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Note

Molecular markers provide evidence for long-distance planting material transfer during plantation establishment of *Dalbergia sissoo* Roxb. in Nepal

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Abstract – We investigated five population pairs of *Dalbergia sissoo* in Nepal for variation of cpDNA and at the isozyme gene locus *Gdh-A*. Each population pair consisted of one natural population and a neighboring plantation. Two or three different cpDNA-haplotypes were observed in each population with a total of eight different haplotypes. The differentiation of cpDNA haplotypes between the group of natural populations and the group of plantations was almost complete. Thus, the plantations did not originate from any of the investigated natural populations, but reproductive material was transported over long distances during plantation establishment. All plantations proved to be fixed at the *Gdh-A* gene locus, which showed considerable polymorphism in natural populations indicating different adaptive potentials of plantations and natural stands.

cpDNA haplotpyes / isozymes / tropical tree / seed transfer / adaptive potential

Résumé – Mise en évidence de transferts à longue distance de matériel de reproduction utilisé pour des plantations de *Dalbergia sissoo* Roxb. au Népal par l'utilisation de marqueurs moléculaires. On a étudié la variabilité d'ADNcp et de l'isozyme du locus *Gdh-A* dans cinq paires de populations de *Dalbergia sissoo* situées au Népal. Chaque paire comportait une population naturelle et une plantation située à proximité ; on a observé l'existence de deux ou trois ADNcp haplotypes dans chaque population avec, au total, huit haplotypes différents. La différenciation pour les ADNcp haplotypes entre le groupe des populations naturelles et celui des plantations était presque générale. Il en résulte que les plantations ne sont pas issues des populations naturelles étudiées ; le matériel de reproduction a été transporté sur de longues distances au cours des opérations de plantation. Aucune variation du niveau du locus du gène *Gdh-A* n'a été trouvée pour toutes les plantations, alors qu'il existe un polymorphisme considérable dans les populations naturelles. Il en résulte un potentiel d'adaptation différent entre plantations et peuplements naturels.

haplotypes ADNcp / isozymes / arbre tropical / transfert de graine / potentiel d'adaptation

1. INTRODUCTION

The significance of forest tree plantations for the production of wood rapidly increases throughout the tropics. The annual increase of the plantation area in tropical Asia has been more than two million hectares during the last decade [3]. Thus, the biodiversity harboured in plantations of tropical trees and the adaptive properties of tree populations used for plantation establishment are topics of growing importance. The genetic diversity and the adaptive potential of plantations mainly depends on the reproductive material used for plantation establishment.

The marketing of forest reproductive material is controlled by legal regulations in industrialized countries [6]. However, comparable regulations do not exist or are not enforced in most developing tropical countries. Costs for procurement is usually the decisive factor for the choice of seeds or other reproductive material. As a result, the origin of material used for plantation establishment is usually unknown in the tropics both for exotics and species native to a planting region. A "narrow genetic base", i.e. low levels of genetic variation, and a poor adaptation of the chosen reproductive material to environmental conditions at the planting site are among the most frequently quoted reasons for disappointing performance or even complete failure of plantations [10].

Dalbergia sissoo Roxb. (Fabaceae) is naturally distributed on the foothills of the Himalayas stretching from Afghanistan to Bangladesh. The species is widely planted throughout its natural distribution, but also worldwide as an exotic [12]. It is a

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Figure 1. Location of the investigated population pairs of *Dalbergia sissoo* in Nepal. A: Hetauda natural population and Hetauda plantation; B: Shivapur natural population and Surai plantation; C: Hattisar natural population and Thakurdwara plantation; D: Godawari natural population and Attaria plantation; E: Pipariya natural population and Shuklaphanta plantation.

moderately fast-growing multipurpose species with an excellent wood for furniture making and many other uses. *D. sissoo* is currently one of the most frequently planted trees in Nepal and has a long history of plantation establishment in the country. More than 40% of all forest trees planted in Nepal and more than 90% of all trees planted in the flat "Terai" region of Nepal are *D. sissoo*. However, many plantations have been seriously affected by a die-back disease of unknown cause in the recent past, and phenotypic characteristics such as stem form and branching habit are often inferior in plantations. The origin of *D. sissoo* plantations in Nepal is usually unknown. Today's occurrence of the species in natural forests in Nepal is restricted to a few fragmented relics of autochthonous populations.

Molecular gene markers can be powerful tools to assess the evolutionary past of populations [9] and to infer the origin of material, which has been transferred by humans. We used molecular markers to conclude on the origin of *D. sissoo* plantations in Nepal. In particular, we addressed the following question: Is there evidence for long-distant transfer of seeds for plantation establishment?

2. MATERIAL AND METHODS

2.1. Plant material

Material was collected in five population pairs (A–E, Fig. 1). Each pair consists of one natural population and one neighboring plantation. The population pairs are located along a east-west transect in the flat "Terai" region of Nepal. The size of populations vary from about one hundred to several hundreds adult trees.

