher was deposited due to uncertain taxonomy and unknown origins of some of these accessions.

Twenty-four accessions are vegetative clones of introduced varieties originally classified in their country of origin; information about taxonomy and geographical origin was taken from NISES (National Institute of Sericultural and Entomological Science, Japan) internet database (http://ss.nises.affrc.go.jp/pub/hmachii/plevdb-e.html). Five accessions (N01, K01, A15, A16, A17) were classified on the basis of unique phenotypical characteristics (i.e., bark colour, leaf shape, tree form, and branch shape) used by their original classifiers (Table 1); information about their origins was not available. Remaining accessions were classified according to old original labelling, because in most cases classification was difficult if done following traditional systematic methods.

Accessions were divided into three main populations according to available information: 26 accessions were included in *Morus alba*, 13 in *Morus latifolia*, and seven in *Morus bombycis*; *Morus nigra* L. and *Morus kagayamae* Koidz. were used in this study as controls. All information available on plant material was reported in Table 1.

DNA extraction

Mulberry genomic DNA samples were isolated from young leaf tissue after overnight storage at 4 °C as described by Fulton et al. (1995) with the following modifications. About 150 mg of leaf tissue were first ground to a fine powder in liquid nitrogen with a mortar and pestle, and then added

Table 1. Information on the *Morus* collection analyzed by AFLP markers.

Accession[a]	Denomination[b]	Species[c]	Origin[d]	Comments[e]
A01	Arancina	*Morus alba* L.**	Unknown	Native, A18-derived
A02	Ascolana	*Morus alba* L.**	Unknown	A25 × A21 hybrid
A03	Cattaneo (female)	*Morus alba* L.	Italy	Native
A04	Cattaneo (male)	*Morus alba* L.	Italy	Native
A05	Florio	*Morus alba* L.**	Unknown	–
A06	Giazzola	*Morus alba* L.**	Unknown	–
A07	Ichinose	*Morus alba* L.	Japan	Introduced
A08	Indiana	*Morus alba* L.**	Unknown	Introduced
A09	Kayriou Nezumigaeshi	*Morus alba* L.	Japan	Introduced
A10	Kayriou Wase Juumonji	*Morus alba* L.	Japan	Introduced
A11	Kokusou27	*Morus alba* L.	Japan	Introduced (1954)
A12	Korin	*Morus alba* L.**	Brazil	Introduced, A22-derived
A13	Morettiana	*Morus alba* L.**	India	Introduced (1815)
A14	Nervosa	*Morus alba* L.**	Unknown	–
A15	Pendula	*Morus alba* L.*	Japan	Introduced
A16	Pyramidalis	*Morus alba* L.*	Unknown	–
A17	Sinuense	*Morus alba* L.*	Unknown	–
A18	Spagna a Frutto Bianco	*Morus alba* L.**	Unknown	–
A19	Spagna a Frutto Nero	*Morus alba* L.**	Unknown	–
A20	Tagowase	*Morus alba* L.	China/Japan	Introduced
A21	Limoncina	*Morus alba* L.**	Italy	Native, A18-derived
A22	Miura	*Morus alba* L.**	Brazil	Introduced
A23	Muki	*Morus alba* L.**	Japan	Introduced
A24	Restelli	*Morus alba* L.**	Unknown	–
A25	Rosa di Lombardia	*Morus alba* L.**	Unknown	–
A26	Sterile	*Morus alba* L.**	Italy	Native, A18-derived
L01	Filippine	*Morus latifolia* Poir.	Philippines	Introduced (1821)
L02	Goshoerami	*Morus latifolia* Poir.	Japan	Introduced
L03	Kasuga	*Morus latifolia* Poir.	Japan	Introduced
L04	Kokusou20	*Morus latifolia* Poir.	Japan	Introduced (1954)
L05	Kokusou21	*Morus latifolia* Poir.	Japan	Introduced (1954)
L06	Kokusou Rosso	*Morus latifolia* Poir.**	Japan	Mutation of L04
L07	Lhou	*Morus latifolia* Poir.**	China	Introduced (1836)
L08	Rosou	*Morus latifolia* Poir.	China/Japan	Introduced
L09	Seijuurou	*Morus latifolia* Poir.	Japan	Introduced
L10	Daikokusou	*Morus latifolia* Poir.	Japan	Introduced
L11	Kayriou Rosou	*Morus latifolia* Poir.	Japan	Introduced
L12	Kokka	*Morus latifolia* Poir.	Japan	Introduced
L13	Kokusou70	*Morus latifolia* Poir.	Japan	Introduced
B01	Akagi	*Morus bombycis* Koidz.	Japan	Introduced
B02	Dateakagi	*Morus bombycis* Koidz.	Japan	Introduced
B03	Enshuutakasuke	*Morus bombycis* Koidz.	Japan	Introduced
B04	Kenmochi	*Morus bombycis* Koidz.	Japan	Introduced
B05	Shimanouchi	*Morus bombycis* Koidz.	Japan	Introduced
B06	Yamanaka Takasuke	*Morus bombycis* Koidz.**	Japan	Introduced
B07	Okaraguwa	*Morus bombycis* Koidz.	Japan	Introduced
N01	Nigra	*Morus nigra* L.*	South Caucasus	Introduced
K01	Platanoide	*Morus kagayamae* Koidz.*	Middle East	Introduced

