CHAPTER V

DISCUSSION

5.1 Species diversity and forest resources

There are two main types of forests in Thailand: Evergreen and Deciduous forests. The Evergreen forest is represented mainly by Hill evergreen forest on the highland parts (above 1000 m), the Semi-evergreen forest where rainfall is between 100-200 cm, and Pine forest on the highlands with poor soil. The Deciduous forest, which is commonly found throughout the country, is broadly divided according to the species composition into Mixed deciduous forest (with and without teak) and the Dry dipterocarp forest in dry area where rainfall is below 100 cm and the soil condition infertile and sandy. Both types are found in northern Thailand.

Hill evergreen forests in northern Thailand dominate 1000-1800 m elevation, with trees of the family Fagaceae forming at least 50% of the main canopy layer and with the mean height of 10-25 m (Santisuk, 2005). This type of forest occupies about one fourth of the total forest in northern Thailand, whereas Mixed deciduous and Dry dipterocarp forests occupy some 60% of the total forest area (Forestry Statistics of Thailand 1996). In the present study, typical Fagaceae rich, Hill evergreen forest is represented by two sites, KPA and KNK. There are only a few kilometers apart, but the site KNK is situated more northerly and at higher elevation (1000-1400 m) than the KPA site (950-1100 m). Although species diversity and composition appeared to be the same at these two sites, the former accommodated plenty of trees of *C. acuminatissima* but the latter had none of it. The most likely reason behind the absence of this species at KPA site is probably the lower elevation as this species is more prevalent above 1000 m. At this KPA, species of *Castanopsis* common at intermediate altitudes were identified, such as *C. indica*. Many trees at this site, particularly *Castanopsis*, have been wrapped for religious purpose hence protected.

These forests are now also protected against major logging - the locals frequently visit the forests mainly for medicinal herbs, and to some extent for edible nuts.

Hill evergreen forest with pine is represented in this study by the sites KBA and KRD which are situated nearby the above-mentioned Hill evergreen forests, but are more exposed and drier. They are also at high elevation: KBA (1050-1200 m) and KRD (1350-1400 m). The canopy of dry hill forests is almost entirely evergreen and usually very open with many gaps. These sites seem to support common species of all three Fagaceae genera, such as the evergreen *C. acuminatissima* common at high altitudes, the large trees of *C. tribuloides* one of the most common and variable Fagaceae species, most of the *Lithocarpus* species discovered in this study, and the most common *Quercus* species from these regions like the evergreen *Q. brandisianus* and the deciduous *Q. kerrii*. Dry hill forests are known to expand beyond their natural limits especially after long-term disturbance particularly by fire, and deciduous species have good ability to establish at such dry sites. At these study sites, however, most species diversity was found to be in the genus *Lithocarpus*. In other words, some *Lithocarpus* species are clearly adaptable to diverse habitats, as shown in this study. Fagaceae which is the main component of Hill evergreen forests is losing ground to pines especially in areas with thin soils and frequent fires.

There is little doubt that Fagaceae in Hill evergreen forests can be viable if undisturbed. The soil quality in the forests understudy seemed to be very good, especially at the site KNK where organic matters were plenty. Active roots of *Castanopsis* were abundant at the upper soil layer and were often associated with ectomycorrhiza. Soil quality in highland areas is very important as lower evaporation rates and scattered dry season rain showers make it possible for sites with good soil to be almost permanently moist. In lowland sites soil quality alone is not sufficient to maintain moisture levels without significant ground water input. Plants that are not confined to areas with permanent supplies of water must develop some mechanism to avoid excessive water loss, and the main mechanisms are deciduous habit (loosing water in the dry season) and sclerophylly (thick leaves with a waxy coating to reduce

water loss). In lowland areas the majority of the plants tolerant to dry conditions adopt the first strategy (e.g. *Quercus*), whereas in highland areas the second strategy is the more common (*Castanopsis* and *Lithocarpus*).