In each population we collected seeds from ten widely separated, randomly chosen, mature seed trees. Seed trees had a diameter of at least 20 cm and were estimated to be at least twelve years old. Seeds of single trees were carefully kept separately. In natural population Hattisar we were only able to sample seeds from six seed trees. We assessed the cpDNA haplotype of one seed per investigated tree. Thus, a total of 96 haplotypes were investigated. Five seeds per tree (total: 480 seeds) were investigated at the isozyme gene locus *Gdh-A*.

2.2. Methods

DNA was extracted from seeds using the QIAGEN DNeasy Plant Mini Kit following the manufacturer's instructions. The extracted DNA was used for PCR-RFLP and microsatellite (cpSSR) analysis.

The isolated DNA samples were amplified with five different universal primers (trn L/F, trn D/T, trn L/T, trn K1/K2, trn T/F) for PCR-RFLP analysis [2, 11]. PCR was conducted according to Demesure et al. [2] with minor modifications. The total volume used for PCR was 25 µL: 2 µL template DNA (20 ng), 2 µL primer (0.5 µM of each forward and reverse), 2 µL 10× PCR buffer with 2.5 mM $MgCl_2$, 6.5 µL distilled H_2O, 12.5 µL Qiagen Master Mix, 1 Unit of Taq-DNA polymerase (Hotstar Taq polymerase from Qiagen). Initial denaturation was at 94 °C for 15 min, followed by 35 cycles, each consisting of 94 °C for 1 min, 51 °C to 64 °C for 1 min, and 72 °C for 2 min. After successful amplification, the PCR products were used for restriction analysis with four to seven enzymes for each primer pair (trn L/F: Taq I, Hae III, Hinf I, Rsa I, Alu I, Hpa II, Cfo I; trn D/T: Taq I, Hae III, Hinf I, Rsa I, Alu I, Trn 9 I; trn L/T: Taq I, Hae III, Hinf I, Rsa I, Alu I, Trn 9 I; trn K1/K2: Hae III, Hinf I, Rsa I, Alu I; trn T/F: Hae III, Hinf I, Rsa I, Alu I, Hpa II, Cfo I). The total volume per sample was 11 µL (7.0 µL PCR product + 1.5 µL enzyme (10 U/µL) + 1.5 µL buffer (10×) + 1 µL distilled H_2O) for the restriction analysis. The restriction probes were incubated at 65 °C for Taq I and Trn 9 I, and for all other enzymes at 37 °C for about 16 h. PCR-RFLP products were separated in a 10% polyacrylamide (PAA) gel with a length of 24 cm. The fragments were visualized by SYBR Gold nucleic acid gel staining and photographed with a digital camera.

Chloroplast microsatellites (cpSSRs) were amplified as described by Weising and Gardner [14] using their universal primers ccmp-6, ccmp-7, and ccmp-10. The preparation of the PCR-mix and the separation and visualization of the fragments followed the procedures described for PCR-RFLPs.

Horizontal starch gel-electrophoresis was used to investigate variation at isozyme gene loci. Only results obtained for glutamate-dehydrogenase (*Gdh*; E.C. 1.4.1.2) are reported here. *Gdh* isozymes were

Table I. Number of observed cpDNA haplotypes and genetic variation at the *Gdh-A* isozyme gene locus (*A*: number of Alleles; H_e: expected heterozygosity) in five natural populations and five plantations of *Dalbergia sissoo* in Nepal.

Pair	Population	cpDNA haplotypes								Gdh-A	
		1	2	3	4	5	6	7	8	*A*	H_e
	Natural populations										
A	Hetauda N	9	1							3	0.097
B	Shivapur	8		2						4	0.317
C	Hattisar	5	1							4	0.436
D	Godawari	4		6						3	0.059
E	Pipariya			9	1					2	0.368
	Plantations										
A	Hetauda P					4	6			1	0
B	Surai				1			7	2	1	0
C	Thakurdwara				4			3	3	1	0
D	Attaria				6			4		1	0
E	Shuklaphanta				5			5		1	0

extracted from seeds and separated in a tris-citric acid buffer (pH 7.4) [4]. Staining followed Vallejos [13] with slight modifications.

2.3. Data analysis

Interpretation of gels was done visually directly on the gels (isozymes) or on the digital photographs (cpDNA fragments). Presence or absence of cpDNA fragments was recorded as (1) and (0), respectively, in a matrix. Trees showing no differences at all investigated cpDNA fragments (PCR-RFLP and cpSSR) were grouped to the same cpDNA haplotype. We refrained from a phylogenetic analysis of different chloroplast haplotypes since we used both PCR-RFLP and cpSSR polymorphisms for this study and due to the restricted geographic range covered by our survey. Phylogenetic relationships among haplotypes may considerably change if data from other regions become available.

Differentiation of haplotypes among populations was measured as F_{ST} [7] for the five natural populations and the five plantations alone, and for all ten populations taken together.

Isozyme phenotypes were interpreted as being controlled by a single gene locus. The number of alleles observed (*A*) was recorded and the expected heterozygosity (H_e) was computed as $H_e = 1 - \sum_i p_i^2$ for each population [1].

