Diversity and genetic structure of teak (Tectona grandis L.f) in its natural range using DNA microsatellite markers

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Abstract Teak (*Tectona grandis* L.f.) is considered to be an extraordinarily durable building timber with a worldwide reputation. Its widespread use has entailed the over-exploitation of natural forests and a large reduction in natural diversity. Fifteen microsatellite markers were used to study the genetic variability and structure of 166 teak trees distributed over the whole natural area of teak. Analysis showed that in the teak natural area there were four main centers of genetic variability. Two clusters were in India and could be considered as main centers of genetic diversity in teak. The third cluster mainly consisting of populations in Thailand and Laos was genetically very distinct from the Indian populations but presented only half as much allelic variability. A fourth cluster from Central Laos showed even less genetic variability. The use of SSR markers for conservation of teak forest diversity is discussed.

Keywords Genetic structure · Genetic diversity · SSR · *Tectona grandis* · Conservation

Introduction

Teak (*Tectona grandis* L.f.) is one of the most valuable timber trees in the world. The teak, a member of the Verbenaceae family, is a diploid species 2n = 36 (Gill et al. 1983). It is indigenous to India, Myanmar, Thailand and Laos. As teak is a species with a wide geographic distribution in South East Asia, the natural populations develop heritable

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adaptations to local environmental factors in order to survive in different ecological conditions.

Extensive variation of stem form, axis persistence, branching, epicormics, buds and buttresses of bole has been found in the populations from India, Myanmar, and Thailand (Bagchi 1995; Bagchi et al. 1989; Bedell 1989; Bendale et al. 2005; Kaosa-ard 1999; Kyaw 2004). The forks are generally connected with flowering precocity (Dupuy and Verhaegen 1993). However, there is large variation and scoring of early flowering is in general not found to be an effective way to estimate the later stem form (Kjaer and Lauridsen 1996).

Leaf measurements of different origin from India reveal twelve intraspecific variations. In Karnataka, a distinct variety known as "Teli" is easily distinguished on the basis of leaf characters and resistance to defoliator, *Hyblaea puera* (Katwal 2003; Rawat et al. 1998; Tewari 1992), but the leaf dimensions show strong instability (Bagchi et al. 1989).

The natural provenances of teak exhibit differences in phenology (Bedell 1989; Kaosa-ard 1999), floral biology and seed biology (Gunaga and Vasudeva 2002, 2003; Nagarajan et al. 1996; Tangmitcharoen and Owens 1997a; Vasudeva et al. 2004). Teak fruit size, weight and viability vary among natural provenances and seed production according to the ecological conditions (Gupta and Pattanath 1975; Indira 2003; Kaosa-ard 1986; Mathew and Vasudeva 2003; Nagarajan et al. 1996; Palupi and Owens 1998; Sivakumar et al. 2002; Tangmitcharoen and Owens 1997b).

In India, teak varies greatly in timber characteristics such as color, grain and texture, but also in physical, chemical, anatomical and mechanical properties (Bhat and Priya 2004; Kaosa-ard 1999; Katwal 2003; Kjaer et al. 1999; Priya and Bhat 1998, 1999; Varghese et al. 2000). Trees from the Western Ghats region with high rainfall are preferred for structural needs like shipbuilding and construction. Teak from Central India, which is known for color (golden yellow, pink colored heartwood), texture, ornamental figuring and decorative grain, is preferred for furniture and cabinet making (Katwal 2003). Desirable as well as undesirable properties in teak heartwood are due to wood extractive content, which is greatly influenced by both genetic and environmental factors (Simatupang 2000).

The neutral genetic diversity of teak from the natural area and introduced populations has been studied with molecular markers. Various methods have been used: isozyme systems (Kertadikara and Prat 1995a, b; Kjaer and Siegismund 1996; Kjaer et al. 1996), Random Amplification of Polymorphic DNA (Gangopadhyay et al. 2003; Katwal 2003; Nicodemus et al. 2003; Parthiban et al. 2003), Amplified Fragment Length Polymorphism (Shrestha et al. 2005) and Sequence Characterized Amplified Regions (Isoda et al. 2000). These markers were used for differentiation and/or identification of clones (Isoda et al. 2000) and to analyze the genetic fidelity of micropropagated clones with respect to subcultural passage (Gangopadhyay et al. 2003). Their principal use was to study the genetic diversity of the natural and exotic populations of teak. Teak shows intraspecific variation both within and between populations and in these molecular studies most of the genetic diversity occurred within populations. The Indian teak provenances were clearly differentiated from the Thailand, Indonesian and African provenances (Kertadikara and Prat 1995b; Nicodemus et al. 2003; Shrestha et al. 2005). Western Ghats and Central Indian regions may be designated as separate breeding zones since these populations are genetically distant and grouped into two distinct clusters (Katwal 2003; Nicodemus et al. 2003). The Berbera population from Orissa near the north-eastern coast of India is an exception, as it seems to have associations with both the Indian and the Thai–Indonesian populations (Shrestha et al. 2005).

None of the population studies with molecular markers have covered the whole natural area of teak. The published results were either obtained from a small number of origins or locations (isozyme, AFLP), or the technique used was considered unreliable (RAPD).

Because of its strength and aesthetic qualities, teak is the tropical hardwood most in demand for the market of furniture, shipbuilding and decorative building components (Pandey and Brown 2000). Consequently, the area of natural teak forests has drastically diminished over the last 50 years and the remaining forests are still under threat from illegal logging and other forms of forest destruction. Due to population pressure and unfavorable biotic factors, teak resources have considerably decreased both in extent as well as in density, quality and quantity over the natural range. To combat loss of biodiversity, programs for evolutionary in situ and ex situ gene resource conservation have been formulated in India, Laos, Myanmar and Thailand (Graudal et al. 1999; Gyi and Tint 1995; Kaosa-ard et al. 1998; Katwal 2003; Rao et al. 1991). In spite of these proposals, human disturbance continues and the impact of these anthropogenic influences on the maintenance of the teak germplasm is unknown (Lowe et al. 2003).

Today, there is an urgent need for teak conservation measures, and this is especially important in the light of likely climatic changes in the years to come. This paper aims to define with neutral molecular markers, and for the first time with SSR markers, the ecological regions of the natural teak area so that decision-makers can take the measures necessary for biodiversity conservation. Fifteen highly polymorphic microsatellite loci were developed from a genomic library enriched for AG/TC repeats (Verhaegen et al. 2005). These loci constitute a powerful tool in investigating the geographical diversity and population dynamics for use in sustainable management of teak forests and for in situ conservation purposes.