Dry deciduous forests occupy low elevation sites that have little or no ground water input during dry season and the soil is often too thin to maintain water reserves. Only those plants which are adapted to dry conditions can survive. The natural vegetation in such areas is often referred to as Dry dipterocarp forest because it is frequently dominated by the major hardwood trees species of Dipterocarpaceae (*Dipterocarpus* and *Shorea*). These forests have a poorer canopy structure than other lowland forests – 60% cover and 15 m mean height are typical, and the tree species diversity is also much lower (Gardner et al., 2000; Santisuk, 2005). A few species of Fagaceae are often present, especially the deciduous *Quercus* spp. At both sites investigated here, i.e. KPS (430-600m) and KHH (490-540m), *Quercus* is indeed prominent particularly the deciduous species *Q. kerrii*. These sites have a long history of logging and fires and therefore a large part of the vegetation today is from self-regeneration. *Quercus* is among the trees that can form a good size stand after such disturbance. Nevertheless some evergreen Fagaceae have also been identified, especially at the KPS site, e.g. *Q. brandisianus*, *Q. lineatus* and *Lithocarpus* spp., indicating high species diversity. Comparing these two sites, the KPS site clearly showed more species diversity than the KHH site. The KPS site location is also more protected and difficult to reach, and hence the species diversity may have been preserved that way. The KHH site is rather homogenous and consists mainly of large stands of deciduous oaks.

The forestry of Thailand has undergone several changes including the commercial logging which was the major forest exploitation from the forties to early sixties. Starting from the late eighties, Thailand has entered the conservation phase as the people have developed awareness of the adverse effects of forest exploitation. Whatever is remaining must be kept for conservation and many areas are now closed

for regeneration of forest trees. Fagaceae species are among the most important trees making the recovery of forests in northern Thailand successful.

5.2 Species diversity and genetic diversification

The most commonly considered facet of biodiversity is species richness – the number of species in a site or habitat. Species are an obvious choice of unit when trying to measure diversity, although not without limitations. Species are also sensible units from a biological perspective: they keep their genes more or less to themselves, meaning that species have independent evolutionary course. Therefore measuring genetic diversity of a species or group of species will not only provide a clearer view of current species diversity but also allows us to make inference about how the diversity has come about. Recent advances indicate that diversity can be expected, on average, to give rise to ecosystem stability (Tilman, 2000). The evidence also indicates that diversity is not the driver of this relationship; rather, ecosystem stability depends on the ability for communities to contain species or functional groups that are capable of differential responses. The effectiveness of strategies for the preservation of diversity is often limited by our knowledge of the mechanisms that maintains diversity.

In an attempt to understand the taxonomic diversity of Fagaceae species in *Khun Mae Kuong* Forest, we examined diversity at the molecular level by analyzing restriction fragment length polymorphism (RFLP) in the 18S-25S ribosomal genes (rDNA) and genome-wide inter-simple sequence repeats (ISSR). The molecular results show clear separation of the genera and of most species, supporting the taxonomic classification. Furthermore, both markers show that the genus *Castanopsis* in this region is genetically diverse, the *Quercus* genus is more homogeneous, but *Lithocarpus* has a split diversity. We find possible indication of gene flow between species belonging to different genera, i.e. between *Castanopsis* and *Lithocarpus* and between *Lithocarpus* and *Quercus*, and this may have been the reason behind such diversity pattern of *Lithocarpus* in this region.

Gene flow can occur between species but the extent of it depends on how effective reproductive barriers are in a given space and time. In the Fagaceae, reproductive isolation among closely related species is generally weak and interspecific hybridization is potentially an important element in the evolutionary history of this plant group (Hardin, 1975; Whittemore and Schaal, 1991; Ferris et al., 1995; Petit et al., 1997; Manos et al., 1999). Most detailed studies have focused on the temperate *Quercus* subgenus *Quercus* – little is known about gene flow in the Asiatic Fagaceae of the tropical rain forests. Nevertheless, scenarios based on chloroplast haplotypes in Southeast Asian *Lithocarpus* have included the possibility of significant fragmentation of the stone oak populations during the Pliocene, leading to independent genetic diversification at regional levels (Cannon and Manos, 2003). Such diversification may have been facilitated by gene flow via interspecific hybridization, especially in the species-rich habitats like in northern Thailand. *Castanopsis* and *Lithocarpus* are closely related and both are pollinating by generalist insects (Manos et al., 2001), and therefore they should be capable of hybridizing sympatrically, and through back-crossing the genetic materials could be transferred between species. Gene flow between *Lithocarpus* and the wind-pollinating *Quercus* is also possible, especially in disturbed forests with lots of gaps, like the KBA and KRD sites. Forest fragmentation or disturbance has been shown to change the movement of pollen and seed dispersal, modifying the gene flow and altering historical patterns of genetic subdivision in tropical trees (Hamilton, 1999).

