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Molecular marker based genetic resolution of *Morus alba* and *M.Indica* for reassessment of taxonomic status

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ABSTRACT

The debate on the taxonomic status of the two most popular cultivated mulberry species of India; *Morus alba* and *Morus indica* has persisted since long time. Subtle morpho-agronomical distinction and cross pollinating nature have added to the confusion in identification. Historical evidence suggests that *M.alba* has been introduced from China to Japan and to rest of the world including India through the famous silk route. *M.indica* is believed to be endemic to the Indian subcontinent. The present study utilizes the DNA markers for screening the genetic resolution of the two species for reassessment of the taxonomic status. DNA profiling of 16 indigenous and exotic varieties belongs to *M.indica* and *M.alba* were analyses for genetic variation by 12 informative RAPD markers. The dominant random primers generated 136 markers, of which 128 (80%) were polymorphic. The UPGMA clustering resulted in four distinct clusters. Based on the results obtained from materials used and interrelationship observed by UPGMA analysis of marker data, it is concluded that, the genetic distinction between the taxa were very less and hence *M.indica* may be considered as a variety under the species *M.alba*. The conclusion is supportive of the findings of the lectotypication and taxonomic studies on these species. Genetic diversity estimates of some of the materials utilized in the present investigation can be used in precise planning of mulberry crop improvement.

KEYWORDS

DNA markers;
Genetic diversity;
Lectotypication;
Morus spp.;
Taxonomic resolution.

INTRODUCTION

Mulberry (*Morus spp.*) is an important crop plant of sericulture and its leaves forms the only source of natural food for the domesticated silk producing worm, *Bombyx mori* L. The art and technique of silkworm rearing was introduced from China through the legendary silk route.

In India, the commonly cultivated mulberry varieties are considered to belong to *M.alba* and *M. indica* along with the naturally occurring wild species viz., *M.laevigata* and *M.serrata* Hooker 1885[1]. However, the varieties belonging to *M.alba* had been introduced from China, to Japan and to other countries including India Kameswara Rao and Jarvis[2]. Linnaeus 1753[3] classified these two species –*M.alba* and *M.indica* under the genus *Morus* along with other five species. Koidzumi 1917[4] and 1923[5], while reviewing the taxonomy of genus *Morus*, did not recognize *M.indica* as a distinct species. *M.indica* varieties are supposed to

Regular Paper

be endemic to India or at least restricted in its distribution to the subcontinent. However, a close examination of collections belonging to these two species shows subtle difference in morphological and reproductive characters. Some of the authors have considered these two species as synonymous, while others treated *M.indca* as variety under *M.alba*. High reproductive compatibility and cross pollination nature has made the species classification difficult and often arbitrary. But varieties belonging to *M.indica* appear to be hardy, invariably lobed from medium to high, poor in leaf yield, and highly acclimatized to tropical conditions. In contrast, the varieties of *M.alba* are generally considered to be succulent, larger leafed, dark green in color with superior leaf quality characteristics and grown extensively in temperate sericulture zones. *M.alba* is considered to be one of the most adaptable and can thrive under extreme cold conditions to the warm humid tropical climate.

Application of DNA marker technology for addressing the problems in plant systematics and genetics is well recognized. With the advent of polymerase chain reaction (PCR), the number of marker techniques available has increased manifolds. PCR based DNA markers like random amplified polymorphic DNA (RAPD) has revolutionized the genetic analysis of plants due to its speed, simplicity, accuracy and are neutral to environmental affects[6]. Conventional marker systems like morphology are of limited value in species identification of mulberry as it exhibits high reproductive compatibility and cross pollination across the members of the genus Dandin and Naik[7]. DNA marker techniques are routinely applied in these days in DNA fingerprinting of mulberry for Naik et al.[8], Naik and Dandin[9], identification of duplicate collections in germplasm Naik and Dandin[10], assessment of genetic diversity and interrelationships among germplasm Chatterjee et al.[11], Awasthi et al.[12], Sharma et al.[13]; Vijayan[14]; Vijayan et al.[15]; Srivatsava et al.[16] and identification of QTLs for water use efficiency (WUE) and root traits Sukumaran et al.[17].