Materials and methods

Population sampling of the natural range for molecular analysis

Based on the seed collection and distribution made by Danida Forest Seed Centre in 1971–1973 (Keiding et al. 1986), 166 trees were identified as representative of the main natural occurrences of the species in India, Thailand and Laos (Fig. 1). The aim of the seed collection was to obtain as broad a representation from the whole range of distribution as possible, covering the more typical and distinctly different types of environments. The seeds from the natural area which were produced by open pollination were sowed and raised in a nursery then planted in three comparative provenance trials in Ghana and Côte d'Ivoire. In 2003, leaf samples of the individual trees were collected in the trials from Téné and Séguié (Côte d'Ivoire) and Tain II (Ghana). In order to represent the maximum variability within each provenance, the samples were collected indiscriminately. Trees could be crooked, forked or buttressed as well as skewed or with many protuberant buds. The number of trees studied and the main characteristics of the populations are given in Table 1. Analysis of the first results led us to group the trees and so to establish four different regions including (i) provenances of South India (15; 16; 20; 3016; 3021; 3022), (ii) provenance of North India (3034), (iii) provenances of Thailand together with two natural provenance of South Laos (10; 12; 13; 3038; 3040; 3054; 3061); (iv) provenances of Central Laos (3055; 3056; 3059) which consist of marginal natural teak provenances.

SSR genotyping and polymorphism revelation

DNA extraction, PCR conditions and electrophoresis conditions were described in another paper (Verhaegen et al. 2005). Automated infrared fluorescence DNA sequencing
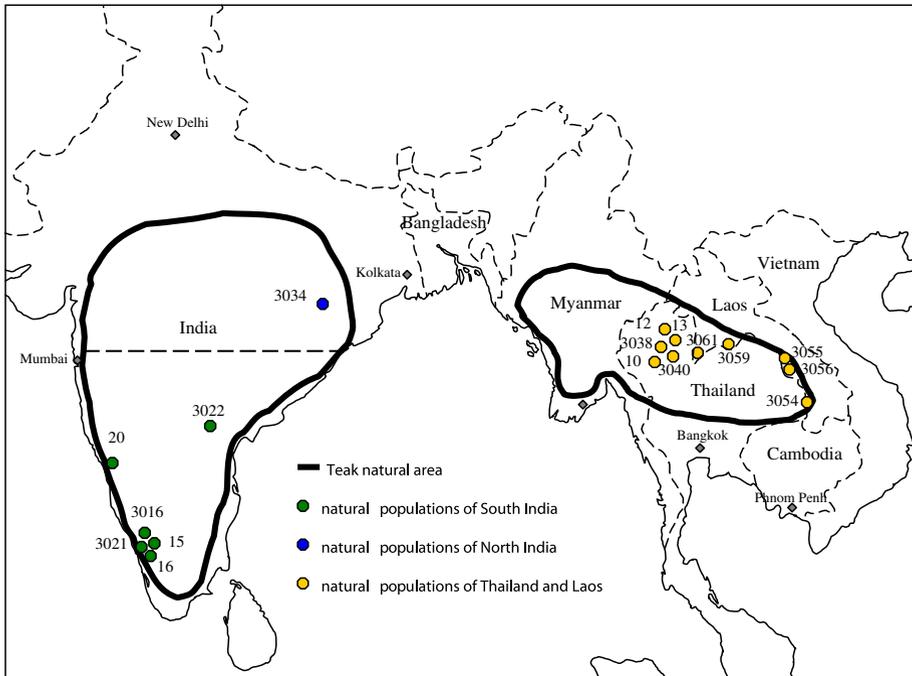
Fig. 1 The natural distribution of *Tectona grandis* L.f. and location of the 17 provenances studied with SSR markers. Six origins in the South of India can be separated from the North Indian origin (dotted line) on the basis of our study and a study carried out with RAPD markers (Nicodemus et al. 2003). The provenances of Thailand and Laos are a part of the East region of the natural area

was used to find the allele variability according to (Steffens et al. 1993). Fifteen microsatellite loci were amplified using PCR in a 15 μl reaction volume containing: 25 ng of genomic DNA in a 0.5X reaction buffer (10 mM Tris–HCl, 50 mM KCl, 2 mM MgCl$_2$), 0.2 mM dNTPs, 0.10 μM of forward primer, 0.06 μM of reverse primer, 0.10 μM of IRdye M13/700 or M13/800 and 0.13 U/μl Taq DNA polymerase (Invitrogen^{TM}). The amplifications were carried out with a thermal-cycler Stratagene® Robocycler gradient 96 under the following conditions: denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 45 s and extension at 72°C for 45 s, and a final extension at 72°C for 5 min. The reverse PCR primers were probed with a 19 base extension at its 5′ tail end with the sequence 5′-CACGACGTTGTAAAACGAC-3′. This sequence is complementary to an IR-labeled universal M13 forward sequencing primer, which is included in the PCR. During PCR, the tailed primer generates a complementary sequence which is subsequently utilized for priming in the amplification reaction thereby generating IR-labeled PCR products. The samples were electrophoresed on an IR DNA analyzer (LI-COR, Inc) which detects primer-labeled extension products at two different wavelengths (IRDye 700 nm and IRDye 800 nm). This allowed loading of a multiplex of four PCR products in one well. The individual trees were genotyped using Saga^{GT} software (LI-COR, Inc).

Table 1 The studied provenances of teak cover a wide area geographically with a range of 1,016 to 2,565 mm of annual rainfall in India, Thailand and Laos

Harvest No	Provenance name	Country	State/Province	Latitude	Longitude	Annual rainfall mm	Climate	Number of studied trees	Trials situation
15	Nellicutha	India	Kerala	11°17′	76°14′	2,565	Very moist teak forest	22	Séguié (Côte d'Ivoire)
16	Nellicutha	India	Kerala	11°17′	76°14′	2,565	Very moist teak forest	18	Séguié (Côte d'Ivoire)
20	Virnoli	India	Karnataka	15°12′	74°37′	2,030	Moist teak forest	7	Séguié (Côte d'Ivoire)
3016	Masale Valley	India	Karnataka	11°55′	76°10′	1,270	Dry teak forest	9	Téné (Côte d'Ivoire)
3021	Nilambur	India	Kerala	11°21′	76°21′	2,565	Very moist teak forest	8	Tain II (Ghana)
3022	Bairluty 1	India	Andhra Pradesh	15°51′	78°45′	1,016	Dry teak forest	7	Tain II (Ghana)
3034	Purunakote	India	Orissa	20°37′	84°49′	1,350	Semi-moist teak forest	10	Téné (Côte d'Ivoire)
10	Mae Huat	Thailand	Lamphun	18°06′	99°	1,200	Dry teak forest	6	Séguié (Côte d'Ivoire)
12	Pong Salee	Thailand	Phayao	19°08′	100°01′	?	?	6	Séguié (Côte d'Ivoire)
13	Huoi Na Soon	Thailand	Phrae	18°07′	100°08′	1,100	Dry teak forest	7	Séguié (Côte d'Ivoire)
3038	Ban Cham Pui	Thailand	Lampang	18°29′	99°49′	1,200	Dry teak forest	16	Téné (Côte d'Ivoire)
3040	Ban Pha Lai	Thailand	Phrae	18°13′	99°59′	1,100	Dry teak forest	11	Téné (Côte d'Ivoire)
3054	Pakse South II	Laos	Pakse	15°07′	105°51′	1,925	Moist teak forest	5	Téné (Côte d'Ivoire)
3061	Pak Lai	Laos	Xaignabouli	18°10′	101°15′	1,200	Dry teak forest	13	Téné (Côte d'Ivoire)
3055	Savannakhet I	Laos	Savannakhet	16°33′	104°45′	1,310	Semi-moist teak forest	7	Tain II (Ghana)
3056	Savannakhet II	Laos	Savannakhet	16°33′	104°45′	1,310	Semi-moist teak forest	9	Tain II (Ghana)
3059	Vientiane Town	Laos	Vientiane	17°56′	102°37′	1,570	Semi-moist teak forest	5	Tain II (Ghana)

