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Structure and Genetic Diversity of Natural Populations of *Morus alba* in the Trans-Himalayan Ladakh Region

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Abstract Sequence-related amplified polymorphism markers were used to assess the genetic structure in three natural populations of *Morus alba* from trans-Himalaya. Multilocation sampling was conducted across 14 collection sites. The overall genetic diversity estimates were high: percentage polymorphic loci 89.66%, Nei's gene diversity 0.2286, and Shannon's information index 0.2175. At a regional level, partitioning of variability assessed using analysis of molecular variance (AMOVA), revealed 80% variation within and 20% among collection sites. Pattern appeared in STRUCTURE, BARRIER, and AMOVA, clearly demonstrating gene flow between the Indus and Suru populations and a geographic barrier between the Indus-Suru and Nubra populations, which effectively hinders gene flow. The results showed significant genetic differentiation, population structure, high to restricted gene flow, and high genetic diversity. The assumption that samples collected from the three valleys represent three different populations does not hold true. The fragmentation present in trans-Himalaya was more natural and less anthropogenic.

Keywords BARRIER · Conservation · Himalaya · Mulberry · SRAP · STRUCTURE

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Introduction

Understanding of population genetic structure is the basic prerequisite for conservation and management of biodiversity (Baverstock and Moritz 1996; Allendorf and Luikart 2007). Habitat destruction is a major problem in the preservation of biodiversity in many terrestrial ecosystems (Young et al. 1996). Population fragmentation leads to reduced genetic diversity and increased genetic differentiation because of high random genetic drift and inbreeding, and reduction in gene flow (Young et al. 1996; Sork et al. 1999; Lowe et al. 2005). These effects can be visualized in light of traditional biogeography and metapopulation theories (MacArthur and Wilson 1967; Levins 1969). A number of studies have shown effects of fragmentation on the genetic diversity and population structure of plant species (Cardoso et al. 2005; Prentice et al. 2006; Yao et al. 2007). Fragmentation of plant populations is caused not only by anthropogenic activities but also by climatic changes, which cause formation of natural barriers between populations like mountain ranges, deserts, and other geographic barriers (Slatkin 1987). Conservation of biodiversity within these fragmented landscapes is a major challenge for policy makers to devise tools for effective conservation. It is therefore necessary to assess patterns of plant response to habitat fragmentation.

Morus alba L. (Moraceae) is a small to medium-sized dioecious, occasionally monoecious, perennial, wind-pollinated, outbreeding heterogeneous tree with wide range distribution from tropical and subtropical to temperate zones in Asia, Europe, North America, Africa, and South America (Kafkas et al. 2008). It is an economically important plant used for sericulture and extensively cultivated in East, Central, and South Asia. It is widely believed that *M. alba* species originated on the low slopes of the Himalayas bordering China and India (Awasthi et al. 2004), and hence evaluating its genetic structure is even more important in Himalaya and its adjoining areas. *Morus alba* is found in the trans-Himalayan Ladakh region at 2,815–3,176 m above mean sea level (amsl) on hilly slopes along the rivers in the Indus, Nubra, and Suru valleys. A long spell of subzero temperature in the region forces the species to remain dormant from October to February. The dormant buds sprout at the onset of spring during March–April along with floral buds. Peculiar features of the species include plasticity of leaf in shape and size and variability in petiole length. Fruiting occurs in the month of July, and the color of the fruit varies from white to purple or red.

Molecular markers have been extensively used to characterize *Morus* species. RAPD and inter-simple sequence repeat (ISSR) markers have previously been used to study the genetic relationships of Japanese and Indian *Morus* cultivars and to assess their molecular variability (Vijayan 2004; Vijayan et al. 2004a). ISSRs are found to be the most commonly used marker system in mulberries (Vijayan and Chatterjee 2003; Vijayan et al. 2004b, c, 2006; Zhao et al. 2006, 2007a, b; Kar et al. 2008). Directed amplification of minisatellite DNA (DAMD), single primer amplification reactions (Bhattacharya and Ranade 2001; Bhattacharya et al. 2005), and SRAP markers have also been used to assess the genetic relationship among mulberry cultivars (Zhao et al. 2009). However, the majority of molecular marker-based studies in *Morus* species are mainly restricted to characterization of

accessions and cultivars. To our knowledge, use of molecular markers for assessment of structure and genetic diversity of natural populations of *M. alba,* especially from the trans-Himalayan region, has not been reported. The present study was therefore undertaken to answer some of the pertinent queries, such as, (i) How are different populations of *M. alba* structured in trans-Himalaya? (ii) What is the level of genetic differentiation? (iii) Is there any geographic tendency in genetic data? (iv) What is the level of fragmentation revealed by population genetic structure? (v) How can genetic information be used to establish effective conservation measures?

