รายงานฉบับสมบูรณ์

โครงการวิทยานิพนธ์

ความหลากหลายทางพันธุกรรมและพลวัตการถ่ายเทยีน

ของ *Shorea obtusa* Wall. ex. Blume

(Dipterocarpaceae)

อาจารย์ที่ปรึกษา

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สนับสนุนโดย

โครงการพัฒนาองค์ความรู้

และศึกษานโยบายการจัดการทรัพยากรชีวภาพในประเทศไทย

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บทสรุป

ไม้เต็ง (*Shorea obtusa*) เป็นพรรณไม้ดัชนีสำคัญของระบบนิเวศป่าเต็งรังในประเทศไทย ผลกระทบจากการลดลงของพื้นที่ป่าและพื้นที่แบ่งแยกเป็นหย่อมขนาดเล็กอาจส่งผลกระทบต่อความหลากหลายทางพันธุกรรมของพืชชนิดนี้ งานวิจัยนี้ศึกษาโครงสร้างทางพันธุกรรม ความหลากหลายทางพันธุกรรม ความสัมพันธ์ทางพันธุกรรม การตรวจสอบความเป็นพ่อ และระบบผสมพันธุ์ของเต็ง โดยใช้เครื่องหมายโมเลกุลไมโครแซทเทิลไลท์ดีเอ็นเอ จำนวน 5 ตำแหน่ง และศึกษาเซลล์พันธุศาสตร์ของเต็งโดยวิธี propionocarmine smear technique ผลการศึกษาพบว่า เต็งเป็นพืชดิพลอยด์ มีจำนวนโครโมโซม 2n = 14 จากการศึกษากลุ่มตัวอย่างเต็งจำนวน 146 ต้น จาก 5 ประชากร ได้แก่ ประชากรจังหวัดชัยภูมิ จังหวัดเชียงราย จังหวัดมหาสารคาม จังหวัดอุบลราชธานี และจังหวัดอุทัยธานี พบว่าความหลากหลายทางพันธุกรรมของประชากรเต็งอยู่ในระดับสูง ด้วยค่าเฉลี่ยเฮเทอโรไซโกตที่คาดหมาย (*He*) เท่ากับ 0.664 ประชากรมีความแตกต่างทางพันธุกรรมในระดับต่ำ ถึงแม้จะ มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ (Fst = 0.030, $p < 0.05$) อาจเป็นผลจากพื้นที่ป่าเต็งรังมีการแบ่งแยกของพื้นที่เกิดขึ้นไม่นาน การวิเคราะห์ความสัมพันธ์ทางพันธุกรรมโดยการสร้างแผนภาพต้นไม้วิวัฒนาการด้วยวิธี UPGMA โดยการวัดค่าความห่างทางพันธุกรรมตามวิธีการของ Nei พบว่าผลจากการสร้างแผนภาพต้นไม้วิวัฒนาการมีการแบ่งประชากรออกเป็น 2 กลุ่ม คือ กลุ่มที่ 1ได้แก่ ชัยภูมิและเชียงราย และกลุ่มที่ 2 ได้แก่ มหาสารคาม อุบลราชธานีและอุทัยธานี ซึ่งสันนิษฐานว่า ประชากรสองกลุ่มที่มีความแตกต่างทางพันธุกรรมนั้นอาจเกิดจากระดับความสูงจากน้ำทะเลที่แตกต่างกันของถิ่นอาศัย จนเป็นสาเหตุทำใ ห้โครงสร้างทางพันธุกรรมของประชากรไม้เต็งเปลี่ยนแปลงระหว่างสองกลุ่มนี้ ข้อเสนอแนะสำหรับการใช้ประโยชน์จากการศึกษาโครงสร้างทางพันธุกรรมไม้เต็งจากการศึกษาครั้งนี้คือ สำหรับแหล่งประชากรที่มีความหลากหลายทางพันธุกรรมสูงควรพิจารณาให้เป็นแหล่งอนุรักษ์พันธุกรรมไม้เต็ง

ผลการศึกษาความสัมพันธ์ทางพันธุกรรม การตรวจสอบความเป็นพ่อ และรูปแบบของระบบผสมพันธุ์ของเต็ง ในสถาบันวิจัยวลัยรุกขเวช จังหวัดมหาสารคาม จากการตรวจสอบจีโนไทป์จำนวน 208 ตัวอย่าง ซึ่งประกอบด้วย ต้นเต็งที่คาดว่าจะเป็นพ่อจำนวน 29 ต้น และต้นกล้าจำนวน 179 ต้น ที่เก็บเมล็ดจากต้นแม่พันธุ์เต็งจำนวน 5 ต้น พบว่าความสัมพันธ์ระหว่างระยะทางและความสัมพันธ์ทางพันธุกรรมของต้นเต็งที่คาดว่าจะเป็นพ่อ จำนวน 29 ต้น มีความสัมพันธ์ในเชิงลบ (r = -0.129, $p < 0.05$) ผลการศึกษาระยะห่างทางพันธุกรรมของ 29 ต้น พบว่ามีค่าต่ำ แสดงว่าเกิดการถ่ายเทยีนอย่างมากในประชากรเต็งในรุ่นที่แล้ว และการแพร่กระจายเของมล็ดพันธุ์ส่งผลกระทบให้โครงสร้างทางพันธุกรรมของประชากรเต็งต่ำ

การตรวจสอบความเป็นพ่อของเต็งพบว่ามีแอลลีลที่อาจจะมาจากต้นเต็งที่มาจากนอกพื้นที่ประมาณ 10.9 เปอร์เซ็นต์ ผลการศึกษาค่าความน่าจะเป็นของความเป็นพ่อ (*Pe*) พบค่า *Pe* เท่ากับ 0.567 และมีค่าความถี่นันแอลลีลมากกว่า 0.05 การวิเคราะห์ค่า effective pollen dispersal distance (δ) พบค่าเฉลี่ย 626.7 เมตร และพบความถี่ของการกระจายของเรณูในประชากรเต็ง ส่วนค่า effective pollen donors per maternal tree (*Nep*) เท่ากับ 3 ถึง 12 ด้วยค่าเฉลี่ย *Nep* เท่ากับ 6 นั่นคือการเพิ่มขึ้นของค่า δ และ *Nep* อาจจะมีสาเหตุมาจากความหนาแน่นของประชากรต่ำ และมีระยะทางในการถ่ายเรณูมากขึ้น

ผลการวิเคราะห์ระบบผสมพันธุ์ของประชากรเต็ง โดยวิธี MLTR พบว่าอัตราการผสมข้ามสายพันธุ์อยู่ในระดับต่ำ (t_m = 0.569) ค่า biparental inbreeding (t_m - t_s) เท่ากับ 0.037) แสดงว่ามีสัดส่วนของการผสมพันธุ์ระหว่างต้นที่บรรพบุรุษร่วมกันต่ำ ค่า correlation of paternity (r_p = 0.340) แสดงว่ามีจีโนไทป์ต้นพ่ออย่างน้อย 4 ต้นอยู่ในรุ่นลูกของประชากรนี้ จากผลการศึกษาอัตราการผสมข้ามสายพันธุ์อยู่ในระดับต่ำของประชากรเต็ง ในสถาบันวิจัยวลัยรุกขเวช อาจเนื่องมาจากการกระจายของต้นเต็งในป่าแห่งนี้มีความหนาแน่นต่ำ

จากผลการศึกษาครั้งนี้สามารถนำข้อมูลโครงสร้างทางพันธุกรรมของเต็ง มาวางแผนการอนุรักษ์แหล่งพันธุกรรม และยังเป็นข้อมูลพื้นฐานในการประเมินสภาพแหล่งพันธุกรรมของกลุ่มไม้ยางหายากที่สมควรอนุรักษ์ไว้ในแหล่งที่อยู่ตามธรรมชาติได้

คำสำคัญ : ไมโครแซทเทิลไลท์, ความหลากหลายทางพันธุกรรม, การถ่ายเทยีน, ความสัมพันธ์ทางพันธุกรรม, การตรวจสอบความเป็นพ่อ, ระบบผสมพันธุ์, ป่าเต็งรัง

Summary

Shorea obtusa is a keystone species of the dry deciduous dipterocarp forest in Thailand. The diversity of this species is under assault from deforestation and forest fragmentations. In this study, genetic structure and diversity, genetic relatedness, paternity analysis and mating system of this species were determined based on a microsatellite marker. Cytogenetic study was also conducted using a propionocarmine smear technique. The results found that *Shorea obtusa* is a diploid species which has a consistent chromosome number of 2n = 14. Population genetic structure and diversity of 146 trees *Shorea obtusa* were collected from five populations (Chaiyaphum, Chiang Rai, Maha Sarakham, Ubon Ratchathani and Uthai Thani) encompassing major forest regions of Thailand. High levels of genetic diversity were found among the five populations with the average *He* of 0.664. Genetic differentiations between populations, although significant, were low ($Fst = 0.030$, $p < 0.05$). This might be indicated that the populations selected were recently part of a continuous population. A tree constructed using the unweighted pair group method with arithmetic average (UPGMA), based on Nei's genetic distance, divided populations into two groups: group 1 (Chaiyaphum, Chiang Rai) and group 2 (Maha Sarakham, Ubon Ratchathani and Uthai Thani) . This separation was consistent with the altitudinal zonation of the populations, thus indicating that altitude might play a significant role in genetic structure of *Shorea obtusa*. Areas of high genetic diversity were identified which could be considered priorities for conservation.