Teak grows naturally mainly in mixed deciduous forests with a distinct seasonal climate (wet and dry seasons)

Molecular genetic data analysis

Genetic diversity within populations and regions was estimated by the number of alleles per locus (Ao), the expected unbiased (H_{nb}) and the observed (H_O) heterozygosity (Nei 1978) using Genetix 4.05.2 software (Belkhir et al. 1996–2004). To check if the differences in sample size and the various spatial scales over which individuals were pooled into provenances and regions affected the diversity estimates, the allelic richness (El Mousadik and Petit 1996) was calculated per provenance and region taking into account the dependence on sample size with an adaptation of the rarefaction index (Hurlbert 1971). The calculation was done with Fstat 2.9.3.2 software (Goudet 2001). The principle is to estimate the expected number of alleles in a sub-sample of $2n$ genes, given that $2N$ genes have been sampled ($N > n$), with n defined as the smallest number of individuals typed for a locus sample.

To construct a phylogenetic tree and represent the differentiation between individuals, the simple matching distance $d_{ij} = 1 - \frac{1}{L} \sum_1^L \frac{m_l}{\pi}$ was calculated with d_{ij}: dissimilarity between units i and j; L: number of loci; π: ploidy; m_l: number of matching alleles for locus l. The individual distance tree was constructed using Darwin 5.0.148 software (Perrier and Jacquemoud-Collet 2006), with the neighbor-joining method of Saitou and Nei (1987). The robustness of each node was evaluated by bootstrapping data over loci for 1,000 replications.

Pairwise genetic distances between pairs of provenances were computed with the Cavalli-Sforza chord measure options (Cavalli-Sforza and Edwards 1967) of the Microsatellite Analyzer (Dieringer and Schlötterer 2003). The distance tree was constructed using the neighbor-joining method (Saitou and Nei 1987). The robustness of each node was evaluated by bootstrapping data over loci for 1,000 replications using the Seqboot program of PHYLIP 3.67 (Felsenstein 2005). The consensus tree obtained using PHYLIP 3.67 was displayed with Darwin 5.0.148 software.

With the genotype data, the 166 individuals of teak were subdivided into genetic clusters using a model-based clustering method to infer population structure and assign individuals to populations with the software package Structure (Pritchard et al. 2000). The program can estimate the number of genetically homogeneous populations (K) that do not require prior information of the number of locations and from which location each individual was sampled. At least six runs of clustering were carried out by setting the number of populations (K) from 1 to 8. For each run, burn-in time and replication number were respectively 80,000 and 400,000. Two models for the ancestry of individuals developed in the software were used. Individuals may have mixed ancestry (admixture model) or come purely from one of the K populations (no admixture model). True number of populations (K) is often identified using the maximal value of L(K) returned by the software. However, for the admixture model we observed, once the real K is reached, L(K) at larger Ks plateaus or continues increasing slightly. For this model and in order to detect the uppermost hierarchical level of structure, the statistic ΔK was calculated based on the rate of change in the log probability of data between successive K-values (Evanno et al. 2005).

Differentiation among all provenances and all provenance pairs was tested using probability tests (Fisher exact tests). Wright's F-statistic F_{ST} (Wright 1951) was estimated for all populations and all population pairs by a 'weighted' analysis of variance (Weir and Cockerham 1984) with Genepop 4.0 software (Rousset 2008).

To investigate the hierarchical structure of genetic variation, an analysis of molecular variance (AMOVA) was done using Arlequin ver 3.11 with 1,000 permutations which tests

the genetic structure by partitioning the total variance into covariance components due to intra-individual differences, inter-individual differences and/or inter-population differences. Components of genetic variance were computed at two hierarchical levels: among populations and among regions of the natural teak area, and among clusters found with the model-based clustering method (Pritchard et al. 2000).

Results

Within-population genetic diversity

The fifteen microsatellite loci were polymorphic across all 166 genotypes and the number of alleles per locus range from 3 for 1TG02 to 19 for 1TA06 (Table 2). The number of rare alleles ranged from 0 to 9 for the loci 1TG02 and 4TD12, respectively. Sixty-eight alleles, from a total of 201 alleles, showed frequency under 1% and 83% of these low frequency alleles were in the seven Indian teak provenances (Nellicutha 15, Nellicutha 16, Virnoli, Masale Valley, Nilambur, Bairluty and Purunakote). The four loci 2TC03, 3TD09, 3TE06 and 4TF02 revealed alleles with low frequencies only in the six provenances of South India (Nellicutha (15), Nellicutha (16), Virnoli, Masale Valley, Nilambur and Bairluty). Two loci (1TA06 and 1TB03) have shown alleles with low frequencies in all four regions (South India; North India; North Thailand and Central Laos). Locus 1TG02 showed three alleles in the seven provenances of India, but in the all other provenances this locus was homozygous with a length fragment of 166 base pairs. Considering each locus, the

Table 2 Genetic diversity among 166 trees of *Tectona grandis* as revealed by 15 SSR loci