[a]Arbitrarily chosen number.
[b]As provided by Sericulture Section of Padova, Livestock Experimental Institute of the Ministry for Agriculture and Forestry Policies.
[c]Classification as described in Materials and methods section (* Classified according unique phenotypical characteristics; **Classified according original labelling provided by Sericulture Section of Padova).
[d]Geographical origin, if known.
[e]Some accessions are derivatives or hybrids, some others are introduced. Year of introduction is reported between brackets, if known.

to 750 μL of microprep buffer prepared as described by Fulton et al. (1995) and previously heated at 65 °C. After incubation at 65 °C for 1 h and centrifugation at 10 000 rpm for 5 min, a 90-min RNase treatment at 40 μg/mL was performed. Each genomic DNA was precipitated with isopropanol and resuspended in 500 μL of Tris-EDTA pH 8 buffer. A phenol–chloroform extraction was repeated twice, first in chloroform: isoamylalcohol:phenol (24 : 1 : 25) and then in chloroform : isoamylalcohol (24 : 1). After ethanol precipitation, the pellet was vacuum-dried and resuspended in 50 μL of Tris-EDTA buffer at 65 °C for 15 min. Samples were then stored at −20 °C.

AFLP analysis

The AFLP (Amplified Fragment Length Polymorphism) protocol was performed as developed by Zabeau and Vos (1993) and Vos et al. (1995). A commercially available kit from Invitrogen Life Technologies (Carlsbad, CA, USA) was used for template preparation and amplification reactions, with some modifications made to adapt the protocol to the mulberry genome. All the experiments were replicated three times to assess the reproducibility of AFLP fingerprints.

The AFLP Core Reagent Kit® was used for restriction of genomic DNA and for ligation of adapters. About 500 ng of genomic DNA from all genotypes was digested with *Eco*RI and *Mse*I endonucleases and the digested fragments were ligated with *Eco*RI and *Mse*I adapters. The digested/ligated mixture was then diluted 1 : 1 with Tris-EDTA pH 8 buffer. Pre-amplification reactions were performed in a final volume of 20 μL with *Eco*RI and *Mse*I primers carrying one selective nucleotide. Twenty cycles were carried out at 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s in an Applied Biosystems GeneAmp® System 9700 (Foster City, CA, USA). The *Eco*RI primer was labelled by phosphorylating the 5′ end with [γ-^{33}P]ATP and T4 kinase, incubating the reaction at 37 °C for 1 h, as described in the manufacturer's instructions. The pre-amplified DNA was diluted 1 : 1 in Tris-EDTA buffer and was used as template for hot-PCRs with a *Mse*I primer carrying three selective nucleotides (M-CAT and M-CTA) in combination with a *Eco*RI radiolabeled primer carrying two selective nucleotides (E-AC, E-AT, E-AA, E-AG). Selective amplification was carried out under the cycling conditions set up by Barcaccia et al. (1999), which begins with one cycle at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s. The annealing temperature was then reduced each cycle by 0.7 °C according to a touch down profile of 13 cycles to reach the optimal annealing temperature of 56 °C. Twenty-three cycles were run to complete the final amplification at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. After amplification, PCR reactions were stopped with equal volume of loading buffer (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol) and denatured at 94 °C for 5 min. AFLP products (4–7 μL) were separated on 5% denaturing polyacrylamide gels with 8 M urea at 80 W constant power using a standard DNA sequencing unit BIORAD Sequigen (Hercules, CA, USA). Gels were dried at 80 °C for 1 h and visualized by autoradiogram after overnight exposure on an X-ray film.

Statistical data analysis

AFLP markers were scored as present (1) or absent (0) for all 48 mulberry DNA samples. Data were recorded as a binary matrix by assigning a progressive number to each comigrating band by comparing sample lanes on the autoradiograms. Statistical data analysis was performed on a selection of all the AFLP markers obtained; best markers were chosen according to reliability, intensity, and clearness of the autoradiogram bands.

The following calculations for genetic diversity analyses were conducted using the software POPGENE version 1.32 (Yeh et al. 1997). Analysis was performed excluding accessions resulted to belong to the same genotype.