Gene flow could be measured accurately and experiments setup to test the impact of the gene transfer, i.e. whether it had any adaptive advantage or what has driven the gene flow. But the study reported here is merely the first step into understanding genetic diversity and diversification, in a region where taxonomic diversity is very large and mostly un-described. The repetitive DNA markers used in this study have been selected with an aim to get an overview of diversity and to put the information into practice, i.e. in the restoration and reforestation activities. For this purpose, they should be powerful and sensitive enough to reveal both genetic

diversity and gene introgression. The 18S-25S ribosomal genes in plants exist in thousand of copies mostly arranged in tandem arrays at particular chromosome loci, and only a very small fraction of the repeat units are required to sustain ribosomal assemblage (Neves et al., 2005). The rDNA-RFLP polymorphism may not reveal true phylogenetic relationships among species investigated, as opposed to using single-copy nuclear genes, because the genes are highly repetitive and the copy number can change rapidly (Alvarez and Wendel, 2003). On the contrary, rapid changes in copy number, amplification in particular, would make gene flow easily detectable without causing overall genomic or phenotypic modifications. Also because of high copy number, the chance of it being transferred via meiotic recombination should be high enough for it to come through, and remain as footprints of introgression. Variation in the ribosomal genes has been used effectively in a number of woody plant species including aspen (*Populus*), spruce (*Picea*), ash (*Fraxinus*) and birch (*Betula*) (Faivre-Rampant et al., 1992; Karvonen et al., 1994; Jeandroz et al., 1996; Anamthawat-Jónsson, 2002). Suitable DNA markers in plants are generally lacking and this is believed to be the single most limiting factor in making molecular population genetic studies in plants lag far behind those in animals (Schaal et al., 1998).

ISSR markers, on the other hand, have been used much more extensively to confer overall diversity in a genome without prior sequence information. The technique is based on the abundance of various polymorphic microsatellite motifs in the plant genomes (Lagercrantz et al., 1993; Zietkiewicz et al., 1994). As the polymorphism is probably genome-wide, it should represent an unbiased variation and hence suitable for phylogenetic analyses. The method is simple and inexpensive but has proven to be applicable for taxonomic inference and genetic diversity assessment in a wide range of organisms. These markers have proven to be good approximates of taxonomic diversity in *Nothofagus* of the Southern Hemisphere (Mattioni et al., 2002), for identification of commercial poplar cultivars (Fossati et al., 2005), useful in estimating loss of genetic diversity in an endangered pine species in China (Zhang et al., 2005) and when combining geographical coordinates and genetic differentiation the markers can detect changes in the gene flow in forest trees (Lu et

al., 2005). Tree species are becoming the focus of increasing conservation concern, with some 9000 species now threatened globally (Newton et al., 1999). Studies of genetic diversity, especially intra- and interspecific variation, can contribute to the development of conservation strategies, by identifying units for conservations. Such variation has increasingly been accepted as a focus for conservation, an approach consistent with the general aim of maintaining the evolutionary potential of species.

5.3 Genomic diversity

Genome diversity among the flowering plants is extensive, mainly due to hybridization and polyploidy. Although a vast majority of plant species are polyploid (Leitch and Bennett, 1997; Soltis and Soltis, 1999), the distribution of polyploid species is not geographically even. Frequency of polyploidy in plants in the Northern Hemisphere has been long known to increase with increasing latitude (Löve and Löve, 1957), and the possible reasons for this trend have been discussed for many decades. The most favored reason is probably that polyploids are better adapted than diploids to extreme climates, or they are better suited to colonize new habitats because of their greater ecological adaptability. The latest evidence from studying Arctic plants, however, has indicated possible correlation between polyploidy and dramatic climate change (e.g. due to glaciation), not directly but via heterozygosity of the genomes (Brochmann et al., 2004).

Based on this line of reason, the frequency of polyploidy among tropical plants should be low as they have not had to cope with drastic environmental changes throughout the Tertiary period, and in the case of tropical woody genera for perhaps even a longer period of time. But tropical and temperate woody angiosperms are not obviously different from one another in terms of ploidy level. In the floras of the temperate zone, trees and shrubs have, on the average, lower frequencies of polyploidy within a genus than perennial herbs, but they have higher basic numbers (Stebbins, 1971). Woody plants of tropical regions resemble those of the temperate zone in the rarity of polyploid series within a genus, and their basic numbers are

similar, with a mode at x = 11, 12, 13 and 14, significantly higher than the mode for temperate herbs, which have basic numbers x = 7, 8 and 9 (Darlington and Wylie, 1955). Stebbins (1971) explained that basic numbers of modern woody genera were derived by ancient polyploidy, and that the original basic numbers of angiosperms, both woody and herbaceous, were x = 6 and x = 7. Although tropical floras are still much more poorly known cytogenetically than temperate ones, it is becoming evident that most woody families of angiosperms include basic numbers of x = 7 – 9, and the great majority of these genera are tropical, in agreement with the widely accepted hypothesis that temperate woody groups have, in general, been derived from tropical ancestors (Briggs and Walters, 1997). The most probable hypothesis is that the (ancient) polyploidy which gave rise to the high basic numbers of woody plants took place at various times during the Cretaceous and the earliest part of the Tertiary period, while the diversification of species on the basis of secondary basic numbers is largely a product of Tertiary and Quarternary periods (Stebbins, 1971). The present-day diploid woody genera are hypothetically ancient polyploid.