Locus name	Accession number	No	$N < 1\%$	A max (%)	H_O	H_{nb}	F_{IS}	F_{ST}	R
1TA06	AJ968929	19	6	205 (31%)	0.67	0.83	0.193**	0.188	4.96
1TB03	AJ968930	14	7	252 (34%)	0.64	0.78	0.174**	0.160	4.19
1TF05	AJ968931	15	4	267 (39%)	0.64	0.77	0.161**	0.221	4.23
1TG02	AJ968932	3	0	166 (84%)	0.24	0.28	0.130^{NS}	0.201	1.83
1TH10	AJ968933	17	5	237 (25%)	0.82	0.86	0.050^{NS}	0.081	5.25
2TB07	AJ968934	11	4	131 (35%)	0.63	0.78	0.194**	0.246	4.15
2TC03	AJ968935	15	5	277 (34%)	0.59	0.81	0.273**	0.235	4.60
3TA11	AJ968936	14	4	279 (44%)	0.52	0.75	0.308**	0.324	4.22
3TB02	AJ968937	16	6	232 (42%)	0.62	0.77	0.200**	0.159	4.53
3TD09	AJ968938	7	1	208 (78%)	0.28	0.37	0.244**	0.148	2.35
3TE06	AJ968939	11	2	218 (61%)	0.33	0.61	0.455**	0.387	3.52
3TF01	AJ968940	18	7	216 (41%)	0.75	0.78	0.035^{NS}	0.112	4.56
4TD12	AJ968941	17	9	141 (32%)	0.57	0.77	0.268**	0.214	4.19
4TF02	AJ968942	14	4	227 (55%)	0.54	0.67	0.195**	0.275	3.85
4TH09	AJ968943	10	4	157 (59%)	0.35	0.58	0.403**	0.369	3.01

No: total number of observed alleles; $N < 1\%$: number of alleles with a frequency <1%; Amax: size (base pair) of the most frequent allele and (%) frequency in the sample; H_O: the observed heterozygosity; H_{nb}: the expected unbiased heterozygosity corrected for small sample size (Nei 1978); F_{IS}: the inbreeding coefficient (fixation index, Fisher) with NS: P value adjusted using sequential Bonferroni (Rice 1989) procedure not significant; **P value significant <0.01; F_{ST} represents the differentiation among the 17 populations within the total population; R is the corrected allelic richness

distribution of allele frequencies was not unbalanced for 10 loci, and one allele presented a frequency higher than 0.5% for loci 4TF02, 4TH09, 3TE06, 3TD09 and 1TG02.

The observed heterozygosity (H_O) and the expected unbiased heterozygosity (H_{nb}) values ranged from 0.24 to 0.82 and from 0.28 to 0.86 for 1TG02 and 1TH10 loci, respectively. All the loci showed a heterozygote deficit. Except for the three loci 1TG02, 1TH10 and 3TF01, the F_{IS} values were highly significant (Table 2). In the studied sample, we found more than 10 alleles per locus, with an exception for the 3TD09 (7) and 1TG02 (3) loci. The allelic richness varied between 1.83 and 5.25 for all the loci.

The mean numbers of alleles per locus per population (A) ranged from 2.07 in Pakse South II to 7.67 in Nellicutha 16, while the allelic richness (R) ranged between 1.94 and 4.46 for Savannakhet I and Masale Valley, respectively (Table 3). The Pearson correlation coefficient between the number of alleles per locus and the allelic richness corrected with a rarefaction index was 0.94 and was highly significant, which demonstrated a strong relationship between these two parameters. The allelic richness of South-east Asian teak provenances (R mean 2.26) was approximately half of that of India (R mean 4.17). These results were confirmed with the regional analysis which showed allelic richness of 6.63 for the South India origins and 3.24 for the North Thailand origins with 71 and 64 individuals, respectively.

Table 3 Summary of intrapopulation genetic diversity at 15 microsatellite loci for 17 natural populations of *Tectona grandis*

Population or cluster	Sample size	A	R	H_{nb}	H_O
Nellicutha (15)	22	6.87	3.93	0.72	0.72
Nellicutha (16)	18	7.67	4.18	0.75	0.76
Virnoli	7	5.20	4.10	0.74	0.73
Masale Valley	9	6.53	4.46	0.78	0.79
Nilambur	8	5.87	4.32	0.75	0.72
Bairluty 1	7	5.67	4.36	0.75	0.74
Purunakote	10	6.20	3.85	0.64	0.63
Mae Huat	6	2.60	2.31	0.38	0.40
Pong Salee	6	2.60	2.32	0.38	0.37
Huoi Na Soon	7	2.47	2.20	0.37	0.32
Ban Cham Pui	16	3.20	2.25	0.37	0.39
Ban Pha Lai	11	3.80	2.78	0.49	0.52
Pakse South II	5	2.07	1.95	0.28	0.32
Pak Lai	13	2.73	2.09	0.32	0.31
Savannakhet I	7	2.13	1.94	0.34	0.35
Savannakhet II	9	2.93	2.24	0.37	0.33
Vientiane town	5	2.60	2.49	0.47	0.35
South India	71	11.47	6.63	0.76	0.74
North India	10	6.20	3.85	0.64	0.63
North Thailand	64	5.07	3.24	0.41	0.38
Central Laos	21	3.80	3.06	0.22	0.26

Results presented for each population and each region of the natural area. A: mean number of alleles per population or cluster; R: corrected allelic richness; H_{nb}: the expected unbiased heterozygosity i.e. expected heterozygosity corrected for the small sample size (Nei 1978); H_O: the observed heterozygosity

Observed heterozygosity (H_O) values ranged from 0.31 in Pak Lai population to 0.79 in Masale Valley population and the expected unbiased heterozygosity (H_{nb}) values ranged from 0.28 to 0.78 in Pakse South II and Masale Valley provenances, respectively. At the regional level, teak heterozygosity was clearly higher in India than in Thailand or Laos.

Analysis of population differentiation

All F_{ST} values were significant, except in the Central Laos populations (Table 4). F_{ST} was 0.22 among regions and among populations. The F_{ST} values were 0.03, 0.04 and 0.12, respectively, within South India, Central Laos and Thailand. All pairwise F_{ST} values were significant at the level $\alpha = 0.05$ except for the Central Laos populations.

With the individual genetic distances, the variation within the populations is so high that it was not possible to separate the 17 populations on the dendrogram (Fig. 2). However, distances among individual trees provided evidence of four clusters:

i)   the provenances of the South of India: Nellicutha 15, Nellicutha 16, Masale Valley, Nilambur, Virnoli and Bairluty, the diversity of the individuals studied by this group being very strong,

ii)   the Thailand provenances: Pong Salee, Mae Huat, Ban Cham Pui, Ban Pha Lai, Huoi Na Soon, Pak Lai, and Pakse The individuals of these populations were compared with the individuals of the South of India,

iii)   the populations of the Center of Laos: Vientiane, Savannakhet I, and Savannakhet II. This group showed a poor individual diversity, individual distances being closer.

iv)   the provenance of the North of India: Purunakote. The few studied individuals presented a very high genetic diversity. This last population seems very different from the other populations.