Genetic relatedness, paternity analysis and mating pattern of *Shorea obtusa* in Walai Rukhavaj Botanical Research Institute (WRBRI), Maha Sarakham province were examined. A total of 208 individuals including 29 candidate parent tree and 179 seedlings of five mother trees were genotyped. Correlation between genetic relatedness and spatial distance among 29 candidate parents within the population was negatively significant ($r = - 0.129$, $p < 0.05$, Mantel test). This correlation was weak, indicating that the spatial genetic structure among 29 trees of *Shorea obtusa* were low. This also implies extensive gene flow in previous generations. The results suggest that long - distance gene flow and seed migration are responsible for weak genetic structure of this species.

Parentage analysis of seedling found that 10.9% of the alleles detected were probably originated from adult trees outside the WRBRI. The probability of paternity exclusion (*Pe*) was 0.576 with the presences of null allele frequency more than 0.05.The average of effective pollen dispersal distance (δ) was 626.7 m and the frequency distribution of pollen movement suggested possibility of the long distance pollen dispersal. The effective pollen donors per maternal tree (*Nep*) range from 3 to 12 with a mean of 6. The long effective distance pollen dispersal and number of *Nep* for isolated maternal trees might be a synergic effect of low tree density and long - distance flight range of pollinators.

The mating system of *Shorea obtusa* population was examined using the multilocus mating system model (MLTR). The results indicated a low rate of outcrossing (t_m = 0.569). The estimate of biparental inbreeding for the total population (t_m - t_s) was 0.037, indicating that a low proportion of mating occurred among close relatives. The correlation of paternity (r_p) was 0.340 indicated that less than four fathers contributed to individual progeny. The low outcrossing rates of this species in WRBRI possibly resulted from its individuals scattered distributed with a low density.

Results obtained from this study could be used for genetically sound conservation and management program for *Shorea obtusa* and also serve as a model for the study of other rare dipterocarps which should be given priority for conservation.

Keywords microsatellite, genetic diversity, gene flow, genetic relatedness, paternity analysis, mating system, deciduous dipterocarp forest

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Genetic diversity and gene flow dynamics of *Shorea obtusa* Wall.ex. Blume (Dipterocarpaceae)

1. Introduction

Tropical forest in Southeast Asia is one of the global biodiversity hotspot (Myers *et al.*, 2000). This forest largely covered by dipterocarps plant species (i.e. family Dipterocarpaceae). Dipterocarpaceae is a species rich tree family with more than 510 species in 17 genera recorded worldwide (Ashton, 1982; Bawa, 1998; Cao, 2006). The genus *Shorea* is the largest and most important genus of Dipterocarpaceae, consists of 188 species. Trees of this genus are widely used for timber and non-timber products (Ingleby *et al.*, 1998; Shiva and Jantan, 1998; Pooma and Newman, 2001; Tennakoon *et al.*, 2005; Chalermpong *et al.*, 2007).

Shorea obtusa Wall. ex Blume (Teng or Siamease sal) is one of the keystone species in dry Deciduous Dipterocarp Forest (DDF) in Thailand. These forests are very important for ecological and economic value (Pooma and Newman, 2001). The diversity of this species is under assault from deforestation and habitat alteration (Bawa, 1998). In Thailand, deforestation for agricultural land use and forest timber products is a major factor threatening biodiversity (Liengsiri, 1999).

Genetic diversity is a critical component for evolutionary process (Bisby, 1995; Finkeldey and Hattemer, 2007; White *et al.*, 2007). Habitat loss and fragmentation increases the chance of genetic drift often resulting in decreased genetic variation and increased inbreeding (Slatkin, 1985; Murawski and Hamrick, 1992; Costin *et al.*, 2001). Information on the extent and pattern of genetic diversity, gene flow, mating system, and population genetic structure is essential for establishing guidelines for the conservation and utilization of the genetic resources of a species (Lee *et al.*, 2000).

Recent improvements in genetic analysis and genotyping methods have increase of the power of molecular markers to address ecological questions. Microsatellites have emerged as the most popular and versatile marker type for ecological applications (Selkoe and Toonen, 2006). There are several studies using microsatellite markers for plant population genetic studies, for example, logepole pine

(Liewlaksaneeyanawin, 2006), white oak (Craft, 2005) and coconut (Perera *et al.*, 2003).

Despite its ecological and economic importance, to the best of our knowledge, information on the genetic diversity of *S. obtusa* is lacking. In this study the microsatellite markers were used to assess genetic variation, population genetic structure and gene flow in natural populations of *S. obtusa* in Thailand. Information gathered from this study will assist conservation management programs and generally enable us to understand the level and pattern of plant genetic diversity in Thailand.

2. Objective

2.1 To investigate chromosome numbers of *Shorea obtusa.*

2.2 To evaluate cross-amplification microsatellite marker of *Shorea curtisii* and *Shorea leprosula* to *Shorea obtusa.*

2.3 To analyze the level of genetic diversity of *Shorea obtusa* using microsatellite marker.

2.4 To estimate gene flow dynamics of *Shorea obtusa* for understanding the reproductive process.

3. Methodology

3.1 Cytogenetic study

Chromosome number of *Shorea obtusa*, *S. siamenesis* Miq. and *S. roxburghii* G. Don in Thailand were examined. Young flower were fixed in Carnoy's solution for 24 - 48 hours. The anthers were smear and stained with propionocarmine. Chromosomes were examined from various stages of meiotic cells, using X100 magnifications with a Carl Zeiss axiostar plus light microscope.

3.2 Population genetic study

3.2.1 Genetic structure and genetic diversity

Samples of *S. obtusa* were collected from five populations; Thungkham community forest at Chiang Rai (CR); 20° 05.201' N, 100° 28.609'E, altitude 404 - 465 m, Khokdongkheng community forest at Maha Sarakham (MK); 15° 42.336' N, 103° 13.771'E, altitude 160 - 170 m, Buengcharoen community forest at Uthai Thani (UT); 15° 33.294' N, 99° 23.741'E, altitude 183 - 313 m, Pakdong community forest at Chaiyaphum (CY); 15° 51.832' N, 101° 31.688'E, altitude 352 - 440 m and Phunoi community forest at Ubon Ratchathani (UB); 14° 32.336' N, 105° 00.533'E, altitude 188 - 218 m (Figure 3.1). Leaf samples were collected from approximately 30 mature trees (> 80 cm dbh) per population each with a minimum distance of 30 m. Samples were kept on ice, and then stored at-20°C until DNA extraction was performed.

3.2.2 Genetic relatedness and paternity analysis

Genetic relatedness and paternity study was conducted in Walai Rukhavaj Botanical Research Institute (WRBRI), Maha Sarakham, Thailand (15° 42.336' N, 103° 13.771'E, altitude 160 - 170 m). Type of forest in this area is DDF with *S. obtusa* as the dominant species. The population area is about 100 ha and the original transplanted trees have produced seed for more than 20 years. *S. obtusa* of all individual trees was determined by observation of flowers, and their spatial positions were mapped by using a handheld global positioning system (GPS). A total of 29 adult individuals were located and mapped (Figure 3.2) and defined as tree of more than 80 cm dbh. A minimum distance of 30 m is kept between sample trees. These trees are randomly distributed within the population with an unequally spatial structure. Five maternal trees (MK8, MK14, MK22, MK25 and MK32) with proficient seed production were preferred as maternal tree. One hundred mature seeds per maternal tree were collected from branch and seeds were germinated as the offspring.

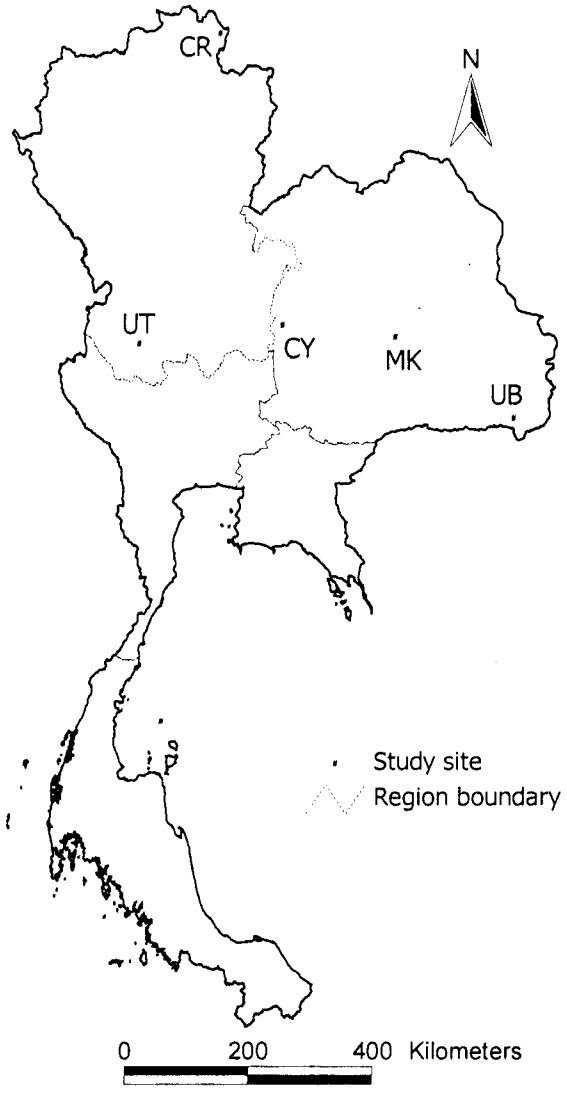

Figure 3.1 Sampling locations of five populations of *Shorea obtusa* in Thailand. CR, Chiang Rai; CY, Chaiyaphum; MK, Maha Sarakham; UB, Ubon Ratchathani and UT, Uthai Thani