Materials and Methods

Study Site and Population Sampling

We collected 56 wild *M. alba* individuals from 14 collection sites spread across the Indus, Suru, and Nubra valleys in Indian trans-Himalaya during 2009–2010 (Table 1; Fig. 1). The altitude of the collection sites ranged from 2,815.4 to 3,176.9 m amsl. Altitude and location of study sites were established using Garmin GPS 72 (Olathe, KS, USA). Valley divisions were considered as separate populations, and a multilocation sampling strategy was adopted. Two accessions of *M. alba* obtained from the Central Sericulture Germplasm Resource Centre, Hosur, Tamil Nadu, India, during 2004 and maintained at the horticulture field of DIHAR, Leh, were used for comparison. The outside mean maximum and minimum temperature recorded during 2001–2011 at 3,235 m amsl in the region was 18.9 ± 9.5 and -5.8 ± 9.8°C, respectively; the mean maximum and minimum relative humidity was 35.54 ± 7.3 and 25.0 ± 3.7%, respectively. The average annual precipitation was less than 200 mm, of which more than 70% was in the form of snowfall (Korekar et al. 2013). A herbarium of mulberry representative samples collected from the three valleys was prepared, and voucher specimens were submitted to Botanical Survey of India, Kolkata, India, to ascertain the *Morus* species status.

DNA Extraction and PCR Amplification

Total genomic DNA was extracted from frozen leaves (5 g) by the CTAB method (Saghai-Maroof et al. 1984) with minor modifications, including the use of 200 mg polyvinyl pyrollidone per sample. SRAP markers developed by Zhao et al. (2009) were adapted in this study. We tested 72 primer combinations [8 forward (Me 1–8) and 9 reverse (Em 1–9); Supplementary Table 1] for amplification in duplicate PCR to ensure reproducible banding patterns. The 55 SRAP primer sets were selected based on proper amplification and reproducibility. Each 20 µl SRAP PCR consisted of 2 mM dNTPs, 10 mM of each forward and reverse primer, 0.75 U *Taq* polymerase, 25 ng template DNA, *Taq* buffer containing Tris with 15 mM $MgCl_2$ and Milli-Q water. Amplification was carried out with the initial cycle at 94°C for 5 min, 5 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 1 min; followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and the final

Table 1 Multilocation sampling of three populations from trans-Himalaya Ladakh region

Collection site	Code	Population	Samples	Altitude (m amsl)	Latitude	Longitude
Skurbuchan	1 SKB	Indus	20	2,894.990	34°25′877N	76°43′106E
Domkhar	2 DM			3,176.930	34°19′340N	76°45′399E
Achinathang	3 ACH			2,892.247	34°30′377N	76°37′618E
Beama	4 BM			2,840.431	34°36′014N	76°30′874E
Sanjok	5 SJ			2,928.213	34°34′458N	76°31′584E
Batalik	6 BT	Suru	16	2,815.437	34°39′376N	76°20′286E
Achkamal	7 AKM			2,901.086	34°33′422N	76°09′554E
Paschym	8 PS			2,872.74	34°31′277N	76°10′897E
Lochum	9 LO			3,034.284	34°28′019N	76°15′280E
Chalunka	10 CH	Nubra	20	2,991.002	34°49′461N	76°58′696E
Bokdang	11 BK			3,009.9	34°48′257N	77°02′696E
Turtuk	12 TU			2,816.352	34°50′84N	76°49′272E
Tyakshi	13 TY			2,970.885	34°53′121N	76°48′285E
Hunder	14 HU			3,176.930	34°19′143N	77°27′824E
South Indian	SI	Reference sample	2			

