

The potential for using molecular markers to facilitate gene management and the *in situ* and *ex situ* conservation of tropical forest trees

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Introduction

As a consequence of depletion of forests due to deforestation and over exploitation, many forest trees and other plants in the tropics have become seriously threatened and are in the focus of conservation concerns. Due to the destruction and fragmentation of their habitats, populations of many species have been reduced and become isolated. These populations generally face considerable risk from the effects of environmental variation, demographic stochasticity and reduced genetic diversity (Meffe and Carroll 1997). The status of genetic resources of forest tree species can be explored by investigating the extent of genetic variation and mating system of each species. The extent and distribution of genetic variation within tree species are of fundamental importance to their evolutionary potential and chances of survival. Therefore, assessments of genetic variation are of key importance for developing effective conservation strategies (Holsinger and Gottlieb 1991; Newton *et al.* 1999). This serves the concept of the World Conservation Union (IUCN) for recognizing genetic diversity as one of the three levels of diversity requiring conservation (McNeely *et al.* 1990).

Concept of conservation

Sound strategy for the conservation of genetic resources of a species starts with the identification of clearly defined conservation objectives. Secondly, genetic resources must be selected mainly based on the available knowledge of spatial patterns of genetic variation. The choice of a conservation method refers to the physical preservation of genetic information, usually by preserving the selected organisms. The final step of a conservation programme is the regeneration of the resource (Hattemer 1995; Finkeldey and Hattemer 1993).

Finkeldey (1998) pointed out that inventories using gene markers are the most important experiments for the selection of genetic resources since centres of genetically differentiated populations can be identified by such inventories. However, it is also possible to identify populations containing otherwise rare or even unique alleles in high frequency. The occurrence of localized common alleles also points towards valuable genetic resources (Brown 1978). Therefore, the combination of different methods is recommended for the identification of forest genetic resources that need to be conserved.

After the genetic diversity within and among populations of any species has been investigated, genetically most diverse populations with relatively high outcrossing rates (for outcrossing species) should be chosen as the sources for gene conservation. Since forest trees live longer than annual or crop plants, high genetic diversity and outcrossing rate would guarantee higher possibility of their survival, viability, longevity, disease and insect resistance for the present and forthcoming generations under the unpredictable and changing environment (Changtragoon and Szmidt 1997).

The combination of marker-aided population genetic analysis and information about adaptive and quantitative traits as well as forest ecosystems would allow comprehensive conservation programmes for individual species in each forest type (Changtragoon 2001).

Type of molecular markers for the detection of genetic variation

Szmidt (1995) pointed out that the biological function of a plant relies on an intimate interplay between three distinct genomes: nuclear, chloroplast and mitochondrial. All these genomes harbour genes, which are vital to growth, photosynthesis, respiration and other biological processes. Therefore, studies of genetic variation should consider all these three components of plant genetic system. Furthermore, each of these components harbours different structure, RNA, regulatory genes and non-coding sequences and has a different mode of inheritance, which may affect the extent of population differentiation among biparental nuclear and uniparental (chloroplast and mitochondrial) inherited genes for the same set of populations. Therefore, the location of molecular markers and their distribution in the genomes as well as their potential adaptive significance (strongly adaptive or near neutral) should be taken into consideration (Karp *et al.* 1996; Szmidt 1995). Therefore, use of molecular markers that can detect the potentially adaptive genetic diversity are recommended (Krutovskii and Neale 2001; Szmidt 1995; Szmidt and Wang 2000). Karp *et al.* (1996) also suggested that in any assessment using molecular markers, attention should first be focused on the specific questions being addressed and on whether or not the choice of markers, sampling strategy and data analysis adequately address it.