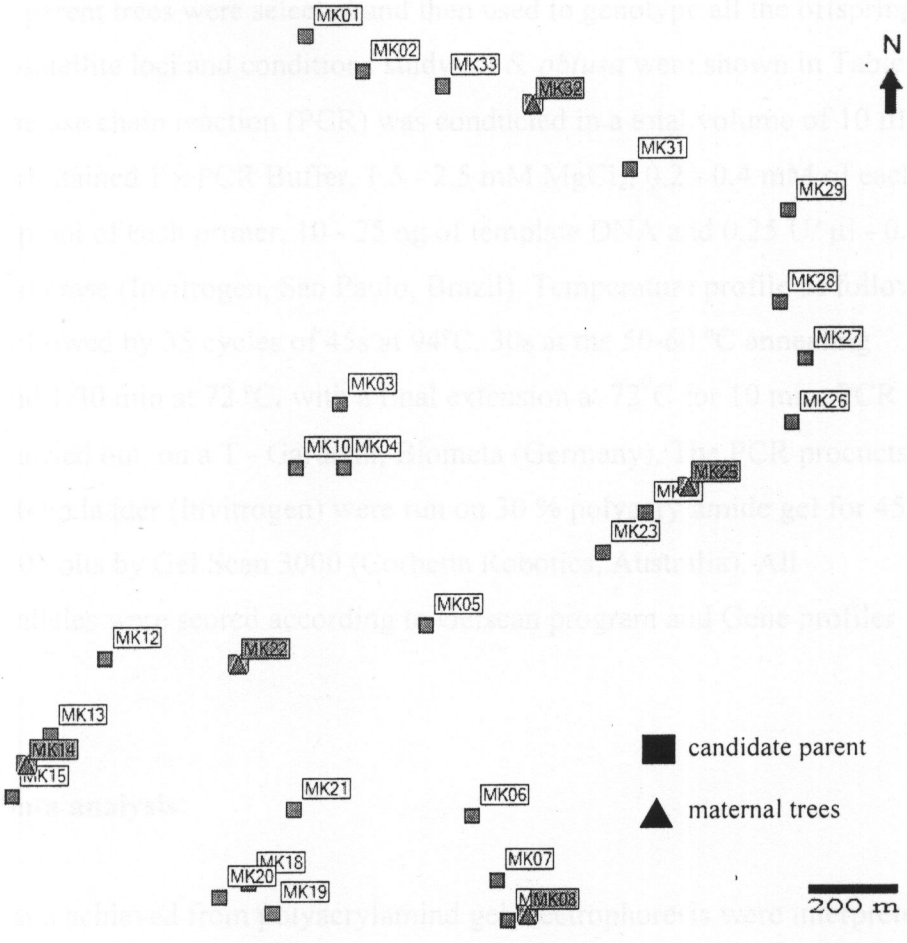

Figure 3.2 Spatial distribution of the 29 S*horea obtusa* individuals in the Walai Rukhavej Botanical Research Institute (WRBRI), Maha Sarakham province (MK)

3.3 DNA extraction and cross - species amplification of microsatellites

DNA was extracted from leaf using a modified 2xCTAB protocol (Doyle and Doyle, 1987). Ten microsatellite loci were screened for polymorphisms. Five primers (shc01, shc04, shc07, shc09 and shc11) developed for *S. curtisii* (Ujino *et al.*, 1998) and for *S. leprosula* (sle074, sle079, sle105, sle111a and sle118) (Lee *et al.*, 2004) were used to test cross - species amplification in *S. obtusa.* The locus that can unambiguously

distinguish all parent trees were selected and then used to genotype all the offspring and parents. Microsatellite loci and conditions study for *S. obtusa* were shown in Table 3.1.

Polymerase chain reaction (PCR) was conducted in a total volume of 10 µl. The PCR reaction contained 1 x PCR Buffer, 1.5 - 2.5 mM $MgCl_2$, 0.2 - 0.4 mM of each dNTP, 0.5 - 1 pmol of each primer, 10 - 25 ng of template DNA and 0.25 U/ µl - 0.5 U/ µl of *Taq* polymerase (Invitrogen, Sao Paulo, Brazil). Temperature profile as follows; 3 min at 94°C, followed by 35 cycles of 45s at 94°C, 30s at the 50-60 °C annealing temperature and 1.30 min at 72 °C, with a final extension at 72°C for 10 min. PCR reaction was carried out on a T - Gardient, Biometa (Germany). The PCR procucts along with a 50 bp ladder (Invitrogen) were run on 30 % polyacrylamide gel for 45 minute at 1,000 volts by Gel Scan 3000 (Corbetta Robotics, Australia). All microsatellite alleles were scored according to Gelscan program and Gene profiler version 4.05.

3.4 Data analysis

The data achieved from polyacrylamind gel electrophoresis were interpreted using population genetic software (see above). The bands obtained from polyacrylaminde gel were scored and genotyped for further statistic analysis as follow:

3.4.1 Genetic structure and genetic diversity

The genetic variation indexes including the percentage of polymorphic loci (*P*), average number of alleles per locus (*A*), the average effective number of alleles per locus (*Ne*), expected heterzygosity (*He*) and observed heterozygosity (*Ho*) were measured within each population using FSTAT 2.9.3 software (Goudet, 1995). The average number of alleles per locus (*N*) was calculated using the equation:

$$N = \frac{\sum n}{L}$$

where Σn: the total number of observed alleles at all loci;

L: the number of observed loci.

The effective number of allele (*Ne*) is estimation of the reciprocal of

homozygosity calculate using the equation according to Kimura and Crow (1964)

$$Ne = \frac{1}{\sum pi^2}$$

where *pi*: frequency of the *i*th allele for the studied locus

Allele frequencies are a measure of the relative frequency of an allele on a genetic locus in a population calculate using the following equation:

$$X_i = \frac{(2H_A + H_B)}{2N}$$

where *Xi*: the frequency of the ith allele

H_A: number of homozygous

H_B: number of heterogous

N: number of all samples

Heterozygosity is the state of having two different alleles of the same gene. Observed heterozygosity (*Ho*), were calculated according to the Nei (1972) using the equation:

$$Ho = \frac{\text{Number of heterozygous individual}}{\text{Total number of genotype per locus}}$$

Expected heterozygosity (*He*), were calculated according to the Nei (1973) using the equation:

$$H_e = \frac{1 - \sum pi^2}{L}$$

where *pi* : the frequency of the i^{th} allele at one locus

L: the total number of loci studies.

Compliance to Hardy-Weinberg equilibrium, linkage disequilibrium and number of "private alleles" (Slatkin, 1985), i.e. the number of allele not present in other

populations were test using Genepop web Version 3.4 (Raymond and Rousset, 1995). Population genetic structure was analyzed by means of Wright's *F*-statistics including *Fis*, the measurement of the heterozygous deficit within population, *Fst* is among populations and *Fit* the deficit of the heterozygous overall populations. Hardy-Weinberg equilibrium was tested using *Fis*. A significant positive *Fis* indicates deficit of the heterozygote and significant negative *Fis* indicated excess of the heterozygote. The *F*-statistics were calculated following the method of Weir and Cockerham (1984) and using FSTAT 2.9.3 software (Goudet, 1995).

Fis, *Fst* and *Fit* calculated using the following equations:

$$Fis = \frac{HT - HS}{HT}$$

$$Fit = \frac{HT - HO}{HT}$$

$$Fst = \frac{HS - HO}{HS}$$

where HO : observed heterozygosity in populations

HT : expected heterozygosity in populations

HT : expected heterozygosity in subpopulaitons

Mantel test (Mantel, 1967) was carried out to test the significance of isolation by distance pattern using Genepop web Version 3.4. Mantel test was calculated based on regressing pairwise population $Fst / (1 - Fst)$ values against the log-transformed geographic distance (km) between the respective pairs of populations (Rousset, 1997). The matrix of geographic distance was constructed by converting GPS coordinates of latitude and longitude to distance in kilometers between all pairs of populations.

Nei's standard genetic distance (Nei, 1972; 1978) was calculated to assess levels of genetic differentiation between populations. The genetic distance estimates, bootstrapping, unweighted pair group method using arithmetic averages (UPGMA) phylogram and consensus tree construction procedures were carried out using the

computer program TFPGA version 1.3; (Miller, 1997). Nei's standard genetic distance (*D*) was calculated using the equation:

$$D = -\ln\left[\frac{G_{xy}}{\sqrt{G_x G_y}}\right]$$

where G_x : the means of Σpi^2 all over loci

G_y : the means of Σqi^2 all over loci

G_{xy}: the means of $\Sigma piqi$ over all loci in the genome

Nei's (1978) unbiased estimate of genetic distance was calculated using the equation:

$$\hat{D} = -\ln\left[\frac{\hat{G}_{xy}}{\sqrt{\hat{G}_x \hat{G}_y}}\right]$$

where $\hat{G}_x$: the average of $(2n_xJ_x-1)/(2n_x-1)$ over the r loci studied

$\hat{G}_x$: the average of $(2n_yJ_y-1)/(2n_y-1)$ over the r loci studied

$\hat{G}_{xy} = J_{xy}$;

n_x: the number of individual sampled from population X;

n_y: the number of individual of individuals sampled from population Y;

J_x: the average of Σx_i^2 over the r loci studied;

J_y : the average of Σy_i^2 over the r loci studied;

J_{xy} : the average of $\Sigma x_i y_i$ over the r loci studied;

x_i : the corresponding sample allele frequency;

y_i :the corresponding sample allele frequency;

3.4.2 Genetic relatedness

The genetic diversity at the five SSRs loci of adult trees and seed population were quantified in term of the number of alleles per locus (*A*), allelic richness (*Ar*) (El-Mousadik and Petit 1996), the mean observed heterozygosity (*Ho*) and gene diversity (*H*) (Nei, 1987) using FSTAT 2.9.3 software (Goudet, 1995). Whether distortion from

Hardy-Weinberg equilibrium resulted from deficient or excessive heterozygosity was tested inbreeding coefficients, *Fis* (Weir and Cockerham, 1984) and their significance was tested by 1,000 permutation. All analyses were performed in Genepop web Version 3.4 (Raymond and Rousset, 1995).