With this dendrogram of the individual genetic distances it was necessary to notice that a sample (i220) of the provenance Ban Pha Lai (Thailand) grouped with the origins of the Central Laos. The samples i107 and i218 as well as i102 seemed to lie outside of their geographical groups, Thailand and North India, respectively.

With the genetic distances of Cavalli-Sforza and Edwards, the 17 provenances separated sharply and the robustness of nodes varied from 575/1000 in 998/1000 (Fig. 3). Only the separations between the populations of Pak Lai and Huoi Na Soon, and the populations of Savannakhet I and Savannakhet II, seemed weaker with, respectively, 431/1000 and 434/1000 bootstrapping values. The phylogram allowed separation of four groups including (i) The population of the North of India (Purunakote) which parted very sharply from all other origins. (ii) The populations of the South of India (Nellicutha 15 and 16, Virnoli, Masale Valley, Nilambur and Bairluty) which formed a single group. The Thailand and Laos provenances formed two others groups: (iii) Central populations of Laos (Vientiane, Savannakhet I and Savannakhet II) which clearly separated from (iv) The group from Thailand provenances and two Laos populations (Ban Cham Pui, Mae Huat, Pong Salee, Pak Lai, Houi Na Soon, Ban Pha Lai and Pakse).

Teak population genetic structure

With the Structure software the no admixture and admixture models were used assuming that the allele frequencies in each population are independent (Pritchard et al. 2000). For the no admixture model, the software defined four genetic clusters in the teak natural area with a probability of 1.00. With the admixture model, the software gave similar estimations

Table 4 Hierarchical analysis of molecular variance, based on 15 SSR markers analyzed on 17 populations of *Tectona grandis* and on regrouped populations defined using a model-based approach and pairwise genetic distances

Source of variation	d.f.	SS	Variance components	%	F_{st}	F_{is}
Among populations	16	416.68	1.1392***	21.8	0.22***	0.005[NS]
Among individuals within populations	149	613.57	0.0213[NS]	0.4		
Within individuals	166	676.50	4.0753***	77.8		
Among India and Thailand-Laos	1	214.48	1.1955***	20.7	0.22***	0.005[NS]
Among populations within groups	15	202.20	0.4951***	8.5		
Among individuals within populations	149	613.57	0.0213[NS]	0.4		
Within individuals	166	676.50	4.0753***	70.4		
Among four clusters	3	305.03	1.2909***	22.9	0.22***	0.005[NS]
Among populations within groups	13	111.65	0.2362***	4.2		
Among individuals within populations	149	613.57	0.0213[NS]	0.4		
Within individuals	166	676.50	4.0753***	72.5		
Total	331	1706.75				
Among South India populations	5	46.88	0.1706*	3.0	0.03*	−0.00005[NS]
Among individuals within populations	65	360.21	0.0000[NS]	0.0		
Within individuals	71	393.50	5.4225[NS]	97.0		
Total	141	800.59				
Among Thailand populations	6	55.02	0.3638***	11.5	0.12***	−0.028[NS]
Among individuals within populations	57	155.0	−0.0778[NS]	−2.5		
Within individuals	64	184.0	2.8750**	91.0		
Total	127	394.02				
Among Central Laos populations	2	9.18	0.1243[NS]	4.7	0.04[NS]	0.155**
Among individuals within populations	18	52.11	0.3880**	14.8		
Within individuals	21	44.5	2.1191***	80.5		
Total	41	105.79				
Among individuals of North India	9	44.4	0.1167[NS]	2.4	–	0.024[NS]
Within individuals	10	47	4.7000	97.6		
Total	19	91.4	4.8167			

Degrees of freedom (df.), Sum of Squares (SS), percentages of variance (%) and estimates of genetic differentiation: among regions, among populations within regions, among individuals within populations and within individuals. For each analysis, we calculated the F_{ST} and the F_{IS} values
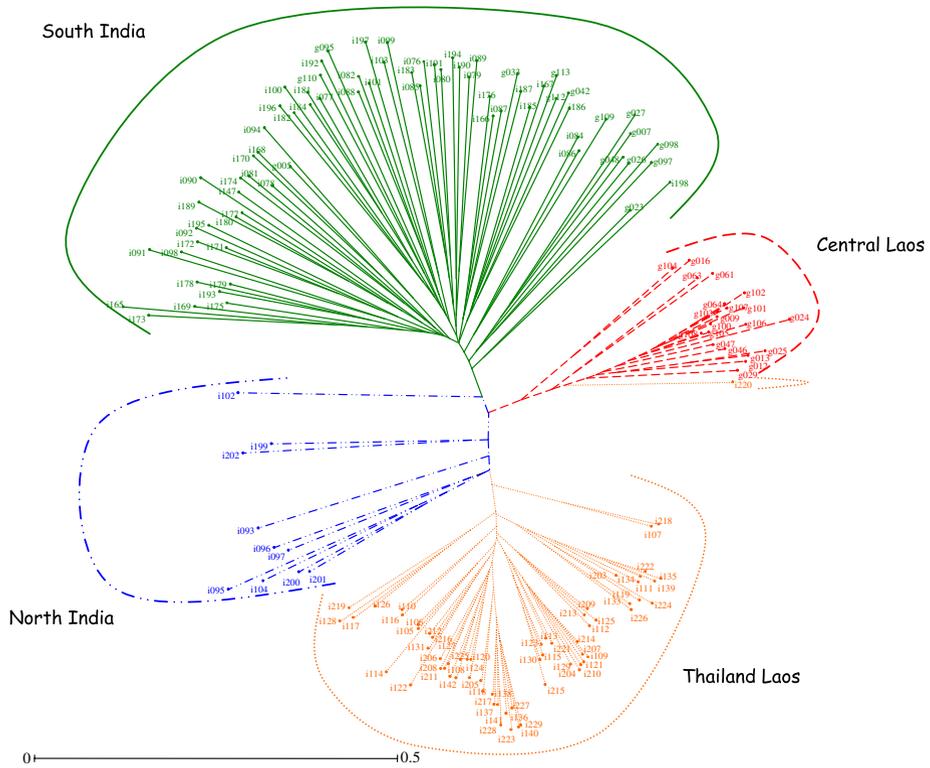
Fig. 2 Neighbor-joining dendrogram based on simple matching method for *Tectona grandis* for 166 individual trees

for K after $K = 4$, the methodology of Evanno et al. (2005) strongly supported $K = 4$ as the correct number of clusters. With the 166 teak samples originating from 17 teak provenances, the Structure software defined four clusters with the two models tested (Fig. 4).

Provenances of the teak were grouped in four clusters which corresponded to (i) South of India with the provenances 15; 16; 3016; 3021; 20 and 3022, (ii) North of Thailand and Laos with the provenances 12; 10; 3038; 3040; 13; 3061 and 3054, (iii) Central Laos with the provenances 3059; 3055 and 3056, (iv) North of India with the provenances 3034.