Different molecular markers have been developed to investigate the genetic variation of plants and forest trees in the past few decades. Isoenzymes are codominant markers that have been used in forest genetics since the 1960s. They are still used in some laboratories due to their low cost and have useful applications, such as estimation of mating system and genetic diversity. Later on, DNA markers have been developed. Since the 1980s, RFLPs (restriction fragment length polymorphisms) have been applied in forest genetics and breeding. In the early 1990s many new DNA techniques and markers were developed such as PCR (polymerase chain reaction) based markers, namely RAPD (randomly amplified polymorphic DNA) and AFLPs (amplified fragment length polymorphisms). Nowadays DNA markers such as PCR-RFLPs, microsatellites (SSRs), AFLPs and RAPD markers are widely used to determine genetic diversity of forest populations because they can detect more polymorphic loci than isoenzymes (Szmidt 1995; Changtragoon 1998). Using microsatellite (SSRs) markers is also an alternative method for estimating mating system and gene flow in forest trees since they are codominant and can detect higher variation than isoenzyme markers. However, the disadvantages of this marker include its high cost and the long time needed to develop one marker. Moreover, microsatellite markers cannot be used across species.

Recently, the innovation of automated DNA sequencing and PCR techniques combined with the worldwide availability of plant and forest tree genes and DNA sequence-databases through internet facilitates the development of new molecular markers for determining genetic variation of forest trees at specific functional and regulatory genes, regions of DNA and (nuclear, chloroplast and mitochondrial) genomes, namely SAPs (specific amplicon polymorphisms), ESTPs (expressed sequence tag polymorphisms) and SNPs (single nucleotide polymorphism). The details of the mentioned molecular markers and their application can be found from Amaral (2001), Harry *et al.* (1998), Karp *et al.* (1997), Kristensen *et al.* (2001), Krutovskii and Neale (2001), Ratnam (2001), Wang and Szmidt (2001), Szmidt (1995) and Szmidt and Wang (2000). FAO (2001) stated that ESTPs are the most informative markers in terms of gene function among the most recently developed ones and are the first genetic markers that offer real potential for detecting adaptive genetic diversity broadly. Many EST sequences are available for several forest tree species, such as *Pinus radiata*, *P. taeda*, *Picea abies*, *Eucalyptus* spp. and *Populus* spp. The example of the application of some molecular markers on investigation of genetic variation in some forest trees is presented in Table 1.

Table 1. Examples of the application of molecular markers in investigation of genetic diversity and variation in some forest trees