The fine-scale genetic structure of all 29 potentially flowering trees was examined by calculating correlation between their genetic relatedness and spatial distance. Genetic relatedness values based on Queller and Goodnight (1989) were calculated for all pairs of 29 adult trees using the Relatedness software version 5.0.8 (http://www.gsoftnet.us/Gsolf.html.) (Goodnight, 2001).

Genetic relatedness (r_{xy}) calculated using the equation:

$$r_{xy} = \frac{\sum\sum\sum(P_y - P)}{\sum\sum\sum(P_x - P)}$$

where *P*: population frequency of the allele present at the current locus allelic position.

Px: frequency of the current allele in the current individual (i.e 0.5 or 1) depending on whether the individual is a heterozygous or homozygote.

Py: frequency of the current allele in the current individual's "partners".

The statistical test for the correlation between the genetic relatedness and spatial distance among all individual was performed using NTsys-pc version 2.1 computer program (Rohlf, 1990). In this study, the 5,000 randomizations were used for Mantel test (Mantel, 1967) which is a realistic minimum for estimating a significance level.

3.4.3 Paternity analysis

Paternity analysis is designed to make the time-consuming task of testing many candidate parents against many offspring a relatively straightforward task, with clearly interpretable results. For each offspring tested, parentage is either assigned to the most-likely candidate parent with a pre - determined level of confidence, or is left

unassigned. Parentage analysis was conducted by Marshall *et al.* (1998) formula. The equivalent average probability of excluding an unrelated individual from parentage given only the genotypes of the offspring is derived below.

For homozygous offspring *AA*, an exclusion occurs if the candidate parent is neither *AA* nor any of the *k*-1 heterozygotes *AX*. For heterozygous offspring *AB*, an exclusion occurs if the candidate parent is neither *AA*, *BB*, any of the *k*-1 heterozygote BX. The heterozygous candidate parent *AB* occurs both in the set of genotypes *AX* and the set of genotypes *BX*. Defining the probability of genotypes *AA*, *AB*, *AX* and *BX* as *p(ii)*, *p(ij)*, *p(ix)* and *p(jx)* respectively and summing across all pairwise genotype combination, the average probability of exclusion at a locus *l*, P_l, can be written:

$$P_l = 1 - \left\{ \sum_{i=1}^{k} \sum_{i=1}^{k} p(ii)p(ix) + \frac{1}{2} \sum_{i \neq 1}^{k} \sum_{x=1}^{k} p(ij)(p(ix) + p(jx) - p(ij)) \right\}$$

Substituting the expected frequencies of the relevant genotypes, assuming Hardy-Weinberg equilibrium, yields:

$$P_l = 1 - \left\{ \sum_{i=1}^{k} p_i^2 \left(\sum_{x=1}^{k} p_i p_x - p_i^2 \right) + \sum_{i \neq 1}^{k} p_i p_j \left(\sum_{x=1}^{k} 2p_i p_x - p_i^2 + \sum_{x=1}^{k} 2p_j p_x - p_j^2 - 2p_i p_j \right) \right\}$$

Where p_i is the frequency of allele *i* as above. Given that $\sum_{i=1}^{k} p_i = 1$,

This simplifies to:

$$P_l = 1 - \left\{ \sum_{i=1}^{k} p_i^2 . p_i(2 - pi) + \sum_{i \neq j}^{k} p_i p_j (p_i + p_j)(2 - p_i - p_j) \right\}$$

By writing

$$\sum_{i \neq 1}^{k} p_i p_j \text{ as } \sum_{i=1}^{k} p_i \left(\left(\sum_{j=1}^{k} p_j \right) - p_i \right)$$

and similarly for other power, P_l becomes:

$$P_l = a_1 - 4a_2 + 4a_3 - 3a_4 + 2a_2^2$$

Where

$$a_n = \sum_{i=1}^{k} pi^n$$

as above.

The overall average probability of exclusion across n independently inherited loci, P, may be calculated in the usual way

$$P = 1 - \prod_{i=1}^{n} [1 - p_i]$$

Paternity analysis of *S. obtusa* was performed by simple exclusion, based on multilocus genotypes for all candidates within the study site. This technique is most powerful when there are few candidate parents and highly polymorphic markers are available (Dow and Ashley 1996). Exclusion probabilities and null allele frequency of each locus in the WRBRI was calculated by CERVUS version 3.0.3 software (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007). Parentage analysis was also conducted by comparing alleles detected between offspring and adults using genotype data at five SSR loci. Alleles at every locus for each offspring were compared with those of adult trees, which did not share any alleles at each locus was excluded as candidate parents.

3.4.4 The mating system

The mating system of 179 offspring in the WRBRI was examined by the mixed mating system model, as implemented in MLTR version 3.23 (Multilocus Mating System Program) (Ritland, 2008). The MLTR estimates from progeny arrays the following inbreeding parameters; the multilocus population outcrossing rate (t_m), the single locus population outcrossing rate (t_s), the outcrossing rates among relative trees or the biparental inbreeding rate (t_m-t_s), the inbreeding coefficient of maternal parents (F) and the correlation of paternity or proportion of full sibs among outcrossed progeny(r_p). In addition, the number of pollen donor to each family, was estimated as $1/r_p$ (Ritland, 1989).

Ritland (2002) described estimation of plant mating systems by the 'mixed mating model. The probability model underlying multilocus estimation of mating system assumes n unlinked loci. In the mixed of mating model, progeny are either self

or randomly outcrossed. Outcrossing also occurs at random to a pollen pool at linkage equilibrium. Estimates of selfing rate are found by maximizing the likelihood of the data with respect to selfing rate (and pollen gene frequencies, if also estimated), using numerical methods or the expectation maximization (*EM*) method. Maternal parentage can be inferred by computing likelihoods of entire progeny arrays across possible maternal genotypes, and choosing the parent genotype giving the highest likelihood. Errors of estimates can be found with the bootstrap method, where entire progeny arrays are resampled.

Multiallelic probabilities are best represented by the Kronecker operator (δ). This operator allowed compact expression for estimators of pairwise relatedness. For mixed-mating probabilities, first consider a single-locus. The Kronecker operator is defined such that if two alleles A_i and A_j are the same (eg, the same band or sequence), then $\delta_{ij} = 1$, while if different, $\delta_{ij} = 0$. Now, for any progeny allele k, define as

$$D_k^{ij} = \left(\frac{\delta_{ik} + \delta_{jk}}{2} \right)$$

This is the probability that allele k is transmitted to the progeny, given parent A_iA_j, This expression will be the backbone of the formulae that follow.

The probability of an outcrosses progeny genotype is the arithmetic average of the probability of the two alternative paternal allele, k and l. As now $k \leq l$ always, the heterozygote probability is multiplied by 2, and any probability is multiplied by $(2 - \delta_{kl})$. The probabilities of progeny conditioned on selfing versus outcrossing, are thus

$$P_{kl}^{ij,s} = (2 - \delta_{kl}) D_k^{ij} D_l^{ij}$$

$$P_{kl}^{ij,t} = \tfrac{1}{2}(2 - \delta_{kl})(D_k^{ij} p_l + D_l^{ij} p_k)$$

Multilocus probability of offspring: If s is the rate of selfing, and $t = 1\text{-}s$ is the rate of outcrossing, the multilocus likelihood of an offspring is:

$$s \prod_{loci} P_{kl}^{ij,s} + t \prod_{loci} P_{kl}^{ij,t}$$

These probabilities are the incorporated into procedure for estimating selfing via progeny arrays.