Only nine individuals of the sample presented a genome composed of two different origins. These were:

– Four trees (g016; g029; i119 and i199) of four different provenances (3059; 3056; 13; 3034) have a small fraction of their genome belonging to another cluster, but their classification corresponds well to the cluster of their provenance origin.

– Four trees (i219; i110; i107; i218) native of the Ban Pha Lai provenance (3040) of Thailand did not fit the cluster 3 and were mainly allocated to the cluster 4 of North India.

– One tree (i102) of Purunakote provenance was mainly allocated to the cluster 1 but a significant part of its genome (34%) corresponded well to cluster 4 of the provenance origin.
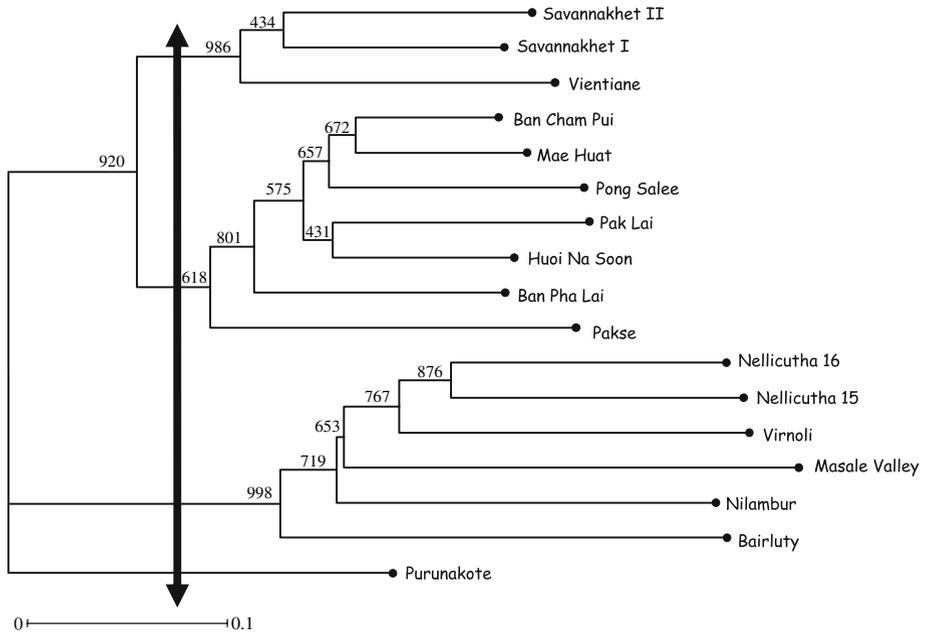
Fig. 3 Neighbor-joining phylogram based on the Cavalli-Sforza and Edwards chord method for 17 natural provenances of *Tectona grandis*. The robustness of each node was evaluated by bootstrapping data over loci for 1,000 replications

For the majority, the level of confidence for a particular sample belonging to a particular origin varied from 56% to 98%.

Discussion

Teak genetic diversity

Different parameters were used to assess the genetic teak diversity. The parameters using the number of alleles (A and R) are complementary to those using allelic frequencies (H), especially for analysis raising conservation issues (El Mousadik and Petit 1996). The differences in sample sizes of the populations (from 5 to 22) led us to use the allelic richness corrected by the rarefaction index (R). We found a very high correlation between A and R ($r = 0.94$) demonstrating that the correction with the rarefaction index has no effect on diversity assessment. This also suggests that rare alleles (which strongly influence measures of allelic richness) are not more scattered in distribution than the other alleles. Individual genetic distances and AMOVA showed a very strong variability (78%) among individual teak samples when all 17 provenances were analyzed. On the other hand the variation among individuals in Indian populations was 97%. These percentages decreased when the populations were grouped into four clusters.

The Indian provenances possessed approximately twice the number of alleles possessed by the Thai provenances and approximately four times more than the Central Laos provenances. This relationship has also been observed on teak with allozyme markers, but
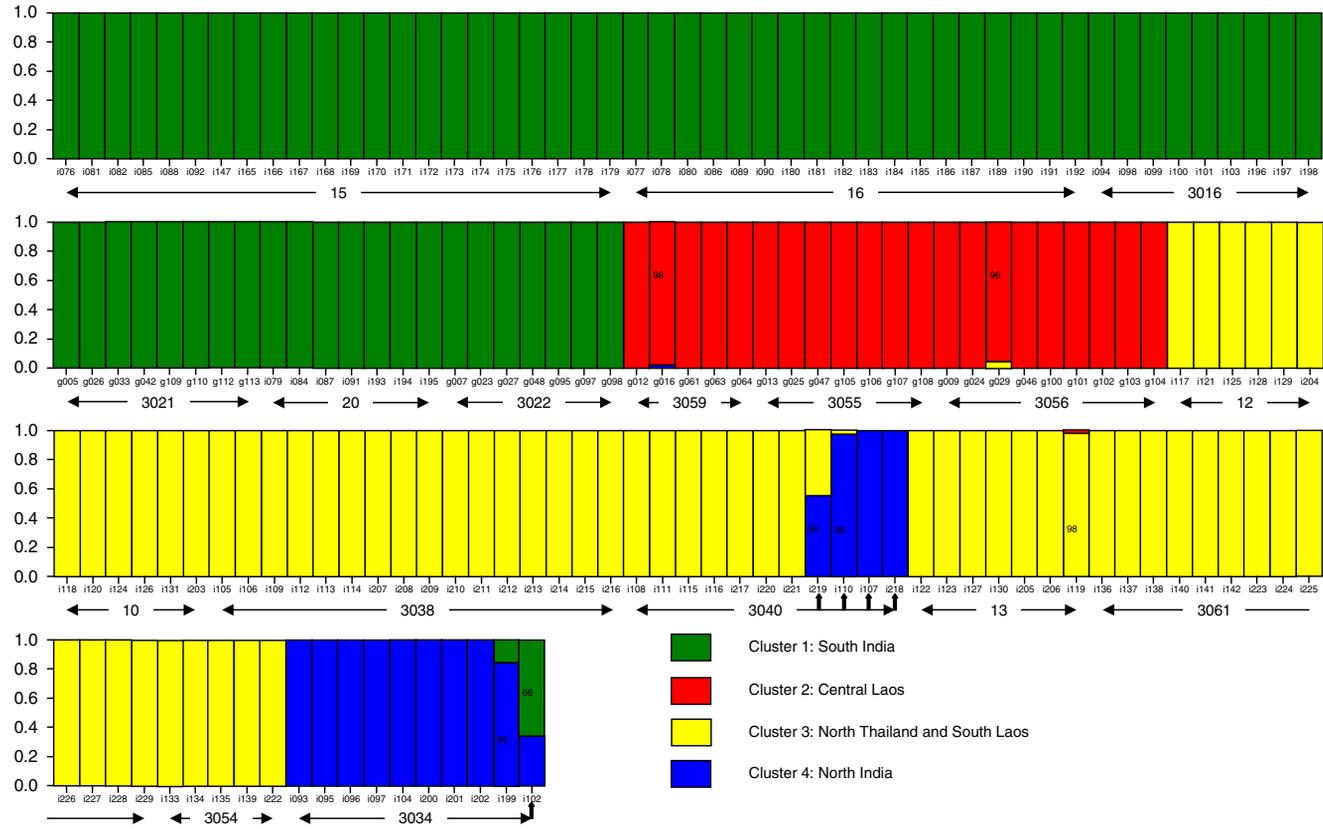
Fig. 4 Estimated population structure of 166 individuals from 17 natural teak populations. Each individual is represented by a vertical bar, which is assigned to four shading patterns that represent the individual estimated affiliation to the four clusters: South India, Central Laos, North Thailand-Laos and North India