Application	Species markers	Molecular	References
1. Genetic diversity and variation			
	<i>Acacia mangium</i>	RFLPs	Butcher <i>et al.</i> 2000
	<i>Acacia mangium</i> & <i>A. melanoxylon</i>	Isoenzyme	Moran 1992
	<i>Azadirachta</i> spp.	Isoenzyme	Changtragoon <i>et al.</i> 1996
	<i>Melaleuca cajuputi</i>	Isoenzyme	Changtragoon and Szmids 1997
	<i>Pinus merkusii</i>	Isoenzyme	Changtragoon & Finkeldey 1995
	<i>Pinus merkusii</i> & <i>P. kesiya</i>	Isoenzyme	Szmids <i>et al.</i> 1996
	<i>Populus balsamea</i>	Isoenzyme	Hamrick <i>et al.</i> 1992
	<i>Pterocarpus macrocarpus</i>	Isoenzyme	Liengsiri <i>et al.</i> 1995
	<i>Quercus robur</i>	Isoenzyme	Muller-Starck <i>et al.</i> 1992
	<i>Abies alba</i>	Cp (RFLPs)	Ziegenhagen <i>et al.</i> 1995
	<i>Pinus attenuata</i>	RFLPs	Strauss <i>et al.</i> 1992
	<i>P. radiata</i>	SSRs	Cato and Richardson 1996
	<i>Pinus merkusii</i> & <i>P. kesiya</i>	Cp (RFLPs)	Szmids <i>et al.</i> 1996
	<i>Pinus</i> spp.	Cp (RFLPs)	Wang & Szmids 1994
	<i>Quercus robur</i> & <i>Q. petraea</i>	RFLPs	Kremer <i>et al.</i> 1991
	<i>Calamus palustris</i>	RAPDs	Changtragoon <i>et al.</i> 1997
	<i>Eucalyptus</i>	RAPDs	Rossetto <i>et al.</i> 1999a
	<i>Melaleuca alternifolia</i>	SSRs	Rossetto <i>et al.</i> 1999b
	<i>Avicennia marina</i>	AFLP & SSRs	Maguire <i>et al.</i> 2002
2. Mating system			
2.1 Selfing rate			
- 50 %	<i>Pinus merkusii</i>	Isoenzyme	Changtragoon & Finkeldey 1995
- 16 %	<i>Pinus sylvestris</i>	Isoenzyme	Yeh 1989
- 10.8%	<i>Pinus sylvestris</i>	Isoenzyme	Szmids 1984
2.2 Pollen contamination in seed orchard			
- 21-89%	<i>Pseudotsuga menziesii</i>	Isoenzyme	Wheeler & Jech 1992
- >50%	<i>Pinus sylvestris</i>	Isoenzyme	Wang <i>et al.</i> 1991
3. Species and clone identification			
3.1 Species			
	<i>Azadirachta</i> spp.	Isoenzyme	Changtragoon <i>et al.</i> 1996
	<i>Populus</i> spp.	Nu (RFLPs)	Wagner 1992
	<i>Pinus densiflora</i> & <i>P. sylvestris</i>	Cp (RFLPs)	Szmids and Wang 1993
	<i>Pinus merkusii</i> & <i>P. kesiya</i>	Cp (RFLPs)	Szmids <i>et al.</i> 1996
	<i>Pinus sylvestris</i> & <i>P. mugo</i>	Cp (RFLPs)	Filppula <i>et al.</i> 1992
	Asian <i>Pinus</i> spp.	Cp (RFLPs)	Wang and Szmids 1993
	<i>Quercus</i> spp.	RAPD	Moreau <i>et al.</i> 1992
	<i>Picea mariana</i> & <i>P. rubens</i>	RAPD	Perron <i>et al.</i> 1995
	American and Mexican pines	RAPD	Furman <i>et al.</i> 1997
	<i>Avicennia</i>	RAPD	Parani <i>et al.</i> 1977
	Mangroves	PCR-RFLPs	Parani <i>et al.</i> 2000
3.2 Clones			
	<i>Acacia auriculiformis</i>	Isoenzymes	Changtragoon and Woo 1996
	<i>Azadirachta indica</i> var. <i>siamensis</i>	Isoenzyme	Changtragoon 1996
	<i>Eucalyptus</i> spp.	RAPD	Kiel & Griffin 1994
	<i>Picea glauca</i>	RAPD	Hong <i>et al.</i> 1992
	<i>Picea sitchensis</i>	RAPD	Van de Ven and McNicol 1995

Potential of molecular markers to facilitate forest gene conservation management

Molecular genetic markers hold great promise for several conservation applications, including approaches to measuring fundamental parameters important in conservation, such as effective population size, past bottlenecks, sex-specific gene flow or founder contribution. They also can be used to infer the historical and geographical relationships between groups (Hedrick 2001). The use of molecular markers has revolutionised studies of mating system, pollen movement, seed dispersal and genetic processes. Results of such studies are of considerable practical significance in relation to conservation and breeding programmes, such as population sampling, seed orchard design and management, controlled pollination methods and clonal forestry programmes for conservation and breeding (Haines 1994). Molecular markers may be used in four types of measurements needed for effective *ex situ* and *in situ* conservation of plants, namely: identity, similarity, structure and detection of genetic background of individuals, accessions, populations and taxa (Karp *et al.* 1996).