Table 3.1 Microsatellite loci and conditions study for *Shorea obtusa*

Locus	Core sequence	Complexity	Type	Primer sequence (5' to 3')	Exp. Site (bp)
Shc01	$(CT)_8(CA)_{10}CT(CA)_4CTCA$	Compound	Imperfect	F:GCT ATT GGC AAG GAT GTT CA R:CTT ATG AGA TCA ATT TGA CAG	152
Shc04	$(CT)_{16}$	Simple	Perfect	F:ATG AGT AAC AAG TGA TGA G R:TAT TGA CGT GGA ATC TG	95
Shc07	$(CT)_8CA(CT)_5CACCC(CTCA)_3CT(CA)_{10}$	Compound	Imperfect	F:ATG TCC ATG TTT GAG TG R:CAT GGA CAT AAG TGG AG	169
Shc09	$(CT)_{12}$	Simple	Perfect	F:TTT CTG TAT CCG TGT GTT G R:GCG ATT AAG CGG ACC TCA G	197
Shc11	$(CT)_4TT(CT)_5$	Compound	Imperfect	F:ATC TGT TCT TCT ACA AGC C R:TTA GAA CTT GAG TCA GAT AC	166
Sle074a	$(CT)_{11}$	Simple	Perfect	F:ATC ACC AAG TAC CTA TCA TCA R:GCA ATGGCA CAC AGT CTA TC	124
Sle079	$(CT)_{11}$	Simple	Perfect	F:GTT GTC TGT TCT TAC CAG GAA G R:GCA TAA GTA TCG TCG CCA	166
Sle105	$(GA)_{12}$	Simple	Perfect	F:CTG TGT CAA AAT CAG TTA GGA CTT ACG AG R:GAG TCG ATT GCT TGT CTT CAC CC	145
Sle111a	$(GA)_{14}$	Simple	Perfect	F:GGA AAC TAC TGG AGC AGA GAC R:GGT GGG TAA TGG AGA ATG AG	153
Sle118	$(GA)_{16}$	Simple	Perfect	F:AAA GCG TAC AAA TTC ATC A R:CTA TTG GTT GGG TCA GAA GG	170

Primers developed for *Shorea curtisii* including Shc01. Shc07, Shc09 and Shc11 (Ujino *et al.*, 1998) and *Shorea leprosula* including Sle074a, Sle079, Sle105, Sle 111a and Sle118 (Lee *et al.*, 2004)

4. Literature Review

4.1 Morphology of *Shorea obtusa*

The genus *Shorea* belongs to the family Dipterocarpaceae *sensu strict* which consists of 19 genera and 470- 580 species (Maury-Lechon and Curtet, 1998). *Shorea* is the largest and most importance genus of Dipterocarpaceae. There are 188 species of the *Shorea*, of these, 21 species were found in Thailand. The genus has been divided into 10 sections; based on the appendage to the connective, anther forms and numbers of pollen sac. *Shorea obtusa* belong to the section *Shorea* with connective shorter than anther, barbate and with ovoid or conical into house glass-shape ovary. The synonym of *S. obtusa* is *S. leucobotrya*. This species have several local names; Teng is the general Thai name and Burmese sal, Siamese sal and Thiya are common name. Other local names including Ngae (north), Chik (northeast), Chan tok (southeast), Pra - chat (Surin), Pra - choek (Buri Ram), La - nai, Lae - noei and Ong - liang - young (Karen) (Pooma and Newman, 2001; Pooma, 2003; The Forest Herbarium Royal Forest Department, 2001).

Shorea obtusa is a medium deciduous forest tree; up to 30 m high but commonly with a height of 15 - 20 m. The outer bark (dead) is dark brown with irregular exfoliated scale, when cut, bark exudes a yellow resin. Buds, stipules, leaf beneath and panicle are cover with light brown tufted hairs. Leaves are simple, petiolate with entire or slightly undulate margin. The shape of the leave is oblong, 10 - 16 cm x 5 - 7 cm, apex usually obtuse. Domatia is pore-like. The venation is pinnate venation with secondary nerves 15 - 22 pairs. *S. obtusa* is a hermaphroditic species with a perfect white to yellow flower. The inflorescences are paniculate. Flower buds shape is elongate. Sepal arrangement is imbricate. Petal shape is linear lanceolate. Stamens are arranged in 1 - 3 whorls contiguous with petals. They are usually appendicular, with dorsifixed 2 - celled anther and 4 pollen sacs. The anthers are basifixed and oblong shape. Numbers of stamen are ca. 30. Ovary is superior, usually pubescent. Its shape is ovoid. The stylopodium can be constricted at middle. Style is very short. Fruiting calyx lobes developing into wing, 3 saccate calyx bases enclosing less than half of the nut, 3 longer lobes 4.5 - 5 x 0.7 - 0.8 cm, 2 shorter lobes ca. 3 - 3.5 x 0.2 cm. Nut is ovoid and

densely pale pubescent. Flowering time is January to July and fruiting is from January to May (Pooma, 2003). The photographs of *S. obtusa* are shown in Figure 2.1.

4.2 Distribution and ecology

Shorea obtusa is an Indo-Burmese species, widely distributed from Myanmar (type locality) to Indo-China except North Vietnam. In Thailand, it occurs throughout the country, except the southeast and not further south than Phetchaburi in the peninsula (Pooma, 2003).

Shorea obtusa is common species in dry DDF, occasionally with oaks and pines, up to 1,300 m in altitude. It is not a gregarious tree, but grows scattered and in association with other species including *Dipterocarpus obtusifolius* (Yang hiang), *D. tuberculatus* (Phluang), *D. intricatus* (Yang krat) and *S. siamensis* (Rang). There are key stone species in DDF. These species are xerophytes (deciduous) species adapted to living in a dry arid habitat (Maury-Lechon and Curtet, 1998; Pooma, 2003).

Shorea obtusa has also been found to be associated with mycorrhizas. They are predominantly ectomycorrhiza. The symbiosis with ectomycorrhiza improves the physiological and important for growth of Dipterocarps, especially in nutrient poor condition (Cao, 2006). This has led to several hypotheses regarding the role of microrrhizas might play in dipterocarp biology. Mycorrhizal associations of *Shorea* appear to be obligating symbiotic. Number of mycorrhiza species are vary among the *Shorea*; 23 species were found in *S. leprosula*, 26 species in *S. parvifolia*, 4 species in *S.curtisii*, 7 species in *S. obtusa*, 4 species in *S. siamensis*, 7 species in *S. roxburghii* and 12 species in *S. henryana* (Ingleby *et al.*, 1998; Shiva and Jantan, 1998; Tennakoon *et al.*, 2005; Chalermpong *et al.*, 2007).

4.3 Timber and non-timber products of *Shorea obtusa*

Most of the dominant trees in forests of Southeast Asia belong to the Dipterocarpaceae. In additionally to the role in ecological aspects, they are the major wood source (Gailing *et al.*, 2003). Tree species of this family are the main timber products in the tropical forest of Southeast Asia and other regions. More than 70% of

the world's demand for ply wood made from hardwoods has been supplied by Indonesia, principally from Dipterocarp species. In Malaysia, the chief export timbers are mainly produced by *Shorea* spp. and they are used chiefly for light construction and furniture. In Thailand, the timbers from *Dipterocarpus* spp. are better known and widely used in general construction and medium grade furniture (Cao, 2006). The wood of *S. obtusa* is widely preferable for more heavy constructional purposes and furniture making because of high quality hardwood.

The major non-timber products from dipterocarps are resins, dammar, camphor, butter fat and tannin which is of great importance and entire forest have been allocated to their exploitations, done generally by digging of a hole in the trunk. Non timber product use is to spread on fishing boats to make them water proofed and for making torches (Pooma, 2003; Cao, 2006).

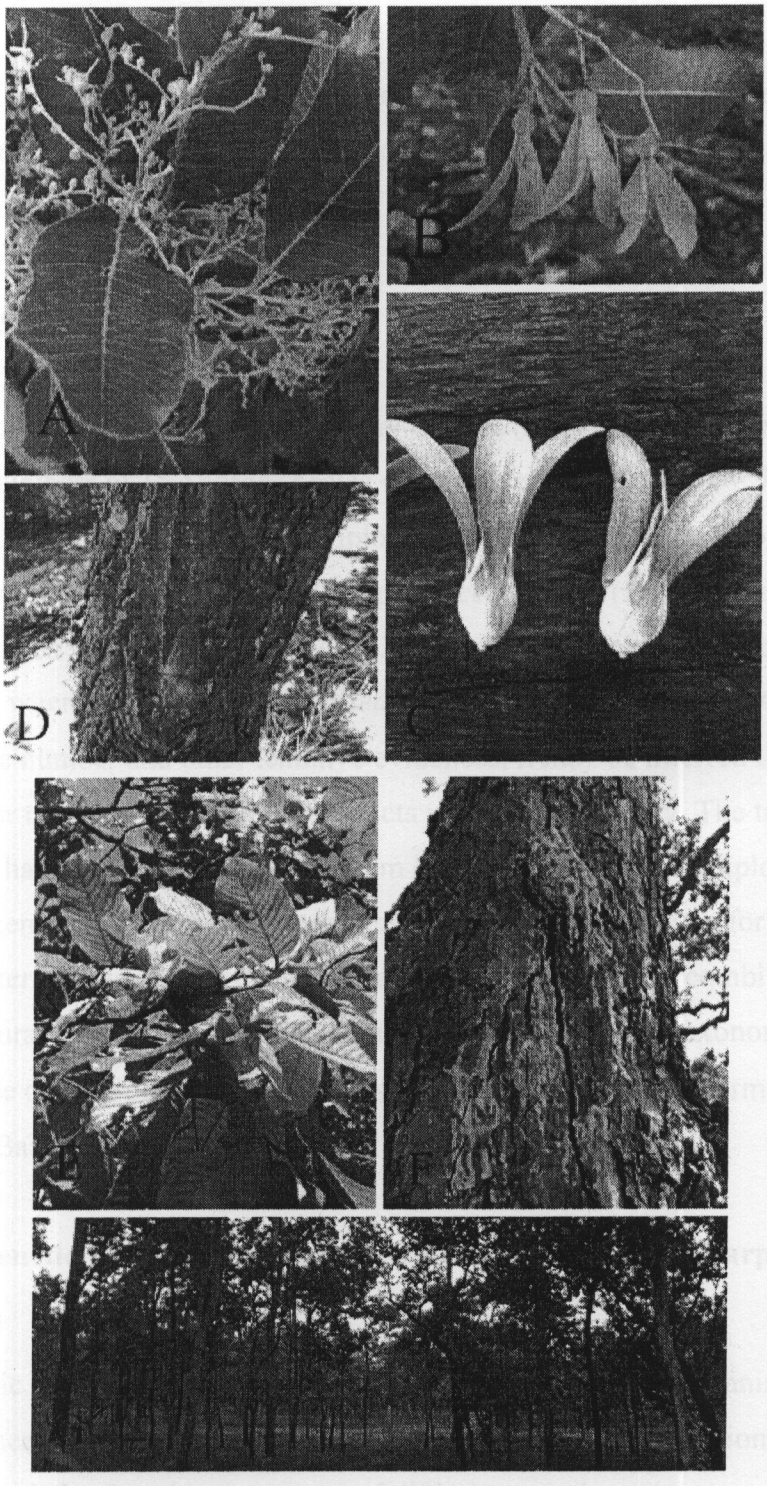

Figure 4.1 Photograph of *Shorea obtusa* A: flower hanging on a tree, B: fruit branch, C: dry fruit, D: bark, E: mature leave, F: bark with yellow resin and G: habitat in Chaiyaphum province

4.4 Conservation status

Shorea obtusa was assigned into the IUCN Red List of Threatened Species 2007 LR/lc ver 2.3 1994 based on IUCN Red List Categories and Criteria (The World Conservation Union [IUCN], 2007).