TABLE 1: List of varieties belonging to *M.alba* and *M.indica*

Sl. no.	Name/Origin of the mulberry variety		Species
1	Kataneo	(Italy)	*M.alba*
2	Tagowase	(Japan)	
3	Kokusou-27	(Japan)	
4	China Peking	(Philippines)	
5	Kyro Negume Geshi (KNG)	(Japan)	
6	China White	(China)	
7	English Black	(France)	
8	Shin Ichinose	(Japan)	
9	Kajli	(West Bengal)	*M.indica*
10	Kaliakutahi	(Jammu & Kashmir)	
11	Sujanpur-5	(Punjab)	
12	Assambola	(Assam)	
13	Almora Local	(Utter Pradesh)	
14	LocalLobed	(Karnataka)	
15	Punjab Local	(Punjab)	
16	Berhampore-20	(West Bengal)	

So far, no attempt has been made to demarcate these two species -*M.alba* and *M.indica* and establish reliable genetic relationships among the species using molecular marker systems. In this background the study will be providing required genetic resolution for clearly assessing the taxonomic status through DNA marker analysis and contribute to the better understanding of this complex genus. The experiment will also provide a reliable estimate of genetic diversity that exists in these two popularly cultivated mulberry species of India.

MATERIALS AND METHODS

Plant materials

Sixteen mulberry varieties belonging to *M.alba and M.indica* (TABLE 1) were used in the study. These varieties were available in *ex-situ* conditions in the mulberry gene bank at Central Sericultural Research and Training Institute, Mysore. These varieties are maintained under standard package of practices recommended by the Institute.

Mulberry genomic DNA isolation

Genomic DNA was isolated using Nucleon Phytopure Kit method (Amersham Biosciences, UK). The DNA was quantified on 0.8% agarose gel stained with ethidium bromide. The DNA stock solution was diluted to uniform concentration of 10ng/µl for PCR amplification.

RAPD amplification

PCR reactions were performed by modified protocol of Williams et al.[6]. The PCR amplification was carried out in a 0.2 ml PCR tube in PTC-200 DNA engine (MJ Research, U.S.A.) with 20µl reaction volumes containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 2.0 mM $MgCl_2$, 0.2µM primer, 0.1mM of each dNTPs, 0.5 U of Taq DNA polymerase (Genei, Bangalore) and 20ng of template DNA. The random primers (TABLE 2) were obtained from Operon Technologies Inc., Almeda, USA. Amplification reactions were car-

Regular Paper

TABLE 2: List of random primers used in the study along with marker polymorphism

Sl. no.	Name of the primes	Sequence 5'-3'	Total no. of markers	No. of monomorphic markers	No. of polymorphic markers	% Of polymorphism
1	OPA 07	-gaaacgggtg-	15	01	14	93.33
2	OPA 09	-gggtaacgcc-	07	00	07	100.00
3	OPA 10	-gtgatcgcag-	09	01	08	88.88
4	OPA 12	-tcggcgattag-	09	01	08	88.88
5	OPA 14	-tctgtgctgg-	11	01	10	90.90
6	OPA 15	-ttccgaaccc-	12	00	12	100.00
7	OPA 16	-agccagcgaa-	15	00	15	100.00
8	OPA 17	-gaccgcttgt-	17	01	10	90.90
9	OPC 05	-gatgaccgcc-	11	01	10	90.90
10	OPC 07	-gtcccgacac-	11	00	11	100.00
11	OPC 08	-tggaccggtg-	14	01	13	92.85
12	OPC 09	-ctcaccgtcc-	11	01	10	90.90
		Total	136	08	128	94.12

ried out by following cycle profiles: initial denaturing cycle at 93^{0}C for 2 min followed by 40 cycles at 93^{0}C for 1min, 35^{0}C for 1min, 72^{0}C for 2 min and a final 7 min extension at 72^{0}C. PCR products were electrophoresed on 1.5% agarose gel Sambrook et al.[18] in 1×TAE, stained in ethidium bromide and the gel image was recorded using the gel documentation system. (Syngene, U.K.).

Data analysis

DNA banding patterns generated by RAPD was scored as '1' for the presence of marker and '0' for the absence. Reproducible RAPD primers were considered for scoring. Dice similarity matrix was generated using the equation, s=2Nxy/(Nx +Ny), where Nxy is the number of shared markers between 'x' and 'y' entries. Nx is the total number of markers in 'x' entry and Ny is the total number of markers in 'y' entry Nei and Li, 1979[19]. Genetic distance (1-s) was calculated and a dendrogram was constructed based on the similarity matrix data set by applying un-weighted pair group method of arithmetic averages (UPGMA). RAPD markers were identified by the source of primers (OP for Operon), kit letter, the primer number and its approximate size in base pairs.