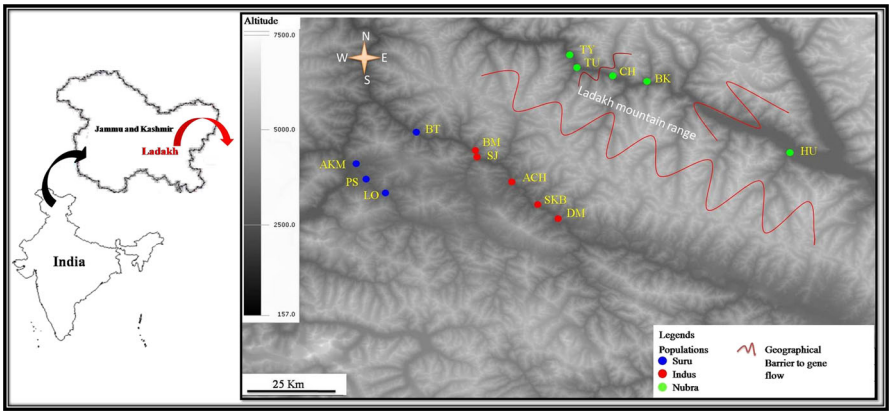

Fig. 1 Sampling sites of *M. alba* in trans-Himalayan Ladakh. Collection site codes as in Table 1. *Inset* location of Ladakh mountain range in northern India

extension at 72°C for 5 min. Amplification products were electrophoresed on 1.5% agarose gel, and molecular size of amplicons was estimated using 100 bp and 1 kb DNA ladders.

Data Collection and Analysis

The banding patterns obtained from SRAP were scored as present (1) or absent (0), each of which was treated as an independent character. POPGENE version 1.32

(Yeh et al. 1997) was used to calculate the different genetic diversity parameters: Nei's genetic diversity (H), Shannon's information index (I), number of polymorphic loci (NPL), percentage polymorphic loci (PPL), gene diversity of total population (H_t), and average gene diversity of subpopulations (H_s). The partitioning of genetic variability at different levels was calculated by analysis of molecular variance (AMOVA) using GenAlEx version 6.3 (Peakall and Smouse 2006) software. AMOVA was calculated at the population and regional levels.

Genetic differentiation coefficients G_{st} and Φ_{PT} were calculated by GenAlEx version 6.3v and POPGENE version 1.32, respectively. Gene flow (N_m) was calculated on the basis of Φ_{PT} with the formula $0.25(1 - \Phi_{PT})/\Phi_{PT}$ (Wood and Gardner 2007). STRUCTURE version 2.3 (Pritchard et al. 2000; Falush et al. 2003, 2007) was used to predict the number of clusters (K) and the probability of individual assignment to each cluster. The parameter sets assumed were admixture allele models with correlated allele frequencies and with no prior population location information. The number of clusters was set from $K = 1$ to 10 with four simulations for each K, and for each simulation we have a fixed burn-in period of 100,000 steps followed by 250,000 Monte Carlo Markov Chain replicates. The results obtained from STRUCTURE were interpreted by the online tool STRUCTURE HARVESTER (Earl and Von Holdt 2012), which implements Evanno's method (Evanno et al. 2005) for the calculation of the correct number of clusters (K). CLUMPP indfile obtained from STRUCTURE HARVESTER was used as input for the CLUMPP (Jakobsson and Rosenberg 2007) program, which permutes replicated matrices into one representative matrix. CLUMPP output was visualized graphically by the DISTRUCT (Rosenberg 2004) program. To test the isolation by distance model (IBD; Wright 1943), a Mantel test was performed by comparing matrices of genetic and geographic distance using GenAlEx version 6.3 software. The matrix of geographic distance was calculated by the Universal Transverse Mercator method using GPS coordinates. The null hypothesis for the IBD model was no correlation between geographic and genetic distance matrices. A specific test was devised to suggest historical barriers to gene flow among collection sites using the program BARRIER version 2.2 (Manni et al. 2004), which uses the geographic coordinates of each collection site and the F_{st} genetic distances calculated in AFLP-SURV (Vekemans 2002) as input. The robustness of estimated barriers was tested by means of 100 bootstrapped distance matrices. The geographic map was constructed using GPS mapping software (http://www.eye4software.com).