4.5 Cytogenetic study

Bawa (1998) and Missouri Botanical Garden (2007) have reported the chromosome numbers of 15 *Shorea* species which have 2n = 14 thus x = 7 as the basic chromosome number. Most *Shorea* are diploid except three species are polyploid including *S. ovalis* Bl (2n = 28), *S. ovalis* ssp. *sevicea* Ashton (2n = 21, ca.27, 28) and *S. resinosa* Foxw. (2n = 21). *Shorea ovalis* ssp. *sericea* is tetraploid with frequent polyembryony whereas *S. ovalis* ssp. *sevicea* and *S. resinosa* are triploid. On the basis of chromosome number and other tentative evidences, it may be inferred that all triploids or near triploids may also be apomicts with polyembryony. The triploid condition may have arisen in some cases from hybridization between diploid and tetraploid congeners. Agamospermy may indeed provide a mechanism for overcoming chromosome sterility, and/or for the stabilization of a heterozygous combination favored by natural selection. Apomictic plants are troublesome for taxonomists because of the multitude of biotypes or micro species that result from agamospermous reproduction (Bawa, 1998).

4.6 Genetic variation and genetic structure of the Dipterocarp species

Genetic variation is the fundamental requirement of living organisms to keep on their existence in heterogeneous and changing environmental condition. The genetic variation can be defined as the occurrence of different genetic variants populations of widespread species (Hamrick and Bawa, 1991). The highly fragmented population structure and self-compatible mating system may also have to contribute to the loss of genetic variation through inbreeding and genetic drift (Boys *et al.*, 2005).

The frequency distribution of genetic types is to compute parameters characterizing genetic variation within or among populations. The proportion of polymorphic loci (PPL), the average number of alleles per locus (A/L), and the expected heterogosity *He* or "gene diversity" are often reported as measures of genetic variation within populations parameters characterizing genetic variation within or among population. The most frequently used method to characterize differentiation among populations is the computation of *Fst* = *Gst* (Nei, 1973), which is the standardized among-population variance in allele frequency (Ouberg *et al.*, 1998; Finkeldey, 1994).

Several genetic markers including allozymes, amplified fragment length polymorphism (AFLP) and microsatellite have been used to assess genetic variation and genetic structure in Dipterocarp species. Chaisurisri *et al.* (2005) described the genetic structure and genetic diversity of some Dipterocarp species in Thailand including *Cotylelobium melanoxylon*, *Hopea odorata* and *Shorea siamensis* based on allozyme markers. They found that the expected heterozygosity (*He*) of *C. melanoxylon*, *H. odorata* and *S. siamensis* were 0.201, 0.172 and 0.030, respectively. In addition, level of genetic variation and genetic structure in a genetic resource area (GRA) of *C. melanoxylon* were assessed and compared to that of three natural populations. The GRA possess higher level of genetic variation than the three natural populations. Genetic differentiation among the three natural populations was low with *Fst* of 0.099. Genetic distance analysis indicated that the GRA was genetically distinct from the other populations. The mean genetic distance among the three natural populations was 0.044 this value has increased to 0.199 after the inclusion of the GRA in the analysis. Increase in genetic diversity among cohorts in the GRA, from seedling cohort (*He* = 0.1554) to mature cohort (*He* = 0.2655), indicated temporal genetic variation in GRA which might cause by loosely connection of temporal breeding web that ensure gene flow throughout a population, which is common to a large population. This study indicated that the establishment of GRA is an effective conservation method.

Lee *et al.* (2000) studied genetic diversity and population genetic structure of *S. leprosulu* using allozyme. Seven natural populations distributed throughout Peninsular Malaysia and one natural population from Borneo was selected. The mean population and species genetic diversity were high *(He* = 0.369 ± 0.025 and 0.406 ± 0.070, respectively). Heterozygosity varied among populations, ranging from 0.326 to 0.400,

with the highest values found in the populations from central Peninsular Malaysia. Correlations among ecological factors (longitude, latitude, and annual rainfall) were not significant ($p > 0.05$), indicating that these ecological variables were not responsible for the observed genetic differences among populations. The Bangi adult population exhibited a higher level of observed heterozygosity but lower fixation indices in comparison to its seedling population. All other seedling populations also showed positive fixation indices *(Fis)*, indicating a general excess of homozygotes. This also may suggest selection against homozygotes between the seedling and adult stages. A low level of population differentiation was detected *Gst*= 0.1 17 with the Lambir population and *Gst* = 0.085 without the Lambir population. Gene flow between populations was not significantly correlated with geographical distances for the populations within Peninsular Malaysia. Cluster analysis did not reflect geographical proximity and gave little insight into the genetic relatedness of the populations. This may indicate that the populations sampled are part of a continuous population with fragmentation having occurred in the recent past.

Changtragoon (2001) studied the genetic diversity of *Dipterocarpus alatus* from 16 natural forests of the Northern, Northeastern, Central and Southern parts of Thailand by isozyme markers. Thirteen isozyme systems were investigated in this species by horizontal starch gel electrophoresis. Eight putative isoenzyme gene loci were identified. The number of polymorphic loci varied from 12.5% to 75%. The average gene diversity and observed heterozygosity were 0.092 and 0.088, respectively. Partitioning genetic variation into within and among population components revealed that 12.8% of the total variation was attributable to differences among population (*Fst* = 0.128). These results suggest that natural population of *D. alatus* in Thailand show considerable levels of genetic divergence that useful for future conservation and management of this species.

Lim *et al.* (2001) studies the genetic structure of five natural populations of *Dryobalanops aromatic* in Peninsular Malaysia namely Lenggor and Ulu Sedili (Johore), Lesong (Pahang), Kanching (Selangor) and Bukit Sai (Terengganu), using SSRs with nine SSRs developed for *Shorea curtisii* and one developed for *D. lanceolata* were tested for cross-species specificity on *D. aromatic*. Seven SSRs can be used to assess genetic variation in *D. aromatic*. The expected mean genetic diversity (*He*) was

high (0.709) with values ranging from 0.684 (Lenggor) to 0.735 (Lesong). Most of the populations showed high and positive fixation indices, indicating an excess of homozygotes. This implies a high level of inbreeding that may be causes by selfing and/or mating between closely related individuals. A genetic diversity analysis showed that 93.3 % of the of the observed genetic diversity was contained within populations and 6.7 % (*Gst* = 0.067) was due to difference between populations. The smallest genetic distance (*D*) was between Bukit Sai and Lesong (0.068), and the largest Ulu Sedili and Lesong (0.477). Mean gene flow (*Nm*) was high (2.94), with the highest value found between Lenggor and Bukit Sai (-6.98). The lowest gene flow occurred between Kanching and Ulu Sedil (1.95). The findings from this study imply that the four natural populations of *D. aromatica* on the east coast of Peninsular Malaysia may have been a single unfragmented population in the recent past. The results of this study also support hypothesis that the Kanching population on the west coast of Peninsular Malaysia originated from the east coast populations. This is supported by the dendrogram derived from the UPGMA cluster analysis of genetic distance, in that the Kanching population is closely related to Lesong and Bukit Sai populations from the east coast.

Konishi *et al.* (2004) studied degrees of genetic variation accumulated in four dipterocarp species in Lambir Hills National Park, Sarawak, Malaysia and compared with those in a man-made forest in Bakam Experimental Reserve using SSRs and AFLP markers. By using 2 to 3 microsatellite loci, 9 to 14 alleles were detected. The average observed heterozygosities for *Dryobalanops lanceolata*, *D. aromatica*, *Shorea beccariana* and *S. macrophylla* in the natural population were 0.400, 0.400, 0.580 and 0.516, respectively, and expected heterozygosities were 0.603, 0.461, 0.685 and 0.745, respectively. In all of the four species, excess homozygotes were observed. Significant deviation from the Hardy-Weinberg equilibrium was detected in *D. lanceolata* and *S. beccariana*. AFLP analysis in the natural population revealed that 94.6% to 99.7% of the fragments were polymorphic. The average heterozygosities for these species were 0.220, 0.232, 0.221 and 0.211, respectively. These values are much larger than those obtained in tree populations such as Japanese beech and Japanese oak populations in temperate deciduous broad leaf forests. Genetic variation in the artificial populations was compared with that in the natural populations. Both the average expected

heterozygosities and the number of alleles in the microsatellite loci were reduced in the artificial populations. Some distortions such as linkage disequilibrium and the deviation from the Hardy-Weinberg equilibrium appeared in the artificial population of *S. beccariana*. The use of seeds collected from a limited number of mother trees to make the experimental site is thought to be the cause of the loss of genetic variation.