The average AFLP marker allele frequency (p_i) for each primer and over all primers was calculated for each species separately and over all accessions. Assessing that gene frequencies of the *Morus* population do not deviate significantly from Hardy-Weinberg expectations, the frequency of *i*th dominant AFLP allele is given by $p_i = 1 - (1 - F_b)^{1/2}$, where F_b is the frequency of a given band corresponding to $p_i^2 + 2p_iq_i$ (Lynch and Milligan 1994; Krauss 2000; Barcaccia et al. 2003).

The observed number of alleles (n_o) and the effective number of alleles (n_e) per locus were calculated according to Kimura and Crow (1964). The degree of polymorphism was calculated over all species and samples using Shannon's information index (I) of phenotypic diversity (Lewontin 1974). Let p_i be the frequency of the ith AFLP marker phenotype, the average diversity can be written as $I = -\sum p_i \ln p_i$.

Genetic diversity (H) and populations differentiation (D_{ST}) statistics of Nei (1973) were used to summarize AFLP-marker data among the tested accessions. Let p_i denote the frequency of the ith marker allele at a given locus, then the genetic diversity (equivalent to the expected heterozygosity) is given by $H = 1 - \sum p_i^2$. Let H_T be the total genetic diversity over all loci and all species considered together and let H_S be the average over all species of H for each species, the proportion of diversity expressed between species (G_{ST}) was estimated as D_{ST}/H_T, where D_{ST} is the among species differentiation computed as $H_T - H_S$ and thus $G_{ST} = 1 - H_S/H_T$.

Genetic distance (GD) estimates among mulberry species were calculated for all AFLP marker loci, irrespective of the marker allele frequency, by using Nei's (1978) unbiased genetic distance coefficient. This parameter is defined as:

$$GD_{ij} = -\ln \sum p_i p_j \Big/ \left(\sum p_i^2 \sum p_j^2\right)^{1/2}$$

p_i and p_j being the frequencies of a given allele in populations i and j. For multiple loci, these values were calculated by summing frequencies over alleles at all loci studied. $GD_{ij} = 1$ if no alleles are shared between populations i and j while a $GD_{ij} = 0$ indicates that the two populations have identical allele frequencies.

The gene flow was estimated by $Nm = 0.5 \times (1 - G_{ST})/G_{ST}$: $Nm < 1$ indicates a local differentiation of populations, while $Nm > 1$ is evidence of a little differentiation among populations (McDermott and McDonald 1993).

Selection of most discriminant AFLP markers was conducted on the basis of the marker allele frequency ($p_i \approx 0.5$) and so using marker loci with the highest values of genetic diversity index ($H > 0.4$).

All the calculations for genetic similarity analyses were conducted by using the appropriate routines of NTSYS-PC software, version 2.11a (Exeter Publishing, Setauket, NY) (Rohlf 2002). Analysis was performed excluding accessions resulted to belong to the same genotype.

Genetic similarity (GS) of Dice (1945) was estimated for all pairs of the accessions, based on the probability that an AFLP marker allele from one sample will also be present in another, using the following formula:

$$GS_{ij} = 2M_{ij}/(2m_{ij} + M_i + M_j)$$

where M_{ij} represents the number of shared amplification products scored between the pair of fingerprints (i and j) considered, M_i is the number of products present in i but absent in j, and M_j is the number of products present in j but absent in i. A sample of *M. alba* plants with distinct genotypes was used to calculate confidence intervals for the mean genetic similarity estimate. For each of the plants of *M. latifolia* and *M. bombycis* was computed the mean genetic similarity estimate over all genotypes of *M. alba*. The observed value of genetic similarity was then compared with the expected interval defined by the lower and upper values. The mean deviation in absolute value between observed and expected genetic similarity estimates was calculated for both *M. latifolia* and *M. bombycis* plants and assayed by Student's criteria (T-test).

An ordination analysis was performed by using the neighbor-joining method developed by Saitou and Nei (1987); neighbor-joining dendrogram was constructed from the symmetrical genetic dissimilarity matrix. We applied coordinate analysis to compute the first two components from the qualitative data matrix. The triangular matrix of genetic similarity estimates was double-centered and then bi-dimensionally plotted based on the extracted eigen-vectors (Rohlf 1972). Selection of AFLP marker alleles showing significant assortment with the fruit color trait was performed by a 2×2 contingency test.