3. RESULTS

Variation of PCR-RFLPs was observed after restriction of the amplified fragment Trn K1/K2 (approximately 2700 bps) with Alu I and Rsa I, respectively. All other primer-enzyme combinations resulted in monomorphic patterns. Variation of cpSSRs was observed for ccmp-6 and ccmp-7, but not for ccmp-10. The observed variation allowed to distinguish eight different haplotypes. All populations proved to be polymorphic with two or three haplotypes identified for each population (Tab. I). The differentiation between haplotypes of natural populations (haplotypes 1–3) and of plantations (haplotypes 4–8) was almost complete. Only one tree of the natural population Pipariya showed haplotype 4, which was otherwise observed in plantations only. The plantation Hetauda showed a diagnostic fragment common to all trees of this population but to no other tree if the Trn K1/K2 fragment was digested with Rsa I.

Differentiation among populations accounted for 50.5% of the total variation of cpDNA haplotypes of all ten populations (F_{ST} = 0.505). The comparable F_{ST}-values were 0.464 for the five natural populations, and 0.286 for the five plantations only.

No variation was observed at the *Gdh-A* gene locus in any of the plantations after investigation of 50 seeds per population (Tab. I). However, all natural populations proved to be polymorphic with two to four alleles at this gene locus. All plantations were fixed on allele $Gdh\text{-}A_3$. This allele was also most common in all natural populations.

4. DISCUSSION

We assume the cpDNA of *D. sissoo* to be maternally inherited since this mode of inheritance has been described for most other angiosperm plants [5]. We observed considerable variation of cpDNA haplotypes within all studied populations. In consequence, the contribution of differentiation among populations to the total diversity of cpDNA haplotypes (F_{ST}) is lower for *D. sissoo* as compared to several other tree species such as European oaks (*Quercus* spp.) [9]. This may be explained by the presumably efficient zoochorous dispersal of diaspores resulting in high rates of seed migration among populations [8].

Differentiation of haplotypes among the two groups of natural populations and plantations is almost complete, i.e. the two groups of populations do not share their haplotypes. The only exemption to this observation is a tree in natural population Pipariya showing haplotype 4, which is otherwise observed in plantations only. It cannot be ruled out that this tree migrated from a neighbouring plantation, possibly plantation Shuklaphanta, to the natural relic forest.

We interpret the observation of almost complete differentiation of cpDNA-haplotypes between plantations and natural populations as a clear indication of substantial long-distant seed transfer by humans in Nepal. Not only the nearest natural population, but all investigated natural populations do not qualify as a possible origin of any of the studied plantations. Currently, we cannot identify the likely origin of *D. sissoo* plantations in Nepal due to the largely unknown, but presumably long history of plantation establishment in the country and due to a lack of comparable data from other regions of the species' distribution. An origin from meanwhile extinct Nepalese populations cannot be ruled out. However, long-distant transfer of reproductive material from other countries is a more reasonable explanation for the differentiation of cpDNA haplotypes. There is circumstantial evidence for a substantial import of *D. sissoo* seeds from India in the past.

Looking only at natural forests, haplotype 1 is most common in the eastern populations (A–C) of the transect, while haplotype 3 is dominating in the two western populations (D and E). Results indicate a clinal variation with decreasing frequencies of haplotype 1 from East to West. However, the number of

investigated populations is insufficient to investigate the spatial structure of cpDNA variation for natural populations in detail. No comparable pattern seems to exist for the plantations.

The observation of a diagnostic PCR-RFLP fragment common to all trees of plantation Hetauda which was not observed in any other population is interpreted as evidence for a different origin of this plantation as compared to the others. The introduction of material with different origin increased the total variation of cpDNA found in plantations.

The observation of differentiation between natural populations and plantations not only at presumably maternally inherited cpDNA, but also at the biparentally inherited *Gdh-A* isozyme gene locus confirms the impact of long-distant seed transport during the establishment of plantations on their genetic structures. The complete fixation of *Gdh-A* gene locus in plantation could be either due to selection or adaptation or for example due to the collection of planting material in a natural stand that was fixed for the same allele. Another cause might be collection of planting material from a reduced number of mother trees in a polymorphic natural stand. Natural populations and plantations could have different evolutionary adaptive potentials at least at this locus.

The susceptibility of plantations to a die-back disease and the frequently disappointing growth and stem form of *D. sissoo* in plantations in Nepal have been explained by the use of presumably poorly adapted reproductive material. Our results support this view by providing evidence for long-distance seed transfer during plantation establishment.

Small relics of autochthonous populations in natural forests are surrounded by large *D. sissoo* plantations in Nepal. We proved considerable genetic differentiation between natural forests and plantations. Thus, unidirectional gene flow through pollen or seed from plantations to natural populations may considerably change genetic structures of the natural relic populations. In this situation, the efficiency of efforts to conserve genetic resources of *D. sissoo* in situ may be limited in Nepal. We propose to implement complementary ex situ conservation methods such as the establishment of provenance resource stands in widely isolated regions, possibly out of the country.

Our results illustrate the utility of molecular methods to investigate the history and adaptive potential of plantations even in the absence of comprehensive data sets on variation patterns in natural populations. Further studies involving material from other regions of the species' distribution may allow to gain additional insights in the human impact on genetic structures due to long-distant seed transfer.

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