RESULTS

The mulberry varieties considered for the study were belonging to the most cultivated species in India. However, mulberry belonging to these species were from different geographical origin and represented from the historically important sericultural regions of the globe. All the varieties included in the study were diploids and the place of the origin is given in the TABLE 1. The two

(Based on the RAPD simiakrity coeficient)

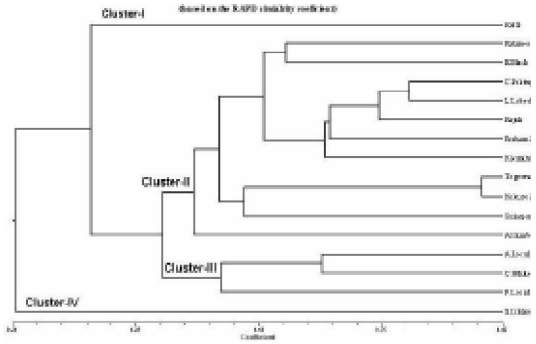

Figure 1: UPGMA clustering of *M.alba* and *M.indica* collestions

species were identified based on the key characters described in Flora of British India Hooker 1885[1] and the published information form the gene bank of CSRTI, Mysore. However, the commercially important growth and yield parameters of the mulberry are highly influenced by the environment and the information on these parameters can be found in published literature under various evaluation programs Rajan et al.[20]; Rajan and Sarkar 1998[21].

A total of 12 informative decamer RAPD primers (TABLE 2) were used for screening 16 mulberry genotypes. PCR amplifications were performed twice and consensus markers from two identical profiles were scored to avoid any ambiguity. A total of 136 markers were scored in the range of 500-3200bp. The number of markers per primer ranged from 7-15 with an average of 11.3 markers per primer. Out of the total, only 8 markers were monomorphic and rest 128 (80%) were polymorphic. The RAPD profiles of all the 16 mulberry varieties belonging to *M.alba* and *M.indica* produced by primers OPA-16 and OPC-05 were shown in the figure 1. On comparison of the RAPD profiles estab-

Regular Paper

lished some variety specific DNA markers (TABLE 3).

Dice of similarity coefficient values were calculated (TABLE 4) based on RAPD markers data set Nei and Li,[19] and 1-s value denotes the genetic distance. Based on the similarity values among 16 mulberry varieties ,the two popularly cultivated species of India Tagowase and Kokuso-27 were genetically close with a similarity of 83.5% and Shin Ichinose and Almora Local were genetically divergent with least similarity (40%). UPGMA clustering based on SHAN routine using RAPD marker similarity coefficient resulted in four distinct clusters (Figure 2). Cluster I was represented by one variety namely KNG and Cluster II was the largest with 11 varieties belonging to *M.alba* (5 nos.) and *M.indica* (6 nos.). Clusters III represented by Almora Local, Punjab Local (*M.indica*) and China White (*M.alba*). Cluster-IV is solely represented by Shin Ichinose belonging to *M.alba*.

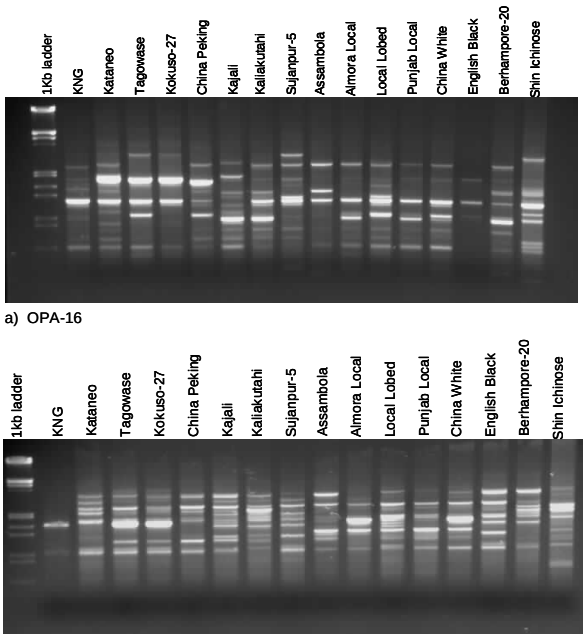

Figure 2 : DNA fingerprints of *M.alba* and *M.indica* collections generated by the random (RAPD) primers (a) OPA-16 and (b) OPC-05