The chromosome number was determined in 24 species of Fagaceae in this study and the results show that they all have diploid chromosome number 2n = 2x = 24, with the basic number being x = 12, and their karyotypes are very similar. Two species of *Castanopsis*, i.e. *C. indica* and *C. tribuloides*, showed 12 bivalents at meiotic metaphase, confirming the basic number x = 12. The exception to this 2n = 24 is that one species, i.e. *Quercus lenticellatus,* has the chromosome number of 2n = 24 in one tree and 2n = 14 with lower basic number x = 7 in another tree. This basic number is confirmed in the karyotype analysis where seven homologous chromosome pairs are shown. Previous studies of *Quercus* spp. in Europe showed the basic chromosome number x = 12 in this genus (D'Emerico et al., 1995; Zoldos et al., 1999). Moreover, when comparing karyotypes of species of *Quercus* in the present study with regards to morphology of metacentric and submetacentric pairs with those from Europe, the karyotpes also showed close similarity. The diploid number 2n = 24 for *Lithocarpus* spp. and *Castanopsis* spp. In the present study is the first chromosome report about these genera. Their karyotypes show many similarities to

those of *Quercus* especially for having the same morphology of metacentric and submetacentric chromosome pairs. According to Soepadmo (1972) there are no records of chromosome number of Fagaceae species covered in Flora Malesiana. Two records on chromosome number of *Castanopsis* can be retrieved from the database IPCN, the Index to Plant Chromosome Number, maintained at the Missouri Botanical Garden website (http://mobot.mobot.org), but none on *Lithocarpus*. Cytogenetic data on tropical and subtropical species of Fagaceae has so far been extremely limited, probably due to difficulty in obtaining plant materials for the conventional root-tip chromosome preparation and difficulty in counting small chromosomes accurately. In the present study, a newly developed chromosome preparation protocol (Anamthawat-Jónsson, 2003b) using leaf buds which are always available in the field and protoplast dropping technique that makes well spread chromosomes suitable for counting, making karyotypes, as well as for molecular cytogenetic experiments.

The basic chromosome number x = 12 found in this study falls within the general range of the basic number mode of most woody plant genera discussed above. The discovery of lower basic chromosome number (i.e. x = 7) in one of the *Q. lenticellatus* trees is most interesting, as this is believed to be an ancestral basic number of angiosperms. No such basic number in Fagaceae has been reported. The only interpretation is that most present-day species of Fagaceae are ancient polyploid, and this region, which is part of species diversity centre of this group of Fagaceae, contains relics of an ancestral type. Other variations in the chromosome number found in this study include aneuploidy and polyploidy in different cells or different plant materials. For example, sample PA25 (*C. calathiformis*) has 2n chromosome number 24 and 28 even on the same chromosome preparation. Two samples: NK 5 (also *C. calathiformis*) and NK10 (*L. vestitus*) have 2n = 36 (triploid number) and 2n = 48 (tetraploid number) from root tips, respectively, but the leaf chromosome number is diploid with 2n = 24 like other plants. These results need to be confirmed. Variation in the chromosome number of *Quercus* has been reported, for example in *Q. petraea* and its closely related species *Q. robur* (Zoldos et al., 1998). The DNA

content of seven species of *Quercus* in France was measured and significant difference in the DNA content between two populations of *Q. petraea* was observed. They verified the chromosome number in one population that showed normal chromosome component of 2n = 24, but some individuals had 2n = 24 + 1, 2, or 3 extra chromosomes, even in difference cells from the same root. The aneuploid variation is not to be unexpected if these species are ancient polyploids having originated from species with the basic chromosome number x = 7. Recent compilation of DNA content data has shown that in most cases of polyploidy the genome size has reduced (Leitch and Bennett, 2004), either via chromosome size reduction or by fixing at a lower 2n number as that might have been in Fagaceae (i.e. 2n = 24 in stead of being 2n = 28). The present study also indicates that polyploidy may still be occurring at certain frequencies even in the cytogenetically stable genera as *Castanopsis* and *Lithocarpus*. Environmental changes, including disturbance due to human activites, may facilitate an establishment of new cytotypes, which is known to have played an important role in plant species evolution.