the difference was less clear. On average the number of alleles decreased between Indian and Thai provenances from 3.1 to 2.2 (Kertadikara and Prat 1995a) and from 1.7 to 1.5 (Kjaer and Siegismund 1996) or remained stable 2.1 (Kjaer et al. 1996). The Thai and Laos population had the lowest degree of genetic diversity and the Indian the highest.

Our diversity parameters ($A = 2.1–7.7$; He $= 0.32–0.78$) were comparable to those of other tree species analyzed with SSR markers. They were higher than those of *Vitellaria paradoxa* ($A = 3.4–4.2$, He $= 0.38–0.44$) (Kelly et al. 2004), *Prunus avium* ($A = 2–7$, He $= 0.47$) (Wünsch and Hormazaa 2002), *Vouacapoua americana* ($A = 3.2–5.1$, He $= 0.34–0.52$) (Dutech et al. 2004), and *Grevillea macleayana* ($A = 3.2–4.2$, He $= 0.42–0.53$) (England et al. 2002), and lower than those of *Symphonia globulifera* ($A = 3.7–16$, He $= 0.67–0.85$) (Aldrich et al. 1998) and *Melaleuca alternifolia* ($A = 20–27$, He $= 0.13–0.92$) (Rossetto et al. 1999).

Differentiation between populations

With individual distances and the clustering method of the genotypic data, all the genotypes represented correspond well to Figs. 2 and 4, except six trees that were found to be ambiguous. Tree i220 was badly represented with the individual genetic distances (Fig. 2), but was well assigned to the Thailand cluster with the Structure software. The genotyping data of this individual presented four homozygous loci common to individual g029 of Laos. This community of fragments involved a weak genetic distance between these two individuals. Tree i102 seemed isolated between the group of South India and that of North India by individual distances, but with the Structure software gave probability values suggesting membership of the South India group and of the North India group of 0.66 and 0.44, respectively. The same observation could be made for trees i218 and i107 which were between the cluster of North Thailand and North India by individual distances, but which were assigned to the group of North India with Structure software. On the other hand, the change of cluster for trees i219 and i110 was problematic. These trees were assigned to the clusters of North Thailand and North India, respectively, on the individual distances dendrogram and by the Structure software with probabilities of 0.56 and 0.98.

It is worth noting that 4 (i107, i110, i218, i219) of the five intermediate trees were collected in the Ban Pha Lai provenance (Thailand) and can be thus considered as migrants from the North India cluster. As *Tectona grandis* is a widely distributed species, the divergence found among some populations may be the outcome of isolation by distance process or of a high mutation rate in the microsatellite loci studied. On the one hand, in a canonical analysis of growth traits, Kjaer et al. (1996) observed that the Thai provenances seem to cluster in two groups, some clustering with Laos provenances and others with Indian provenances. On the other hand, homoplasy of SSR alleles and the analysis of these data may fail to contribute to an informative phylogeny because of the high mutation rates, irregularities and asymmetries in mutations, and degradation of microsatellites over time with the substitution and insertion of other nucleotides (Goldstein and Pollock 1997). Our teak results showed a high differentiation between the Indian and Thai provenances, which suggests genetic isolation for a substantial time scale (also supported by the high F_{ST} value $= 0.22$).

The majority of teak SSR loci presented a heterozygote deficit. This result was expected because F_{IS} were calculated for all the samples and included the Walhund effect. At the regional level, our study revealed a heterozygote deficit only in the Laos populations ($F_{is} = 0.155$). Three hypotheses may account for this deficit. The first is the occurrence of null alleles (alleles that are never amplified because of mutations in the flanking primer

sequence (Callen et al. 1993)). A second explanation is the Walhund effect, which occurs when a subdivided population contains fewer heterozygotes than predicted despite the fact that all subdivisions are in Hardy-Weinberg equilibrium. The last explanation is selfing, which seems to be the most logical explanation for heterozygote deficiency in situations such as the Laos populations (also supported by the lowest mean number of alleles and expected heterozygosity).

Both F_{ST} values and percentage of variance obtained by AMOVA indicated strong differentiation between the four clusters ($F_{ST} = 0.22$***) and less differentiation between populations within clusters ($F_{ST} = 0.03$, 0.12 and 0.04 among South India, Thailand and Central Laos populations, respectively). This result was expected and confirms that gene flow is very limited between populations of different clusters and is greater between populations of the same cluster.

By using various methods of analysis, we showed that in the natural teak area there were four clusters that agreed with three main centers of genetic variability. The first two clusters identified were found in India (North India and South India). With RAPD markers, the UPGMA dendrogram had revealed two major clusters each for the Western Ghats (Kalakkad, Nilambur, Mudumalai, Dandeli, Walayar, Tholpetti, Topslip) and Central Indian (Bardipada, Alappalli, Seoni) regions (Nicodemus et al. 2003). By comparing published information with our results, we concluded that in India the separation between two clusters of genetic teak variability was situated approximately at latitude 19°25′ north. Studies of more populations in this vicinity using molecular markers will be needed to place the boundary between the Indian clusters more precisely. With regards to the number of alleles, the observed heterozygosity and the inbreeding coefficient, both Indian clusters could be considered as two main centers of genetic diversity of teak. The third cluster was formed with the populations of Thailand and Laos and the fourth cluster was composed only of three provenances from Central Laos (Vientiane and Savannakhet I and II). Even if the third cluster in Thailand can be considered as a third center of genetic diversity, it is important to note that this group was genetically very sharply separated from the Indian and presented only half the allele variability of the Indian clusters. The cluster of Central Laos is a small group with reduced genetic variability that could be attached to the third cluster. This cluster could be attributed to natural populations having undergone a genetic bottleneck or to human intervention, which would have established these populations by plantation.