DISCUSSION

India has a long history of mulberry cultivation. Silk culture spread to India through the legendary silk route, which dates back to 140 BC. Even though few of the species of mulberry are truly native to India, however the origin of some of the cultivated forms of mulberry is not clear. Hooker 1885[1] and Brandis 1906[22] had reported the occurrence of four mulberry species namely, *M.indica, M.alba, M.laevigata* and *M.serrata* in India. Among them *M.laevigata* and *M.serrata* are truly

TABLE 3: List of mulberry variety specific DNA markers identified

Sl. no.	Name	Variety specific marker
1	Kaliakutahi	$OPC\text{-}08_{800}$
2	Sujanpur-5	$OPC\text{-}08_{750}$
3	Punjab Local	$OPA\text{-}09_{900}$, $OPA\text{-}12_{2100}$
4	Berhampore-20	$OPA\text{-}15_{2400}$
5	Kataneo	$OPA\text{-}15_{750}$
6	Tagowase	$OPA\text{-}12_{1100}$
7	Shin Ichinose	$OPA\text{-}07_{2000}$, $OPA\text{-}12_{750}$, $OPA\text{-}15_{1900}$, $OPA\text{-}15_{1250}$, $OPA\text{-}15_{500}$, $OPA\text{-}16_{2800}$, $OPA\text{-}17_{2000}$, $OPA\text{-}17_{1450}$, $OPA\text{-}17_{1100}$, $OPA\text{-}17_{800}$, $OPC\text{-}05_{600}$, $OPC\text{-}08_{500}$, $OPC\text{-}09_{2700}$

TABLE 4: Dice similarity coefficient based on RAPD marker data of *M.alba* and *M.indica* collections

S.no	Accessions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	KNG	1.000															
2	Kataneo	0.541	1.000														
3	Togowase	0.581	0.681	1.000													
4	Kokuso27	0.566	0.632	0.835	1.000												
5	C.Peking	0.598	0.731	0.629	0.620	1.000											
6	Kajali	0.505	0.672	0.614	0.589	0.738	1.000										
7	K.kutahi	0.500	0.637	0.610	0.569	0.687	0.748	1.000									
8	Sujanpur5	0.553	0.661	0.645	0.684	0.614	0.632	0.702	1.000								
9	Assambola	0.527	0.627	0.629	0.584	0.667	0.667	0.609	0.620	1.000							
10	A.Local	0.545	0.539	0.512	0.545	0.613	0.613	0.589	0.646	0.611	1.000						
11	L.Lobed	0.583	0.748	0.732	0.676	0.783	0.783	0.736	0.748	0.630	0.645	1.000					
12	P.Local	0.584	0.603	0.590	0.577	0.643	0.625	0.584	0.598	0.542	0.602	0.672	1.000				
13	C.White	0.625	0.618	0.605	0.661	0.655	0.605	0.600	0.673	0.544	0.720	0.682	0.693	1.000			
14	E.Black	0.530	0.694	0.707	0.676	0.686	0.643	0.652	0.689	0.645	0.562	0.745	0.525	0.651	1.000		
15	Berham20	0.538	0.626	0.613	0.587	0.756	0.693	0.719	0.595	0.613	0.611	0.729	0.624	0.655	0.642	1.000	
16	S.Ichinose	0.432	0.493	0.514	0.511	0.537	0.567	0.622	0.467	0.492	0.400	0.558	0.414	0.455	0.542	0.519	1.000

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wild with different geographical origin and sexually distinct. However, *M.indica, M.alba* are grown throughout India for silkworm rearing and to produce fertile hybrids. There is no authentic report of natural population of *M.alba* or *M.indica* in India. Watt 1891[23] and Parker 1956[24] reported that the origins of these species are from China and extensively cultivated through out the plains of India up to an elevation of 3300m. The varieties of *M. indica* are cultivated and are also grown as garden plants in house holds in India especially in the plains and hill stations. Occurance of *M.alba* trees were found in the mountainous terrain viz., Lhasa near Tibetian border, valleys of Kashmir, North-west Himalayas had been reported Ravindran et al.,[25]. *M.indica* varieties are sturdy, relatively poor in leaf yield and have longer style than the *M.alba* varieties. However in cultivated forms of mulberry, the variation in the style length makes it difficult to classify species based on its morphology. Attempts had been made by Hirano et al.,1980[26] to classify some of the Japanese varieties of mulberry under *M.alba, M.latifolia* and *M.bombycis* based on the isozyme and protein profiles and concluded these species are genetically close and hence to be treated as subspecies of a species.