The physical mapping of the 18S-25S gene in *Quercus* spp. showed variation in both the number and chromosomal location of the 18S-25S rDNA loci: some species have two sites (one pair) and others have four sites (two pairs). The two pairs of the 18S-25S rRNA gene in *Q. kerrii* are subterminal. These results are different from the previous study of eleven *Quercus* spp. in Europe, which showed highly conserved map both in number (four sites: two major and two minor sites) and position (major loci were subterminal whereas minor loci were paracentromeric, Zoldos et al. 1999). These molecular cytogenetic results indicated that *Quercus* spp. in Europe and Asia are genomically different, which supports the taxonomic division that *Quercus* spp in Europe belong to the subgenus *Quercus* while the subgenus *Cyclobalanopsis* comprises species from Southeast Asia (Manos et al., 2001).

The physical mapping of the 18S-25S rRNA gene in *Lithocarpus* and *Castanopsis* is the first report of these genera. *Cantanopsis* spp. showed variation of this gene in terms of number of loci. There are four groups of *Castanopsis* separated

by the number of the 18S-25S rDNA loci in the genome: 1) having two sites (one pair), 2) having three sites (un-paired), 3) having four sites (two pairs), all major sites, and 4) having four sites (two pairs), one pair major and one pair minor loci. Most of *Lithocarpus* spp. showed four sites (two pairs) of the 18S-25S rRNA gene, except one species, *L. vestitus*, showed six sites with five major and one minor loci, all un-paired. The un-paired loci and the odd number of sites in three samples from these two genera clearly indicate hybridity and/or polyploidy, which confirms the cytological analysis. The species *L. vestitus*, which has six sites of the 18S-25S rRNA gene, also has tetraploid chromosome number in the root tip cells as mentioned above. The two samples of *C. calathiformis*, NK5 and PA25, which show three sites of the 18S-26S rRNA gene, also have unusual chromosome numbers (NK5 being triploid in root cells and PA25 having 2n = 24 – 28 indicating recent polyploidy).

The physical mapping of the 5S rRNA gene in all three genera under study has shown that the maps, based on both number and chromosomal position of this gene, are highly conserved. There are two sites (one pair) in the genome and are localized at paracentromeric region, as reported also in *Quercus* spp. from Europe (Zoldos et al., 1999). However, when considering the localization of both 18S-25S and 5S rRNA genes together, the ribosomal gene mapping as a whole produces species-specific pattern useful for tracing species origin or for taxonomic identification. This application has been shown in many species, for example in differentiating closely related *Picea* species (Brown and Carlson, 1997), and finding ancestry of *Hordeum* genome (Taketa et al., 2005). Based on these rDNA maps, there is a clear indication of hybridization and gene flow between the Fagaceae genera in this study. For example, *C. fissa* has the typical rDNA map of *Lithocarpus*, i.e. the two ribosomal genes are locating next to each other. Other evidence, molecular and morphological, also indicates that *C. fissa* is more closely related to *Lithocarpus* than to other species of *Castanopsis* (Manos et al, 2001).

The investigation of genetic diversity by using neutral molecular markers may provide useful information on co-ancestry, gene flow and ecology over species and

populations, but the very issue of genetic identity gets confounded when the variation in the population is on account of intraspecific cytological variation (Lavania, 2002). The molecular cytogenetic technique provides new tools for the analysis of plant genomes. This technique of fluorescence *in situ* hybridization is on of the most appropriate methods for physical mapping of DNA sequences on chromosomes, facilitating identification of individual chromosomes with different DNA markers. The physical mapping of genes can reveal the organization of a genome and identify relationship of plant species, especially where they are involved in interspecific hybridization and polyploidy (Anamthawat-Jonsson and Heslop-Harrison, 1995).

Tree species are becoming the focus of increasing conservation concern, with some 9000 species now threatened globally (Newton et al., 1999). Studies of genetic diversity, especially intraspecific and interspecific variation, can contribute to the development of conservation strategies, by identifying units for conservations. However, molecular markers may not screen cryptic, cytotypic and/or ploidy variation that influence genetic architecture and reproductive potential. Microscopic visualization of chromosomes offers distinct advantages over molecular characterization on the gel and filters in certain specific situations; typical examples are on account of cryptic structural variation and/or numerical chromosome variation in autopolyploids/aneuploids and diploids (Lavania, 2002). It is important to know the structure and behavior of chromosomes and genomes to elucidate evolutionary potential of population, and also to underpin the structural rearrangements, which in the end can help complement conservation plans.