The application of molecular markers to facilitate genetic conservation in the tropics should be made in two steps. Firstly, they can be used to evaluate the status of genetic background of *ex situ* plantations and *in situ* sites that are established based on conventional silvicultural practices for any forest tree species. They can be used to check whether they contain correct clones and ramets and have sufficient genetic diversity for the conservation as representatives of the species' gene pool. If not, remedial measures could be taken according to the information guidelines provided by molecular genetic investigation.

Secondly, they can be applied to evaluate the status of genetic resources of species for which conservation plots have not yet been established, but that are planned to be included in conservation programmes. This can be done by determining the mating system, genetic variation within and among populations and as well as gene flow. In this manner, molecular markers can be used to guide as to how and where to collect samples for *ex situ* conservation and to determine/identify suitable sites for *in situ* conservation. However, to maximize the latter application, the method should be combined with an ecogeographic survey and measurement of adaptive traits.

The potential of the application of molecular markers for the management of forest genetic conservation could be summarized as below:

1. To clarify the identity of taxa and their relatedness as well as to infer their evolutionary histories
2. To correctly identify clones and ramets in genebanks to avoid mislabelling, duplication and contamination
3. To evaluate the amount, extent and distribution of genetic diversity within and between populations
4. To estimate mating system (selfing and outcrossing rate) and gene flow
5. To evaluate the status of genetic resources as the criteria for *ex situ* and *in situ* conservation from genetic information provided
6. To maximize the efficiency of management of conservation by combining adaptive traits, ecogeographic and genetic survey for both collection programs for *ex situ* conservation as well as for identifying sites for *in situ* conservation

Therefore, the answer to questions such as how to manage, which and how many materials should be manipulated and where the genetic resources should be established or protected, depends on whether the genetic background of particular species which we want to conserve is really known (Changtragoon and Szmidt 1993).

Case studies of the application of molecular markers in Thailand

Two case studies of the application of molecular markers in some forest trees as a guideline for genetic conservation management in Thailand are presented:

Genetic diversity of *Pinus merkusii* in Thailand

A genetic inventory in 11 natural populations of *Pinus merkusii* in Thailand revealed only little genetic diversity at 14 isozyme gene loci (average $d_T = H_e = 0.058$) as shown in Table 2. Allelic differentiation among populations was also small ($d = 0.034$), but higher than the differentiation reported for many other conifers, if measured as a proportion of the total variation ($F_{st} = 0.104$). Genotypic structures of seed samples were characterized by a deficiency of heterozygotes relative to Hardy-Weinberg expectations in most populations, while the genotypic structures of seed trees, which represent the adult forest stand, did not differ significantly from Hardy-Weinberg proportions. Estimation of outcrossing rates revealed extraordinary high proportion of selfing ($0.017 < t_m < 0.65$) for 9 out of 10 analysed populations, which accounts for the high inbreeding coefficients (Table 3). Scarcity of foreign pollen available for fertilization of ovules due to low population density, poor synchronization of flowering periods and over-mature character of most stands resulting in limited flower production are probable reasons for this result (Changtragoon and Finkeldey 1995).

Table 2. Genetic variation within *Pinus merkusii* populations in Thailand

Population	N	PPL (95%)	A/L	Ho	He
Ban Wat Chan 1	126	21.4	1.6	0.019	0.038
Ban Wat Chan 2	108	21.4	1.6	0.037	0.048
Khun Yuam	126	7.1	1.4	0.006	0.029
Omkoï	139	21.4	1.7	0.042	0.076
Pitsanuloke 1	180	21.4	1.8	0.063	0.088
Pitsanuloke 2	156	14.3	1.4	0.032	0.048
Nong Khu	150	21.4	1.6	0.051	0.070
Pooniyom	120	7.1	1.3	0.030	0.039
Huey Tha	138	35.7	1.6	0.061	0.109
Kong Chiam	108	21.4	1.5	0.044	0.055
Buntarik	180	14.3	1.6	0.025	0.042
Average	139.18	18.8	1.6	0.037	0.058