Kameswara Rao and Jarvis 1986[2] made a special attempt to designate lectotypes for *M.alba, M.tartarica* and *M.indica*. *M.tartarica* was treated as synonymous with *M.alba* and following Bureau's 1873[27] treatment, *M.indica* was recognized as a variety of *M.alba*. The authors gave key differences that existed between the two species. *M.alba* has style free at the base with glabrous or glabrescent style arms, sepals of male flowers is ovate and upper surface of the leaf is smooth, but lower surface has tufts of hair on the axils of major veins. Leaf apex is acute and margin dentate. In comparison, in *M.indica*, style is connate, style arms downy, sepals of the male flower is obovate, leaf surface is rough and tufts of hair on the axils of major veins on the lower surface is clearly absent. The leaf apex is acuminate to caudate with coarsely serrate margin. Under cultivated conditions they express monoecious features against the normal dioecious nature. The most important difference between the two taxa is presence or absence of connate style. This character is most dependable and allows the identification of female specimen as *M.alba* or *M.indica* irrespective of the variation in the length of the connate part of the style and hairiness of the style arm. But, it is often difficult to distinguish male specimens of these taxa as the shapes of the sepals are sometimes variable and is not a well marked character.

DNA markers have been increasingly used to resolve the taxonomic problems. The present study is an effort towards obtaining further insight into the taxonomic status of the cultivated varieties belonging to the *M.alba* and *M.indica*. Some of the varieties have originated from open-pollinated hybrid selection/cross-pollinated hybrid selection with the possibility of mixing of genomes from different species. Thus the taxonomic considerations of cultivated varieties are more complex and should not be approached routinely. RAPD marker analysis revealed that Tagowase and Kokuso-27 originated from Japan were the closest entries with 83.5% similarity and belongs to *M.alba*. Shin Ichinose (*M.alba*) originated from Japan and Almora Local (*M.indica*) originated from India were the most distant (genetic distance=0.600). Grouping based on UPGMA broadly resulted in four clusters. The cluster I has a single representation of the variety KNG (*M.alba*) from Japan and quite distinct. The cluster II is most complex and represented by 5 varieties of *M.alba* and 6 varieties of *M.indica*. The varieties of *M.alba* is either form Chinese or Japanese origin. Cluster III is represented by two varieties belonging to *M. indica* (Almora Local and Punjab Local) and a variety-China White belonging to *M.alba*. Shin Ichinose variety evolved out of inter-specific hybridization and classified (based on key characters) under *M.alba* forms a distinct cluster IV. The study further does not support the claim of molecular marker discriminating the cultivated mulberry varieties in accordance with the geographical origin Sharma et al.[13]. The RAPD marker analysis based UPGMA clustering has not succeeded in clearly delineating the two cultivated species. The sexual isolation was recognized with the species having lowest taxonomic unit. However, reproductive isolation is yet to be found among the cultivated species of mulberry. Categorization of cultivated varieties needs to be analyzed in terms of its economic value to the industry rather than purely based on some of the morphological traits. The grouping or classification of the cultivated varieties is required mainly for identification and proper utilization by the end user or by the breeder.

The study highlights the complexity in the evolution of cultivated varieties of these two species. The pure materials (wild population of *M.alba* and *M.indica*) of

the species are very much needed for the study, which is lacking. As understood presently, there is no pure or wild population of *M.indica* in India or elsewhere. Based on the result obtained from materials used and interrelationship observed by UPGMA clustering obtained from the DNA marker analysis, it is concluded that, there is not much genetic distinction between the taxa under consideration and hence *M.indica* may be considered as a variety under the species *M.alba*. This also supports the taxonomic analysis of *M.indica* by Kameswara Rao and Jarvis 1986[2]. The relative estimates of the genetic diversity based on RAPD markers also support the above conclusion. Genetic diversity estimates of some of the common varieties utilized in the present investigation can be used in precise planning of the mulberry crop improvement.

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