Results

Genetic diversity and differentiation

AFLP markers allowed to obtain reproducible fingerprints and informative polymorphisms in the mulberry DNA samples (Figure 1). The six primer combinations, selected on the basis of their

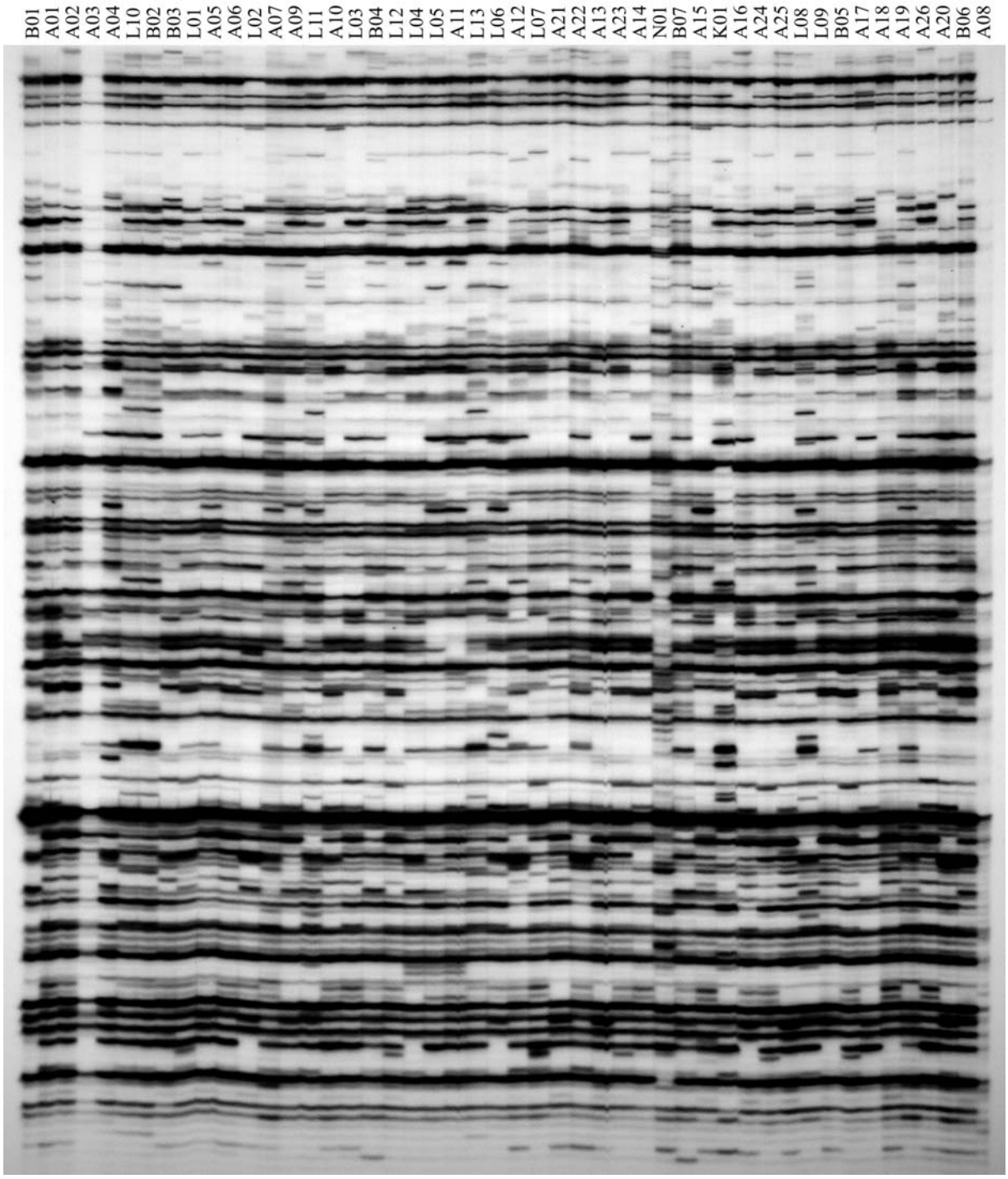

Figure 1. AFLP fingerprints generated by *Morus* cultivars using combination E-AC/M-CAT.

Table 2. Descriptive statistics of the three primary mulberry species including total number (N_{pl}) and proportion ($\%_{pl}$) of polymorphic loci, observed (n_o) and expected (n_e) number of alleles per locus, Shannon's information index (I), Nei's genetic diversity (i.e., within-population heterozygosity, H), and Dice's genetic similarity estimate among pair wise plant comparisons (GS). For each parameter, the overall value and standard deviation (S.D.) are also reported.

	N_{pl}	$\%_{pl}$	n_o	n_e	p_i	I	H	GS
Morus alba	169	87.1	1.87	1.42	0.329 ± 0.022	0.390	0.255	0.884
Morus latifolia	145	74.7	1.75	1.42	0.364 ± 0.024	0.369	0.244	0.869
Morus bombycis	139	71.7	1.72	1.43	0.355 ± 0.023	0.376	0.251	0.845
Overall	151	77.8	2.00	1.44	0.343	0.421	0.273	0.872
S.D.	15.9	8.2	0.00	0.33	0.301	0.204	0.025	0.031

electrophoresis pattern and amplification product number, recovered as many as 662 AFLP markers over the 48 *Morus* accessions. On average 110 marker alleles per primer combination were scored. A total number of polymorphic loci was 244 (72.2%) over the 338 selected (see Materials and methods). At the species level, the total number of polymorphic loci was 169 (87.1%) for *Morus alba*, 145 (74.7%) for *M. latifolia*, and 139 (71.7%) for *M. bombycis* (Table 2), being 151 overall the three primary species analyzed.