Results

Genetic Diversity

Initially, 72 SRAP primer pairs were screened. Of those, 55 pairs gave proper reproducible amplification and were considered for further analysis. Of the 348 amplicons produced, 329 (94.3%) were polymorphic. The average number of bands produced by each primer was 6.33, with 5.98 polymorphic bands (Supplementary Table 2). The genetic diversity parameters at the level of sampling sites were highest for the Paschym site (NPL = 178, PPL = 51.15%, $H = 0.2027 \pm 0.2155$,

I = 0.2961 ± 0.3047) of Suru and lowest for the Chalunka site (NPL = 74, PPL = 21.26%, H = 0.0828 ± 0.1687, I = 0.1215 ± 0.2419) of Nubra valley (Supplementary Table 3). Genetic diversity parameters at the population level are presented in Table 2. The average gene diversity of subpopulations (H_s), which reveals the actual gene diversity present within the population, was highest in Suru (0.1834 ± 0.0241) and lowest in Nubra (0.1234 ± 0.0121). The total gene diversity, however, of the population (H_t) was highest in Nubra (0.2447 ± 0.0385) and lowest in Indus (0.2064 ± 0.0352).

Putative Genetic Barrier Prediction

The barrier prediction analysis using Monmonier's maximum difference algorithm revealed three likely barriers to gene flow. We considered only those barriers with 100% bootstrap value. The first barrier (aa) was assigned isolating the peripheral Hunder collection site from the rest of the collection sites. The second barrier (bb) separated the Nubra valley population from the Indus and Suru valley populations. The third barrier (cc) was detected within the Nubra population between the Turtuk-Tyakshi and Chalunka-Bokdang collection sites (Figs. 1, 2).

Genetic Structure

A Mantel test ($R_{xy} = 0.409$, $P = 0.020$; Supplementary Fig. 1) showed a weak positive correlation between genetic and geographic distance across the sampled region. The Mantel test rejected the null hypothesis, as correlation between genetic and geographic distances was established. AMOVA at the population level based on sampling locations showed a peculiar pattern. The Nubra population displayed 67% variability within sampling locations and 33% among sampling locations, whereas the Indus and Suru populations showed more than 90% variability within sampling sites (Table 3). AMOVA at the regional level displayed 80% variability within sampling sites and 20% among sampling sites.

To investigate the population structure at the regional level, we applied a Bayesian model-based clustering algorithm implemented in the STRUCTURE program, which estimates the shared population ancestry of individuals purely on the basis of genetic data without considering population location information. The log probability of the data and Evanno's method of cluster determination detected six ($\Delta K = 6$) clusters, suggesting that the 58 individuals partitioned into six clusters (Fig. 3). STRUCTURE analysis separated samples from south India as an outgroup and divided Ladakh populations into five clusters. The Indus and Suru populations revealed admixture, while Nubra displayed clear-cut structuring.

Genetic Differentiation and Gene Flow

Overall genetic differentiation coefficients ($\Phi_{PT} = 0.20$; $G_{st} = 0.1026$) showed high and significant differentiation. G_{st} varies from 0.2231 (Suru) to 0.4958 (Nubra) and Φ_{PT} from 0.02 (Suru) to 0.325 (Nubra). Both genetic differentiation coefficients revealed higher fixation in the Nubra population and lower in the Suru. The number

Table 2 Genetic diversity across three populations of *M. alba* in Ladakh

Population	Sample size	NPL	PPL	H_s (mean ± SD)	I (mean ± SD)	H_t (mean ± SD)	Gst
Indus	20	233	66.95	0.1396 ± 0.0183	0.2035 ± 0.2874	0.2064 ± 0.0352	0.3236
Suru	16	257	73.85	0.1834 ± 0.0241	0.2685 ± 0.2998	0.2360 ± 0.0353	0.2231
Nubra	20	259	74.43	0.1234 ± 0.0121	0.1805 ± 0.2750	0.2447 ± 0.0385	0.4958
Average		249.67	71.74	0.1488 ± 0.2531	0.2175 ± 0.2874	0.2290 ± 0.0363	0.3475
Species level	56	312	89.66	0.2286 ± 0.0262		0.2547 ± 0.0320	0.1026