With the Structure software, the admixture and the no admixture models were used, because the correlated allele frequency model could predispose to overestimation of K, and it was decided to run the software with the independent frequencies model (Pritchard et al. 2000).

The number of homogeneous genetic groups remained the same with the various models used. The no admixture model clearly indicated $K = 4$ with a P value of 1, but the admixture model required the use of Δk to finally find the same number of clusters. These results showed that in the teak natural area, the genetic structure found with the microsatellite markers was very strong. This structure has never been highlighted by other studies. Kjaer et al. (1996) found no clear separation between three provenances: from southern India, central India and Thailand. Other literature showed incomplete groupings of provenances without extension to the whole teak natural area. Isozyme markers have distinguished three southern Indian provenances (Sakrebail, Virnoli, Thithimathy) and two Thai provenances (Tam Bah Thai, Mae Huat) (Kertadikara and Prat 1995b), or one southern Indian provenance (Sadiuaval) and two Thai provenances (Ban Cham Pui, Mae Huat) (Kjaer and Siegismund 1996). AFLP markers do not distinguish the Thai and Indian

populations, but comparison between Indian populations suggests that the northwestern Allapally plain population is distinct from the two southern Indian populations (Shrestha et al. 2005).

Conservation implications

In India the demand for teak has increased several fold during the last five decades, resulting in extraction of trees from old plantations and from natural forest. Extraction of best teak from forest has resulted in the loss of good genotypes (Katwal 2003). Genetic teak conservation in India is urgent because most of the natural teak forests have been gradually converted into teak monoculture. The gene diversity has been reduced with each round of teak plantations as seeds are collected from selected trees of the existing plots. Transformation of the natural forests into plantation caused numerous problems including site deterioration due to repeated fires, heavy grazing and water erosion, poor quality of planting stock raised from genetically inferior seeds, and attack by the teak defoliator *Hyblaea puera* (Prabhu 2003). In the absence of preservation plots and protected forests, the choice is limited to identifying appropriate plantations for long-term conservation (Katwal 2003). In the state of Andhra Pradesh, due to population pressure and unfavorable biotic factors, the teak resources of the state have considerably decreased in extent as well as in density, quality and quantity. Constant fires in the summer season and overgrazing have damaged the existing stock and prevented natural regeneration (Rao 2003). In the state of Karnataka, the demand for timber being quite high due to the continued growth of urban centers, the natural forests were progressively transformed into teak plantations (Kumar 2003). From the eighteenth to nineteenth century, teak exploitation damaged and depleted the natural teak forests of Kerala (Prabhu 2003).

In India the biodiversity of teak conservation stands may be estimated within each genecological zone taking into consideration the extent of population differentiation within each zone (Katwal 2003).

The SSR molecular markers showed that the differentiation between the various teak populations of the South of India remained slight ($F_{ST} = 0.03$) and that there was no heterozygote deficit. This result was important for the definition of genetic units of conservation in this part of the natural area.

Natural teak forests in Thailand decreased from 2,234,300 ha in 1954 to about 150,000 ha in 2000, mostly due to the demand for agricultural land and constructional wood by the increasing human population (Kijkar 2003). Deforestation during the 1970s and 1980s was extremely high and caused disappearance of natural teak forests. Teak may also be found in other national parks and/or wildlife sanctuaries, as well as in the national forest reserves. These areas may be partly illegally encroached and the real natural teak forests should not exceed 150,000 ha. Since January 1989, after the complete ban on forest concessions in Thailand, and given the current conservation attitudes of the public in Thailand since 1990, forest encroachment has gradually decreased to a satisfactory level, and the target of 150,000 ha of teak forests is expected to be achieved. Nevertheless, it is anticipated that density of teak trees within these areas may be reduced due to illegal felling as the price of teakwood is still increasing and the government cannot supervise all areas thoroughly with limited resources (Kijkar 2003). The Thai teak forests are under pressure and have suffered from overexploitation and conversion to agricultural land. Only fragments of the original teak forests now remain, mainly in a few protected National Parks (Suangtho et al. 1999). Large areas of teak forest do still exist in Thailand, but outside

protected areas logging takes place to such a degree that in a few years almost no straight trees will remain. Thus the conservation status of the species is gradually deteriorating.

A conservation plan for teak in Thailand has been developed with the aim of protecting this precious genetic resource for future use. The conservation plan is based on so-called genecological zonation where variation in ecological conditions within the distribution area is investigated and uniform zones are established based on available data. A network of conservation stands based on this zonation is recommended rather than a few populations (Graudal et al. 1999).

In our study the F_{st} estimated with the molecular markers of the Thai populations was raised enough for teak ($F_{st} = 0.12$). This would indicate that the microsatellite markers could help in the delimitation of the genetic units for the conservation of the teak gene pool in Thailand, and particularly that a multiple population approach would be advisable, notably because of the apparently limited gene flow.

In Myanmar the state economy, together with the social system, employment and economy of the rural communities, depends largely upon the natural teak-bearing forests (Dah 2004). Due to the increase in population and demand on forest products and land for agriculture, unauthorized human interventions including shifting cultivation, agricultural expansion and illicit logging have resulted in forest depletion and degradation with declining production, especially of teak (Dah 2004).

Effective protection and cultural treatments together with compensatory and enrichment plantings are in operation within the natural forests of Myanmar to restore and enhance the natural stock of teak while extensive plantations are being established in depleted natural teak habitats to replenish the natural forests and enlarge the wood capital (Dah 2003).

Molecular genetic studies, carried out on many forest tree species around the world, are contributing to a better understanding of patterns of variation and supporting the development of improved management practices, and monitoring species turnover in time and in space. Studies of intraspecific variation can contribute to the development of conservation strategies, by identifying appropriate units for conservation (Newton et al. 1999). Integrating new tools, such as modeling simulations or GIS, with molecular research will improve our knowledge of landscape patterns of genetic diversity within species distribution, and help develop resource management plans (Kjaer et al. 2004).

The genetic variability of teak in its natural area remains at an acceptable level, but the risks incurred by the species are major due to overexploitation, anthropological pressure, fire, loss of the most valuable trees through international and national demand, and the conversion of natural populations.

The molecular data, in particular the microsatellite markers, can be of great use in defining the best methods of genetic conservation and insuring tracking of future evolution of variability. They can also be very effective to combat illegal logging or to certify wood provenance. On the other hand, it is necessary to note that the molecular data should be completed by phenotypic and ecological data. Various sources of information will be necessary to protect and manage the genetic variability of teak in its natural area.

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