Descriptive statistics over all AFLP marker loci for each *Morus* species and over all *Morus* accessions are reported in Table 2. Within each species, the observed number of marker alleles per locus (n_o) ranged from 1.72 to 1.87 and it was 2.00 overall, while the effective number of marker alleles per locus (n_e) varied from 1.42 to 1.43 within each species and was 1.44 over all accessions (Table 2).

The Shannon's information index over all cultivars and AFLP marker loci was I = 0.421, varying from 0.369 to 0.390 (Table 2). Comparisons of genetic diversity estimates indicated that the sample of *M. latifolia* accessions was the most genetically uniform (H = 0.244).

Nei's genetic diversity estimates were calculated for all AFLP loci and for each of the three *Morus* species analyzed. The total genetic diversity (H_T) was moderately high, being equal to 0.273 (Table 3). The average value of the within-species genetic diversity was $H_S = 0.250$, specifically, 0.255 for *M. alba*, 0.244 for *M. latifolia*, and 0.251 for *M. bombycis*. Estimates of this index for the most discriminant AFLP marker loci are reported in Table 3. The extent of differentiation between the three *Morus* groups was as low as $D_{ST} = 0.023$, and, thus, the proportion of the among-species genetic diversity computed over all AFLP marker loci was also low at $G_{ST} = 0.084$. The fixation index values referred to the most discriminant marker loci ranged from 0.006 ($E + AT/M + CAT_{496}$) to 0.098 ($E + AT/M + CAT_{79}$) and it was lower than 0.04 for most of the AFLP loci investigated (data not shown). The absence of genetic differentiation among the three *Morus* species over most of the AFLP loci was confirmed by the gene flow estimate that on average resulted as high as Nm = 5.468 (Table 3).

Genetic similarities, genetic distances and ordination analyses

Cluster analysis was used to construct a neighbor-joining dendrogram displaying the relatively high level of genetic variation detected among the 48 *Morus* accessions (Figure 2). Most of them (28) were clustered into a subgroup with an average genetic similarity of 0.878; this cluster (group A) consisted mainly of accessions classified as *Morus alba* (19), and only of six and three accessions classified as *Morus latifolia* and *Morus bombycis*, respectively. Eighteen other accessions (group B) scattered along the same branch without forming distinct clusters, while *M. nigra* (N01) and *M. kagayamae* cv. Platanoide (K01) clearly clustered separately (Figure 2). Within both main subgroups A and B multiple accessions with distinct names and histories corresponded to a single genotype. As a matter of fact, 37 distinct genotypes were identified among the 48 accessions. In the main subgroup, eleven accessions (i.e., A12 and A22; B05, A23, A24 and L12; L03 and A26; A13, A21 and A25) appeared to belong to four genotypes, while in the second subgroup only 14 genotypes

Table 3. Genetic diversity and differentiation statistics of AFLP marker loci along with gene flow estimates of mulberry species.

	Marker loci[a]											
Species	E + AC/ M + CAT_{79}	E + AT/ M + CAT_{102}	E + AT/ M + CAT_{108}	E + AT/ M + CAT_{109}	E + AT/ M + CAT_{187}	E + AT/ M + CAT_{202}	E + AA/ M + CAT_{226}	E + AA/ M + CAT_{227}	E + AA/ M + CAT_{329}	E + AT/ M + CTA_{470}	E + AT/ M + CTA_{496}	E + AT/ M + CTA_{541}	E + AT/ M + CTA_{564}	Overall values
M. alba	0.494	0.483	0.483	0.500	0.475	0.494	0.494	0.495	0.475	0.465	0.500	0.475	0.475	0.255
M. latifolia	0.444	0.444	0.498	0.444	0.488	0.444	0.488	0.444	0.498	0.498	0.498	0.444	0.444	0.244
M. bombycis	0.414	0.483	0.488	0.414	0.483	0.488	0.488	0.488	0.483	0.483	0.483	0.414	0.483	0.251
H_S	0.451	0.451	0.490	0.453	0.482	0.476	0.490	0.476	0.487	0.482	0.494	0.444	0.467	0.250
H_T	0.500	0.494	0.496	0.500	0.496	0.496	0.498	0.500	0.499	0.500	0.497	0.485	0.500	0.273
D_{ST}	0.049	0.024	0.006	0.047	0.015	0.020	0.007	0.024	0.012	0.018	0.003	0.041	0.032	0.023
G_{ST}	0.098	0.048	0.012	0.094	0.029	0.040	0.015	0.047	0.024	0.036	0.006	0.083	0.065	0.084
Nm	4.608	9.988	42.595	4.844	16.643	11.964	32.561	10.067	20.136	13.527	84.020	5.495	7.240	5.468

[a]Only genetic diversity estimates of the most discriminant AFLP marker loci are reported.

were identified out of the 18 accessions, as can be seen in Figure 2.