NPL number of polymorphic loci, *PPL* percentage of polymorphic loci, H_s average gene diversity of subpopulations, *I* Shannon's information index, H_t gene diversity of total population, *Gst* genetic differentiation coefficient

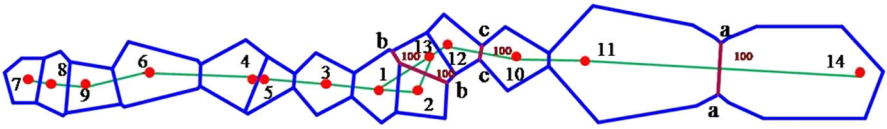

Fig. 2 Genetic barriers predicted by BARRIER (version 2.2). *Line a*, *b*, and *c* indicate genetic barriers; collection sites are numbered as in Table 1

of migrants per generation calculated on the basis of Φ_{PT} displayed a higher migration rate in the Suru population, with 12 migrants per generation, whereas no migrants were observed in the Nubra population ($N_m = 0.52$; Tables 2, 3).

Discussion

SRAP Marker System

The SRAP marker system has several advantages over other molecular marker systems, such as simplicity, reasonable throughput rate, disclosure of numerous codominant markers, ease of isolation of bands for sequencing, and, most important, targeting of open reading frames (Li and Quiros 2001). Budak et al. (2004) showed that SRAP analysis is more powerful than SSR, ISSR, or RAPD markers for detection of genetic diversity among closely related cultivars. According to Ferriol et al. (2003), the information derived from SRAP markers is more concordant to the morphological variability and to the evolutionary history of the morphotypes than that of AFLP in genetic diversity analysis. This marker system has been recognized as a new and useful molecular tool in assessing population structure and genetic polymorphism in many plant species (Ding et al. 2008). Therefore, SRAP markers were used in the present study to evaluate the genetic diversity and population genetic structure of wild *M. alba* populations. The 55 SRAP polymorphic markers used were highly informative. The level of polymorphism obtained (94.3%) was higher than previous reports using RAPD, ISSR, and AFLP studies (Bhattacharya and Ranade 2001; Vijayan et al. 2004c; Awasthi et al. 2004; Kafkas et al. 2008). The 55 combinations of primer pairs produced a high number of polymorphic bands

Table 3 AMOVA for 14 subpopulations of *M. alba* distributed in three valleys of Ladakh

Group	Partitioning	DF	SSD	MSD	Estimated variance	Variance (%)	Φ_{PT} (p value)	N_m
At population level								
Indus	Among collection sites	4	179.7	44.925	2.781	8	0.076 (0.04)	3.04
	Within collection sites	15	507	33.8	33.8	92		
	Total	19	686.7		36.581	100		
Suru	Among collection sites	3	144.813	48.217	0.911	2	0.02 (0.029)	12.25
	Within collection sites	12	535.5	44.625	44.625	98		
	Total	15	680.313		45.536	100		
Nubra	Among collection sites	4	389.35	97.33	16.026	33	0.325 (0.01)	0.52
	Within collection sites	15	498.5	33.23	33.23	67		
	Total	19	887.85		49.259	100		
At regional level								
	Among collection sites	13	954.393	73.415	9.190	20	0.2 (0.01)	
	Within collection sites	42	1,539.5	36.655	36.555	80		
	Total	55	2,493.893		45.845	100		

(329) among 56 individuals, which indicates the utility of SRAP markers for genetic diversity evaluation of natural *M. alba* populations.

Genetic Diversity

Accurate estimates of genetic diversity are useful for optimizing sampling strategies and for conservation and management of various tree species (Hamrick and Godt 1996). Analyses in the present study revealed overall high genetic diversity at the species level, meaning that there is a large difference in intrapopulation gene diversity and total gene diversity. However, genetic diversity in the Nubra population was low as revealed by H_s (average gene diversity of subpopulations). This signifies that the Nubra population is structured. The Indus population displayed low genetic diversity, although it was higher than Nubra. The Suru population showed the highest level of genetic diversity and the least difference in H_t and H_s values.