N : Sample size

PPL : Percentage of polymorphic loci

A/L : Average number of alleles per locus

Ho : Average observed heterozygosity

He : Average expected heterozygosity

Table 3. Estimation of outcrossing rates of *Pinus merkusii* in Thailand

Population	tm
Ban Wat Chan 1	0.444 ± 0.322
Ban Wat Chan 2	0.593 ± 0.202
Khun Yuam	0.017 ± 0.013
Omkoï	0.422 ± 0.120
Pitsanuloke 1	0.644 ± 0.154
Pitsanuloke 2	0.395 ± 0.095
Nong Khu	0.455 ± 0.115
Pooniyom	0.767 ± 0.145
Huey Tha	0.468 ± 0.077
Kong Chiam	0.843 ± 0.087
Buntarik	0.400 ± 0.088

Recommendations for genetic conservation of *Pinus merkusii*

In situ conservation

In situ conservation of *P. merkusii* is very important. Priority populations to be conserved are:

- Huey Tha (highest diversity)
- One more population from the Northeast (Nong Khu or Kong Chiam)
- One population from the North (e.g. Ban Wat Chan)

Ex situ conservation

Ex situ conservation has a complementary role to play. Several *ex situ* conservation stands should be established, because:

- Protection is easier if the locations are properly chosen, and
- Inbreeding might be lower than in natural populations due to a high density of stands and better flowering (uniform age structure).

It is suggested that 3-6 *ex situ* conservation stands should be established, at least one in each main region (North, Central, Northeast Thailand). Seeds should be harvested from approximately 20 trees of 3 populations within each region. Since the genetic diversity is low, this number is regarded as sufficient to contain most of the genetic information of *P. merkusii* in Thailand. Seeds from the same region should be bulked and be planted in small *ex situ* conservation stands (1 ha each), preferably at two sites, which are easy to protect within each region. Thus, only 6 ha of *ex situ* plantations needs to be established (Changtragoon and Finkeldey 1995; Changtragoon 2001).

Genetic diversity of teak (*Tectona grandis*) in Thailand

Fifty-one RAPD loci were identified and used to evaluate genetic diversity in fifteen natural populations of teak (*Tectona grandis*) in Thailand. Partitioning of genetic variation into within and between population components revealed that about 21% of the total variation was attributable to differences between populations. The number of polymorphic loci in most of the investigated populations was very high with the average of 70% of polymorphic loci (Table 4). Significant differences in allelic frequencies were found for most pairwise comparisons between populations (Changtragoon and Szmidt 2000). The outcrossing rates ranged between 82–97% (Table 5). However, within a population, there were differences in outcrossing rates among families. Despite the high average outcrossing, there was some inbreeding in each family and population, which should not be ignored (Changtragoon 2001).

Table 4. Genetic diversity estimates *Tectona grandis* populations investigated in Thailand

Population	Sample size	Polymorphic loci in %	Gene diversity
Pongsaree,Chiengrai	45	58.8	0.205
Mae Saaeab,Phrae	41	82.4	0.343
Chiengdoa,Chiengmai	20	78.4	0.335
Hod,Chiengmai	33	82.4	0.367
BanmaiMaetha,Lumpang	20	80.4	0.373
Thumpathai,Lumpang	18	68.6	0.334
Pratupa,Lumpang	34	84.3	0.345
Maesaraeng,Maehongson	30	84.3	0.365
Lansang 1,Tak	30	80.4	0.347
Lansang 2,Tak	20	62.8	0.278
Maemue 1,Tak	21	82.4	0.353
Nampad,Utradit	18	50.9	0.226
Thaepnimit,Utradit	36	92.2	0.371
Kangpalom, Kanchanaburi	20	27.4	0.110
Wangnamwon, Kanchanaburi	22	72.6	0.297
Average	27	72.6	0.310

Table 5. The estimates of outcrossing rate in *Tectona grandis* in Thailand.