Genetic similarity (GS) within single *Morus* species ranged from 0.845 (*M. bombycis*) to 0.884 (*M. alba*) being intermediate in *M. latifolia* (0.869) (Table 2).

Genetic similarity estimates between each pair of three *Morus* species are reported in Table 5 along with the dendrogram of genetic distances in Figure 3. The between-species mean genetic similarity estimates based on pair-wise AFLP marker fingerprint comparison were very similar ranging from 0.861 to 0.874. Based on AFLP marker allele frequencies, the most genetically related species were *M. alba* and *M. latifolia*, whereas *M. bombycis* clustered apart (Figure 3). The mean pair-wise genetic distance estimate was 0.023 between *M. alba* and *M. latifolia*, and 0.030 and 0.028 with respect to *M. bombycis*.

On the whole, the high coefficients of genetic similarity as well as the low estimates of genetic distance among species suggest that the 46 accessions received as *M. alba*, *M. latifolia* and *M. bombycis* share a common gene pool and show little genetic differentiation. This finding was confirmed by the principal-components analysis performed from the mean genetic-similarity matrix. The scatter diagram plotted according to the first two components was ineffective to ordinate centroids of the 35 *Morus* genotypes according to the three species. The first three components with eigenvalues of 2.294, 1.913 and 1.433, respectively, were able to explain 49.2% of the total variation. In particular, the first two components, although accounting respectively for 20.0% and 16.7% of the total variation, were not associated with *Morus* species (Figure 4).

Confidence intervals, as calculated for the 21 distinct genotypes of *M. alba,* were 0.878 (lower) and 0.924 (upper) at $P = 0.05$ enabled allowing us to statistically test *M. latifolia* and *M. bombycis* accessions for similarity to this group. We determined that five of the 13 *M. latifolia* accessions (L12, L01, L02, L09, and L03) and three of the 7 *M. bombycis* accessions (B05, B03, and B06) did not depart significantly from the expected values. On the basis of AFLP fingerprints, these eight accessions can be classified as '*M. alba*-like' genotypes. The mean deviation in absolute value between observed and expected genetic similarity