High genetic diversity at the species level was in accordance with previous studies (Cao et al. 2006; Zhou et al. 2012; Pometti et al. 2012). The percentage of polymorphic loci (PPL) across these studies revealed high genetic diversity within the species. Bhattacharya and Ranade (2001) reported 85% PPL using RAPD and

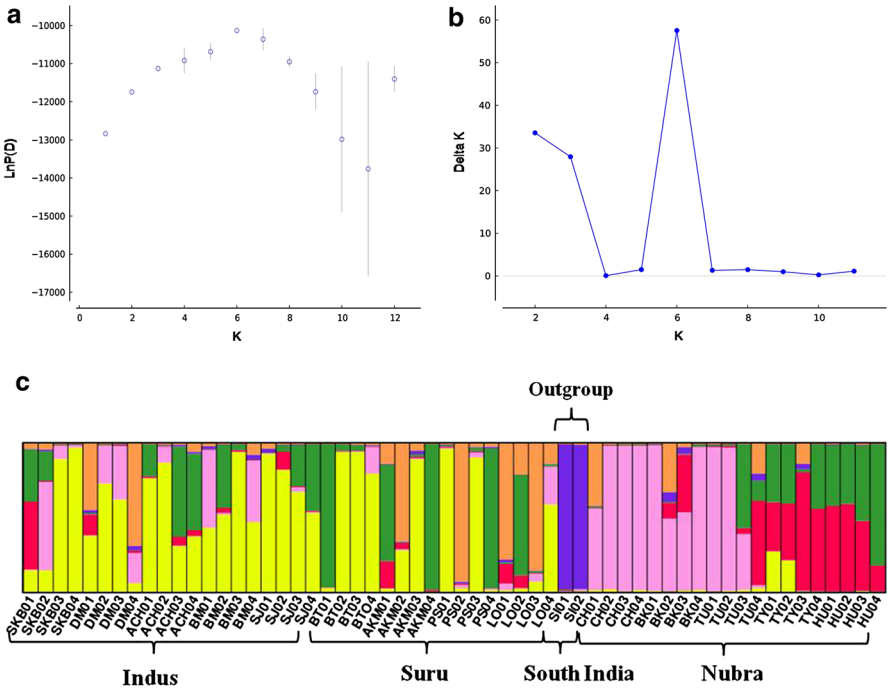

Fig. 3 STRUCTURE analysis *M. alba* populations. **a** Relationship between K and LnP(D). **b** Relationship between K and delta K. **c** Membership probability of assigning individuals of all populations to different clusters when $K = 6$. Collection site codes as in Table 1

91% using DAMD markers in various *Morus* species. Srivastava et al. (2004) reported 74.13% PPL in *M. alba* using ISSR markers and 60.75% using RAPD. Vijayan et al. (2004c) reported PPL of 86% and H_t of 0.27 for ISSR markers and PPL 78% and H_t 0.23 using RAPD in *M. alba* and other *Morus* species. Studies in Turkey (Kafkas et al. 2008) and China (Zhao and Pan 2004; Zhao et al. 2007a) also reported high genetic diversity. Genetic diversity within natural populations is directly linked to their mating system, life form, pollen dispersion syndrome (relative strength of pollen vs. seed dispersal), mechanism of seed dispersal, geographic range, and also their effective population size (Hamrick 1983; Hamrick and Godt 1996). *Morus alba* is an outbreeding and wind-pollinated species. Pollen release is fastest in the plant kingdom, which is about half the speed of sound (Taylor et al. 2006). Outbreeding woody species have higher within-population genetic diversity than plants with inbreeding and herbaceous life forms (Hamrick and Godt 1996). The proportion of gene flow via pollen dispersal compared with seed dispersal is higher in wind-pollinated trees (Squirrell et al. 2001). Moreover, pollen often disperses farther than seeds and in greater quantities (Kremer et al. 2012). The ability of plants to disperse pollen grains at very high velocities is an ancient evolutionary adaptation that enhances wind pollination in unfavorable environmental conditions (Taylor et al. 2006), such as the trans-Himalaya region, which is characterized by prolonged subzero temperatures, low precipitation, sandy

soils with low water-holding capacity, low relative humidity, intense solar radiation, low atmospheric pressure, high wind velocity, and rugged terrain. Schuster et al. (1994) reported high genetic diversity in outbreeding tree species in cold and arid environments. Spatially, cold arid land is characterized by differences in substrate, soil structure, slope, and aspect that create a mosaic of microhabitats and vegetation patterns (Parker 1991). Theoretical and empirical studies have indicated that such environmental heterogeneity can lead to maintenance of high levels of within-population genetic variation (Hedrick et al. 1976; Ewing 1979; Gillespie and Turelli 1989; Yeaman and Jarvis 2006). So it is not unreasonable to believe that *M. alba* has maintained high levels of genetic diversity due to environmental heterogeneity.