Ng *et al.* (2004) studied the spatial distribution pattern, spatial genetic structure and of genetic diversity were carried out in two tropical tree species with contrasting breeding systems and different ploidy levels using a 50-ha demographic plot in a lowland dipterocarp forest in Peninsular Malaysia. *S. leprosula* is a diploid and predominantly outcrossed species, whereas *S. ovalis* ssp. *sericea* is an autotetraploid species with apomictic mode of reproduction. Genetic diversity parameters estimated for *S. leprosula* using microsatellite were consistently higher than using allozyme. Level of genetic diversity of *S. leprosula* in three diameter classes (BIG, MED and SMA) based on seven SSRs were 0.70, 0.71 and 0.69, respectively with mean of *He* = 0.70 and *S. ovalis* ssp. *sericea* were 0.67, 0.64 and 0.62 respectively with mean of *He* = 0.64. Whereas *He* of *S. leprosula* based on allozyme were 0.49, 0.51 and 0.48 respectively with mean of *He* = 0.49. In comparisons with *S. leprosula* and other tropical tree species, *S. ovalis* ssp. *sericea* also displayed relatively high levels of genetic diversity. This might be explained by the lower pressure of genetic drift due to tetrasomic inheritance, and for autotetraploids each locus can accommodate up to four different alleles and this allows maintenance of more alleles at individual loci. The observed high levels of genetic diversity in *S. ovalis* ssp. *sericea* can also be due to a random retention of more heterogeneous individuals in the past, and the apomictic mode of reproduction might be an evolutionary strategy, which allows the species to maintain high levels of genetic diversity. The spatial distribution pattern analyses of both species showed significant levels of aggregation at small and medium but random distribution at the big diameter-class. The decrease in magnitude of spatial aggregation from small to large-diameter classes might be due to compensatory mortality during recruitment and survival under competitive thinning process. Spatial genetic structure analyses for both species revealed significant spatial genetic structure for short distances in all the three diameter classes. The magnitude of spatial genetic structure in both species was observed to be decreasing from smaller to larger diameter classes. The high spatial

genetic structuring observed in *S. ovalis* ssp. *sericea* at the small diameter class is due primarily to limited seed dispersal and apomictic mode of reproduction. The similar observation in *S. leprosula*, however, can be explained by limited seed and pollen dispersal, which supports further the fact that the species is pollinated by weak fliers, mainly of Thrips and Megalurothrips in the lowland dipterocarp forest.

Cao *et al.* (2006) studied the genetic diversity within and among population of *S. leprosula* and *S. parvifolia* from Indonesia. Genetic variation was assessed by AFLP markers in 12 natural populations (six for each species) from Sumatra and Kalimantan (Borneo), and for *S. leprosula* in addition in one plantation from Java. Levels of genetic variation (*He*) were estimated based on the assumption of Hardy-Weinberg structures in both populations. Variation is higher for *S. leprosula* in comparison with *S. parvifolia*. At the population level, a higher level of genetic diversity was revealed for *S. leprosula* with a percentage of polymorphic loci (PPL) of 53.32 % and expected heterozygosity (*He*) of 0.16, in comparison with *S. parvifolia* showing PPL of 51.79 % and *He* of 0.14. At this species level, *S. leprosula* showing PPL of 92.86 % and *He* of 0.21, while *S. parvifolia* showing PPL of 85.71 % and *He* of 0.21. Low variation was observed in both *S. parvifolia* populations sampled in Borneo. The population Asialog Sumatra showed the highest variation for both species. Variation levels measured as *He* decreased in the same order for the five locations where both species were sampled (Asialog Sumatra, Pasir Mayang Sumatra, Batu Ampar Borneo, Sari Bumi Kusuma Borneo and Nanjak Makmur Sumatra). Considerable differentiation was not only observed among species, but also among populations. The proportion of the total genetic variation within species due to differentiation among populations is high for *S. leprosula* (*G*st = 0.20) and even higher for *S. parvifolia* (*G*st = 0.31) indicated that 25 and 31 % of total genetic diversity in *S. leprosula* and *S. parvifolia*, respectively, were attributed to the differences among populations. Populations of *S. leprosula* from Borneo are clearly separated from populations of the same species from Sumatra. The cluster diagram suggests the origin of the planted population (Haurbentes, Java) as Sumatra. Conservation of genetic resources of Dipterocarps needs to take into account the strong genetic differentiation among populations at presumably neutral AFLP loci. The presence of numerous diagnostic characters occurring in high frequency in only one or a few populations suggests that conservation in as many populations as possible is important. The

identification of centers of genetic diversity of Dipterocarps seems feasible since ordering of populations with regard to their variation (*He*) revealed the same trend for both species, with a single location (Asialog) clearly showing the highest diversity for *S. leprosula* and *S. parvifolia.*

The determinant of population genetic structure and genetic diversity within population reported in *Shorea* spp. and other Dipterocarp plants using several different genetic markers were shown in Table 2.1.

Table 4.1 The level of genetic variation and genetic differentiation in Dipterocarp species

Species	Marker	*He*	*Fst/GSt*	Reference
Cotylelobrium melanoxylon	Isozyme	0.201	0.099	Chaisurisri *et al.* (2005)
Dipterocarp alatus	Isozyme	0.092	0.128	Changtragoon (2001)
Dryobalanops aromatica	SSRs	0.709	0.067	Lim *et al.* (2001)
	SSRs	0.461	-	Konishi *et al.* (2004)
Dryobalanops lanceolata	SSRs	0.603	-	Konishi *et al.* (2004)
Hopea odorata	Isozyme	0.172	-	Chaisurisri *et al.* (2005)
Shorea beccariana	SSRs	0.685	-	Konishi *et al.* (2004)
Shorea leprosula	SSRs	0.70	-	Ng *et al.* (2004)
	Isozyme	0.49	-	Ng *et al.* (2004)
	SSRs	0.369-0.406	0.085,	Lee *et al.* (2000a)
	AFLP	0.16	0.25	Cao *et al.* (2006)
Shorea macrophylla	SSRs	0.745	-	Konishi *et al.* (2004)
Shorea siamensis	Isozyme	0.030	-	Chaisurisri *et al.* (2005)
Shorea parvifolia	AFLP	0.14	0.31	Cao *et al.* (2006)
Shorea ovalis ssp. *sericea*	SSRs	0.64	-	Ng *et al.* (2004)

4.7 Plant mating systems

Mating systems is one of the primary determinants of the pattern of genetic variability in natural populations (Hamrick, 1982; Bisby, 1995; Finkeldey, 1999). Knowledge of mating systems is useful for forest conservation, tree breeding and targeted seed collection for environmental reforestation strategies (Bittencourt and Sebbenn, 2007). Study of plant mating systems has been dominated by comparisons of the relative frequency of selfing and outcrossing (Barrett and Harder, 1996).

Outcrossing combined with extensive movement of pollen and seed can lead to a high degree of genetic variation within populations but reduce differentiation among populations. Selfing and limited mobility of pollen and seed can have the opposite effect of reducing variation within, but promoting differentiation among population (Bawa, 1998). Selfing rates increase when effective population size is small or plant density is low. The outcrossing rate, defined as the proportion of outcrossed progeny produced by an individual mother or population, is the most localized form of within-population pollen gene movement (Sork *et al.*, 1999). Moreover, outcrossing rate (t_m) can be quantified between zero and one; zero representing complete selfing and one indicating 100 % outcrossing.

Kitamura *et al.* (1994) compared outcrossing rates of *Dryobalanops aromatic* in primary and secondary forest but found no significant differences. The rate of outcrossing ranged from 0.794±0.059 to 0.856±0.059. Murawski and Bawa (1994) studied mating system of an endemic Sri Lankan Dipterocarp, *Stemonoporus oblonggifolius*, was examined using allozyme analyses. The multilocus outcrossing rate was 0.898±0.022, with evidence for apomixes in one tree. Other factors potentially contributing to the high observed allozyme diversity in this species would be large effective population sizes, introgression of genes from sympatric species.

Murawski *et al.* (1994) studies outcrossing rated of two endemic *Shorea* species from Sri Lankan, based on allozyme markers. The rate of outcrossing of *S. congestiflora* and *S. trapezifolia* were 0.874±0.021 to 0.617±0.033, respectively. A high rate of outcrossing of *S. congestiflora* (t_m = 0.874) suggest that self-incompatible may be variable among individual. *S. trapezifolia* exhibited a mixed-mating strategy with only 54 and 62 percent of seed resulting from outcrossing in two succeeding years.

Differences between the outcrossing rates of the two species may reflect average differences in the degree of self-incompatibility.

Murawski *et al.* (1994) studied mating system of *S. megistophylla*, an endemic canopy tree from Sri Lanka was quantified by allozyme analysis of progeny arrays using a mixed mating model. Two adjacent populations were compared, one in forest that was selectively logged about 20 years ago, and the other in undisturbed primary forest. The selectively logged population had a lower multilocus outcrossing rate (t_m = 0. 71) compared to that of the undisturbed forest (t_m = 0.87). Only the progeny from the logged population showed evidence of either biparental inbreeding or Walund effect and the genotypic frequencies violated the assumptions of the mixed-mating model. Apomixis was detected in one isolated tree in the Royal Botanical Garden in Peradeniya by a multilocus test of progeny genotypic frequencies relative to the maternal genotype. However, significant levels of apomixes were not discerned in the natural (logged and unlogged) population. These findings indicate that a reduction in population density of *S. megistophylla* following selective logging can significantly elevate the proportion of seeds produced through inbreeding. Adventives embryonic may also increase in isolated trees that lack the chance to outcross.