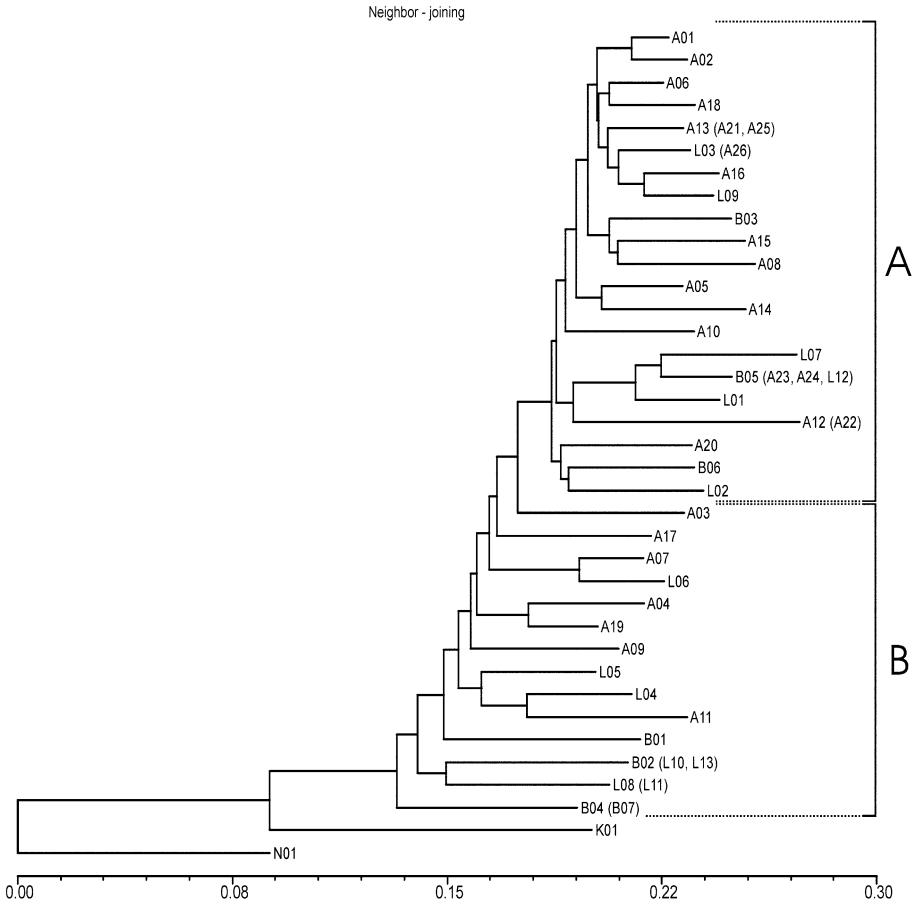

Figure 2. Dendrogram of *Morus* accessions based on AFLP data using the Dice's genetic distance matrix of dissimilarity and the Neighbor-joining clustering method. Identical accessions are reported within brackets.

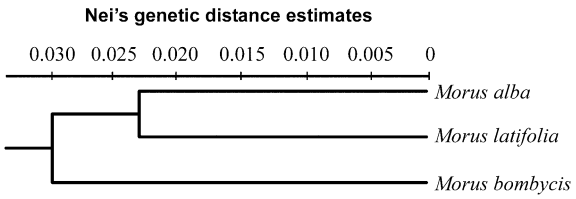

Figure 3. UPGMA dendrogram of genetic distance between mulberry species.

estimates for the remaining accessions was 0.026 for *M. latifolia* and 0.041 *M. bombycis* cultivars.

The AFLP marker allele distribution and the fruit color trait (white vs. black) over all 48 *Morus* accessions analyzed were compared by a 2 × 2 contingency test in order to select markers showing non-independent assortment with the trait to be used in assisted selection programs. This approach enabled the selection of 10 AFLP marker alleles showing a significant relationship with the trait (Tables 4 and 5). In particular, the marker allele coded as E + AC/M + CAT_{49} proved to be the most reliable one scoring the highest chi-square value ($\chi^2 = 10.025$***).

Discussion

The major finding of this work concerns the possibility of discriminating the *Morus* genebank accessions with multilocus, dominant PCR-based AFLP markers. Considering the high level of polymorphisms (87.1% overall accessions) assessed by AFLP fingerprints, and their reliability and reproducibility, the establishment of a molecular reference system in

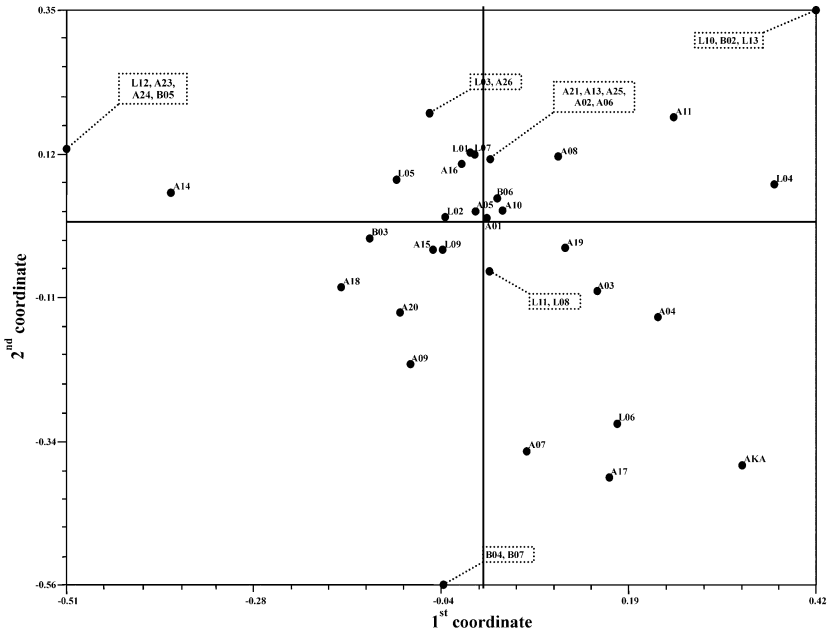

Figure 4. Centroids of *Morus* accessions according to the first and second principal coordinates obtained from the mean genetic similarity matrix.

Table 4. AFLP marker alleles showing assortment with the colour trait.

	Black fruit		White fruit		
Marker	Present	Absent	Present	Absent	χ^2
E + AC/M + CAT_{49}	29	3	2	5	10.025***
E + AC/M + CAT_{76}	0	32	2	5	4.659**
E + AT/M + CAT_{101}	3	29	4	3	5.951**
E + AT/M + CAT_{186}	15	17	0	7	3.535**
E + AT/M + CAT_{189}	4	28	4	3	4.549**
E + AA/M + CAT_{264}	2	30	4	3	7.853**
E + AT/M + CTA_{480}	22	10	1	6	4.971**
E + AT/M + CTA_{510}	2	30	3	4	4.001**
E + AT/M + CTA_{527}	3	29	4	3	5.951**
E + AT/M + CTA_{565}	2	30	4	3	7.853**

,*significant at the $P = 0.01$, $P = 0.001$ levels, respectively.