Population Structure and Geographic Barrier

Geographic isolation is one of the major constraints to gene flow via both pollen and seeds, which results in genetic differentiation of populations (Pfeifer and Jetschke 2006). A Mantel test displayed weak positive correlation between the genetic and geographic distance matrix. Therefore, an isolation by distance model does not fit completely in the present study. Genetic clustering based on a Bayesian model predicted six clusters; only five clusters were of interest, as one cluster represents an outgroup formed by south Indian genotypes. The five clusters displayed an interesting pattern. Individuals from the Indus and Suru populations show coancestry, while the Nubra population structured into subpopulations. The Indus and Suru populations clearly fit the admixture model we considered during the STRUCTURE analysis. Coancestry in Indus and Suru genotypes indicated the presence of a good migration pattern, which was confirmed by a significant number of migrants per generation (3 for Indus and 12 for Suru). BARRIER programs predicted four gene pools; the first consists of only the Hunder collection site, the second of the Indus and Suru populations, the third Bokdang and Chalunka, and the fourth Turtuk and Tyakshi. When we combined STRUCTURE and BARRIER, four genetic populations emerged. The Indus and Suru populations merged into one population, and Nubra structured into three genetic subpopulations (Turtuk-Tyakshi, Bokdang-Chalunka, and Hunder). Structuring in Nubra was also confirmed by a high genetic differentiation coefficient ($\Phi_{PT} = 0.325$; $G_{st} = 0.4958$) and nil migrants ($N_m = 0.52$) per generation. The Nubra population showed isolation by distance, as nearby collection sites merged into the same subpopulation but distant collection sites formed separate subpopulations. The Indus and Suru populations showed coancestry, because the overall Mantel test correlation was weak. AMOVA results were also in concordance with the STRUCTURE and BARRIER results. The Indus and Suru populations exhibited over 90% variability within samples, which displayed high gene flow and outbreeding, whereas the Nubra population showed 33% variability among subpopulations and 67% within subpopulations, indicating structuring in the Nubra population. A pattern appeared in STRUCTURE, BARRIER, and AMOVA results that clearly demonstrated gene flow between the Indus and Suru populations, while there is a geographic barrier between Indus-Suru and Nubra populations that effectively hinders gene flow. The geographic barrier predicted between the Indus-Suru and Nubra populations by BARRIER was

supported by the existence of the Ladakh mountain range (Fig. 1). The separation of the Nubra and Indus-Suru populations may be the result of a past vicariance event. These results showed that the Ladakh mountain range is an effective barrier to gene flow. Migration of mulberry is also influenced by the human migration pattern. Historically, the Ladakh range was a great barrier to human movement. The mountain range therefore plays an important role in shaping the genetic structure of *M. alba* in trans-Himalaya.

Divergence within the Nubra valley population may be due to the separation of the Hunder subpopulation from other Nubra sampling sites by 40–60 km distance. The long distance may act as a geographic barrier, and hence isolation by distance was observed. The Bokdang-Chalunka subpopulation is located on the eastern side of the Shyok River; the Turtuk-Tyakshi site is on the western side (Fig. 1). The Shyok River may be the natural barrier that corresponds to a historical barrier predicted by the BARRIER program and that separates Bokdang-Chalunka and Turtuk-Tyakshi subpopulations. This may be responsible for the occurrence of structure and two geographic barriers among collection sites in the Nubra valley. Our initial classification of three populations based on three valleys does not hold true. Actually there are four gene pools or genetic populations of *M. alba*.

Genetic Differentiation and Gene Flow

Correct estimation of population differentiation is essential in conservation biology, as it gives an idea of how populations are genetically isolated from each other and to what extent (Balloux and Lugon-Moulin 2002). In the present study we used two types of genetic differentiation coefficients to approximate the genetic differentiation scenario. G_{st} as derived by Nei (1973) can be defined as the ratio of the inter-subpopulational gene diversity to the total gene diversity. Φ_{PT} (Peakall and Smouse 2006) is a measure of population genetic differentiation for binary data and is analogous to Wright's F_{st}.