Population	tm	ts
Banmai Maetha, Lampang	0.968 + 0.008	0.942 + 0.009
Hod, Chiangmai	0.884 + 0.020	0.884 + 0.022
Kaeng Palom, Kanchanaburi	0.903 + 0.030	0.897 + 0.011
Mae Saeab, Phrae	0.859 + 0.059	0.886 + 0.036
Pongsaree, Chiangrai	0.975 + 0.021	0.959 + 0.014
Pratupa	0.958 + 0.014	0.917 + 0.006
Mae Saerang	0.823 + 0.104	0.852 + 0.054
Wangnamwon	0.922 + 0.045	0.917 + 0.029

tm = multi-locus estimate, ts = single locus estimates

Recommendations for conservation of teak

These results suggest that natural populations of *T. grandis* in Thailand are highly differentiated genetically implying that multiple sources of materials from at least one population of each province in the northern and central part of Thailand for both *in situ* and *ex situ* conservation purposes may be required. Based on the fact that there is a high outcrossing rate, it is suggested that teak seed collection should be made separately by family and population basis. Seed sources and years of collections should be well registered so that based on ancillary information, seed sources could be screened before preparing seedlings for *ex situ* gene conservation. For example, seed sources from highly inbred families could jeopardize the long-term fitness of progeny by lowering the seedling survival percentage or by increasing the susceptibility to pest and disease incidence. Therefore, care needs to be exercised in eliminating such seed stocks from *ex situ* gene conservation units (Changtragoon 2001).

Constraints and limitation

There are some limitations, which should be considered before starting any programme employing molecular markers for genetic conservation of tropical forest tree, especially in the developing countries. First of all, the questions and objectives of a study should be clearly specified. Second, understanding the nature of each molecular marker is necessary whether they are codominant or dominant markers and which markers can be determined in nuclear and/or organelle (chloroplast and mitochondria) genomes. Therefore, the choice of markers and purpose of their application should be properly matched. Thirdly, knowledge on how to analyse and manage the molecular data as well as how to interpret the results should be sufficient. Last of all, laboratory facilities such as equipment, water quality, electricity, expertise and manpower as well as financial support should be considered in order to decide whether it is realistic to use those markers for a particular purpose.

The question that is often raised in international forest genetics conferences is which techniques and markers would be the best for use in forest population genetics and conservation. It is not difficult to answer this question, but to find the financial support to facilitate and handle the equipment and laboratory needed for these techniques is more difficult especially for developing countries due to lack of financial support and expertise. The greatest disadvantage in developing countries, such as Thailand is the cost of equipment and chemicals, which are about 2-4 times higher than in developed countries due to extra transportation costs and taxes (Changtragoon 1998).

Future plans and possibilities

The priority forest tree species in Thailand as well as in the Asia Pacific region should be identified at different levels according to their importance and conservation status: first their ecological and economic value and, second, their status of distribution and existence; whether they are specific to a certain location, whether they are fragmented, threatened or endangered. As soon as the species are identified, the survey of genetic variation and

mating system as well as gene flow of particular species can be planned and initiated. The survey will take about at least 2 years. However, this will largely depend on which molecular markers are used and as well as on the budget, human resources and facilities provided. One should keep in mind that most of the available molecular markers can detect only neutral genetic variation. Therefore, use and application of molecular markers, which can detect potentially adaptive genetic diversity is encouraged. In addition, combining molecular markers with other methods, such as ecogeographic surveys and adaptive traits (mostly morphological characterization) measurement for assisting in the selection of tropical forest trees for *ex situ* and *in situ* conservation and management, is also recommended.

As new molecular markers have been developed very fast but are still relatively costly, close collaboration and training within and among the developing countries in the region would be an alternative way to initiate the application of molecular markers for the management of gene conservation programme of tropical forest trees in the Asia-Pacific region more efficiently.

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