Table 5. Matrix of Dice (1945) genetic similarity estimates (below diagonal) and Nei (1978) genetic distance estimates (above diagonal).

	Morus alba	*Morus latifolia*	*Morus bombycis*
Morus alba	–	0.023	0.030
Morus latifolia	0.874	–	0.028
Morus bombycis	0.861	0.861	–

the genus *Morus* seems to be feasible for the precise identification of single genotypes, and suitable for the evaluation of the extent of natural hybridization that can occur among populations.

High genetic similarity coefficients within single *Morus* species (0.845–0.884), and comparable estimates between each pair of the three species analyzed (0.861–0.874) suggest little genetic differentiation and possible natural hybridization among the three primary species considered in this study. Sharma et al. (2000) took into account several mulberry species and reported a wide range of genetic similarity estimates (0.58–0.99) referred to the genus *Morus* as a whole. Thus, no comparison between these data and our data is possible at a single species level.

The partition of the genetic variation over the three *Morus* species was unexpected. In fact, our findings pointed out a proportion of the among-species genetic diversity as low as $G_{ST} = 0.084$, meaning that about 92% of the total genetic diversity found among *Morus* accessions was due to DNA polymorphisms within a species, while only 8% of the total variation was highlighted among species. The low extent of differentiation between the three *Morus* groups, D_{ST}, and the relatively high gene flow estimates, Nm (Table 4), further

confirmed that accessions received as *M. alba*, *M. latifolia* and *M. bombycis* were not genetically differentiated and share a common gene pool. Additionally, neither cluster analysis nor principal-components analysis was able to subdivide and clearly group accessions, except for N01 and K01 genotypes, expressly used as controls, which clustered apart.

Conventional systematic studies based on different methods including morphological and agronomic traits, grouped *Morus* species in different ways. Since spontaneous and artificial hybridization is possible, and due to continuous variation of most phenotypic characteristics, the taxonomy of the genus *Morus*, especially for *M. alba*, *M. latifolia* and *M. bombycis* species, is not well defined. As a consequence, phenotypical characteristics have a low diagnostic value for identifying interspecific hybrid constitutions, assessing introgression patterns, or defining genetic variation structure and relatedness at the species level. The difficulty experienced in the ordination analysis of accessions received as *M. alba*, *M. latifolia* and *M. bombycis* suggests that some of them could be hybrids or introgressants. This would also be supported by morphological and molecular evidence since mulberry genotypes showing intermediate morphological traits and common molecular polymorphisms were found.

Furthermore, recent findings indicate that most of the mulberry cultivars are naturalized because they have been established, adapted and persisted in areas far away from their origin, making their classification very difficult and unreliable when based solely on morpho-phenological traits (Sharma et al. 2000). Our data are in agreement with these assumptions and indicate that some of the introduced accessions showing distinctive phenotypes, clearly differentiated from those revealed in the original habitat where they have been selected and adapted, hide an identical genotype. In fact, AFLP fingerprints allowed to identify 37 genotypes out of 48 accessions analyzed. In other words, the collection analyzed includes several mulberry accessions labelled with distinct names that actually correspond to the same genotype. This was the case of L12 and B05 which most likely are the same *M. alba* genotype instead of *M. latifolia* and *M. bombycis* distinct genotypes, respectively, as reported by original classification. Also L03 cannot be *M. latifolia*, being genetically identical to A26 classified as *M. alba* and known to derive from A18 of *M. alba*. Moreover, statistical analysis pointed out that a few additional accessions classified as belonging to *M. latifolia* or *M. bombycis* closely resemble *M. alba* in marker composition and polymorphism, and so can be considered as '*Morus alba*-like' genotypes (e.g., L01, L02, L09, B03, B06). It is known that the adaptation of *Morus* genotypes in areas far away from their origin is common. As a consequence, such a result is not striking because some of the *Morus* genotypes introduced in Italy during last few centuries under a given name might have changed their phenotype under different selective environmental and anthropological pressures that most likely led to a variation of their morphological traits and then led final users to rename them. Additionally, misleading local nomenclature and incorrect germplasm recordkeeping may have contributed over the years to an erroneous or confused classification of cultivars.

Concluding, traditional methods used for taxonomic classification may not be reliable and in some case may cause a wrong and deceptive classification. In this context, AFLP markers could provide a useful tool to unequivocally identify single genotypes, to rationally conserve genetic resources, to eliminate duplicate accessions within germplasm collections and to monitor the level of genetic diversity within (and relatedness among) *Morus* species through DNA fingerprinting.

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