Overall, genetic differentiation was high in this region. The Nubra population especially showed greater genetic differentiation, revealing high among-population diversity. High differentiation in the Nubra population confirmed the population substructuring revealed by STRUCTURE, BARRIER, and AMOVA. However, the Suru population showed high gene flow within the population with low genetic differentiation. According to Waples and Gaggiotti (2006), when $N_m = 1–5$, genetic differentiation is strong to moderate. Therefore, gene flow also showed strong differentiation in the Nubra, moderate in the Indus, and negligible in the Suru populations.

The main evolutionary forces leading to genetic differentiation between natural populations are generally considered to be natural selection, random genetic drift, and limited migration (Baines et al. 2004). These results clearly showed the contribution of restricted to moderate gene flow to genetic differentiation. Natural selection can be assumed, as these plants are growing in a variety of microhabitats in this heterogeneous climate. Genetic drift also cannot be ruled out in such an unpredictable climate. Natural selection, genetic drift, and gene flow do not act in isolation in natural populations (Andrews 2010). So, the apparent genetic

differentiation is the result of the interplay between all three factors of natural selection, genetic drift, and gene flow. Yeaman and Jarvis (2006) suggested that migration–selection balance is important in maintaining genetic diversity in heterogeneous environments. The present study revealed high overall genetic diversity and a significant amount of differentiation. So it appears that genetic diversity is maintained because migration and genetic differentiation both are present and balancing each other. Andrews (2010) also stressed that balancing selection, in contrast to directional selection, maintains genetic polymorphism in populations. It means, despite significant structuring and differentiation, that *M. alba* still maintains a high level of genetic diversity, in concordance with similar studies on tree species (Suarez-Montes et al. 2011; Melendez-Ackerman et al. 2005).

Implications for Conservation

The negative effect of fragmentation on genetic diversity is the main basis for conservation efforts made in the recent past (Ouborg et al. 2006). To formulate effective conservation strategies it is essential to study patterns of plant response to habitat fragmentation. The present study displayed high levels of genetic diversity with significant population genetic structure and genetic differentiation in *M. alba,* but the genetic structure predominant in the Nubra population and therefore the population from the Nubra valley should be given priority while devising management procedures. Considering the significant genetic structure and limited gene flow in the Nubra valley, conservation strategies should aim at in situ conservation, since habitat fragmentation and small population size make it vulnerable to the loss of genetic diversity caused by the effects of genetic drift and inbreeding. Besides, the balance between genetic differentiation and migration proved to be an important factor in maintaining genetic diversity. Conservation measures should therefore be such that they conserve both heterogeneous landscapes and historical levels of gene flow. All *M. alba* plants grow wild in the trans-Himalayas with no conservation measures, so here we propose the establishment of new populations in areas like farm fields and areas under governmental protection. Another important measure is to conduct surveys to uncover more individuals and populations in other localities. Additionally, in regions like the Nubra valley, where the possibility of habitat destruction or exploitation of plants for local consumption is high, it is necessary to establish sustainable management plans through local government agencies and non-government organizations. Considering the critical situation of *M. alba*, probably the safest way to preserve the species is through all of the methods mentioned above.

In conclusion, a mixed population structure appeared in this study. The Nubra population clearly displayed structuring with three subpopulations. The Indus and Suru populations did not display any significant structuring. The Ladakh mountain range proved an important barrier to gene flow and shaped population genetic structure of *M. alba* in trans-Himalaya. The results shed light on the role of geographic barriers in speciation. Genetic diversity has an important role in plant survival, as it provides disease resistance, adaptability to a changing climate, or

some other trait necessary to survive in the ever-changing world. The present study reported overall high genetic diversity at the species level. There is balance between migration and genetic differentiation that helps in maintaining genetic diversity. These results also have evolutionary significance, as the Himalayan region is considered a center of diversity for the mulberry. Furthermore, our results provide a basic genetic profile for conservation and responsible exploitation of the extant germplasm of this species to improve the genetic base for breeding. SRAP markers are highly reproducible and efficient markers for assessment of genetic structure and diversity among wild populations.

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