Lee (2000) studied mating system of *Dryobalanops aromatic* in three different forest types and a seed orchard was quantified by allozyme analysis of progeny arrays using a mixed mating model. The primary forest (Bukit Sai) had the highest multilocus outcrossing rate (t_m = 0.923 ±0.035), followed by logged forest (Lesong; t_m = 0.766±0.056) and artificial forest (FRIM; t_m = 0.661±0.066) with seed orchard showing the lowest (Tampin; t_m = 0.551±0.095). A high rate of outcrossing in primary forest (t_m = 0.92) may indicated that the species is self-incompatible, but a lower value in the seed orchard (t_m = 0.55) might suggest further that the self-incompatibility system is weak. The outcrossing rate was greater in the primary forest (t_m = 0.92) than in loged forest (t_m = 0.77). It is argued that this might be a consequence of the lower density of flowering trees and alteration of pollinatior foraging behavior in logged forest. Higher values of correlated mating (r_p) and biparental mating (t_m-t_s) in primary forest (0.08 and 0.39, respectively) in comparison with logged rates of outcrossing in artificial forest (t_m = 0.67) and seed orchard (t_m = 0.55) might be attributed to lack of flowering synchrony and insufficient number of pollinators. The high level of correlated mating (r_p = 0.43)

and biparental mating (t_m-t_s = 0.12) in the seed orchard may further suggest that the seed orchard was established using related seed source.

Lee *et al.* (2000) studied mating system in a natural population of *S. leprosula* from Malaysian lowland dipterocarp forest was quantified by allozyme analysis using the multi-locus mixed-mating model. The population was found to be predominantly outcrossed (t_m = 0.837 ±0.066). Variation in individual multi-locus outcrossing rates (range = 0.55-1.00) may have reflected variation in individual self-compatibility, heterogeneity in the pollen pool, differences in the mating neighborhood of individuals, or population substructure. Departure from the mixed-mating model was evident from the differences in pollen and ovule allele frequencies. Pollen pool heterogeneity and significant levels of biparental mating (t_m - t_s = 0.127, p < 0.01) indicated that the population was most probably genetically sub structured. The inbreeding coefficient based on Wright's fixation index for maternal trees (F_a = -0.078) was lower than that obtained for progenies (F = 0.089), suggesting considerable selection against selfed progenies during growth of seedlings to reproductive size. Incidence of multiple seedlings was observed in a small proportion of seeds, but genetic analysis and genotype comparisons showed that this was not due to apomixis and may have been caused by multiple fertilization.

Chaisurisri *et al.* (2005) described genetic diversity and mating system in Huay Kha Khaeng Wildlife Sanctuary, Thailand, according to the level of disturbance for *Shorea siamensis* using allozyme marker. They found that outcrossing rate (t_m) were ranging from 0.852 - 0.909 with mean of t_m = 0.918±0.046. The study indicated the potential application of genetic diversity index to monitor forest degradation as well as potential to monitor level of forest ecosystem service in order to maintain their adaptive potential and recoverable potential of the population.

Mating system of several Dipterocarp species were reported in Table 2.2. Analysis of mating system shows high rates of outcrossing. It is also clear that there is a considerable potential for selfing in almost all species examined so far.

Table 4.2 Outcrossing rates of Dipterocarp species

Species	Outcrossing Rate (tm) (± standard error)	References
Dryobalanops aromatica	0.794±0.059	Kitamura *et al.* (1994)
	- 0.856±0.063	
	0.923±0.035	Lee (2000)
Shorea congestiflora	0.874±0.021	Murawski *et al.* (1994)
Shorea megistophylla	0.713±0.025	Murawski *et al.* (1994)
	- 0.866 ±0.058	
Shorea leprosula	0.837±0.066	Lee *et al.* (2000)
Shorea trapezifolia	0.617±0.033	Murawski *et al.* (1994)
Shorea siamensis	0.918±0.046	Chaisurisri *et al.* (2005)
Stemonoporus oblongifolius	0.898±0.022	Murawski and Bawa (1994)

Leiengsiri *et al.* (1998) suggested that the differences in outcrossing rated (t_m = 0.719-0.959) among 11 population of *Pterocarpus macrocarpus* (Fabaceae) were attributable to the degree of habitat disturbance and the density and distribution of flowereing trees. Similary, in *Senna multiuga* (Fabaceae) which is pioneer species in a neotropical forest, the Brazilian Atlantic. The outcrossing of the two populations was significantly different, one located in a reserve area (RD1) was t_m of 0.540 and the other (RD2) about 15 km away was t_m of 0.838. Although, RD2 population of S. *multiuga* is located in a more disturbed area than is RD1 population. The density of these populations was not measured. Thus, the degree of environment disturbance probably did not affect in mating system of this species. This variation may be an important adaptation to ensure reproduction in different environmental conditions (Ribeiro and Lovato, 2004). Whereas, recent studied in gene flow pattern and mating system in *Quercus semiserrata* (Fagaceae) in Khun Wang Royal Agriculture Research Center,

Thailand, show high outcrossing rates (t_m = 0.995), high level of gene flow from other population and heterogeneity in the pollen received by an individual may be sufficient to prevent loss of genetic diversity through genetic drift of *Q. semiserrata* (Pakkad *et al.*, 2008). In addition, the estimated of biparental inbreeding (t_m-t_s) of oaks in natural population such as *Q. semiserrata* (t_m-t_s = 0.013) (Pakkad *et al.*, 2008), *Q. humboldtii* (t_m-t_s = 0.033) (Fernandez-Manjarres and Sork , 2005) and *Q. velutina* (t_m-t_s = 0.022) (Fernandez- Manijarres *et al.*, 2006) , suggest that level of biparental inbreeding are generally low in fragmented population and probably the mating system of these species is resilient to reductions in population size and support the hypothesis that oaks are virtually complete outcrossers.

Gene flow is the transport of genetic information via pollen and seed. Thus gene flow is the force which is responsible for the distribution of genetic information within populations (intrapopulation gene flow) and among populations (interpopulation gene flow). The efficiency of gene flow by pollen and seeds is of fundamental importance with regard to the reproduction-effective population size. Population size is importance for spatial pattern of genetic variation and genetic differentiation between populations. Gene flow among population counteracts process of genetic differentiation due to selection and drift among populations (Gailing *et al.*, 2003).

Hamilton's rule is the concept of inclusive fitness was developed to provide a framework for understanding the evolution of behaviors that affect the finesses of individual other than the performer of the behavior. The level of genetic relatedness among pairs of individual *r* is defined as the expected proportion of allele that is identical -by-descent that is shared among those individual. The *r* in Hamilton's rule can be expressed as a genetic correlation or regression coefficient (Queller and Goodnight, 1989).

Paternity analysis is one of the most direct and reliable method for assessing contemporary, pollen-mediated gene flow in plant (Sork *et al.*, 1999), because of their critical role in determining the genetic structure of population, transmitting diversity across generation, and influencing the rate and direction of microevolutionary (Wang *et al.*, 2007). The estimation of pollen dispersal is provided by paternity analysis, which relies on a sampling of mother plants, along with a sample of their offspring, as well as an enumeration and genetic characterization of the surrounding males. Paternity

analysis method attempt to detect (for each offspring) whether paternity of the seed can be attributed. Then, for the subset of offspring for which a credible on site father has been found, the position of the mother and the father has been found, the position of the mother and the father can be used to estimate the parameter of the pollen dispersal function chosen (Marshall *et al.*, 1998; Austerlitz *et al.*, 2004; Pakkad *et al.*, 2008).

Highly variable molecular markers, especially SSRs, have facilitated a direct genetic approach to measuring gene flow, based on parental analyses (Ouborg *et al.*, 1999) and relatedness can be calculated within colonies. The SSRs approach also allows the determination of parentage by exclusion analysis, especially the determination of paternity if maternity is already know. Estimate of genetic relatedness and paternity have been obtained in several species. Sato *et al.* (2006) reported parentage analysis of seeding within a 20-ha study site in *Cercidiphyllum japonicum*. They found that 28.8 % of seeding was fertilized by pollen from trees outside the study site. The average pollination distance within the study site was 129 m, with a maximum of 666 m. Thus, long-distance dispersal is common in this species. Ratnam and Seng (2003) studied genetic relatedness of selected mother trees of *S. leprosula* (24 trees) and *Dipterocarpus cornutus* (10 trees) was investigated using four SSRs loci in two seed production areas in a 1 ha seed stand of *S. leprosula* and a 0.9 ha seed stand of *D. cornutus* in Compartment 17, Labis Forest Reserve, Segamat, Johor. A total of 24 and 32 saplings in the vicinity of selected mother trees of *S. leprosula* and *D. cornutus*, respectively was collected for parentage analysis. The results revealed that extensive gene flow occurred in *S. leprosula* and *D. cornutus*. Based on SSR polymorphisms, four mother trees of *S. leprosula* (i.e. SM1, SM9, SM15 and SM21) and three mother trees of *D. cornutus* (i.e. DM2 or DM3, DM5 and DM8), are not closely related and therefore could be used as potential seed sources for an advanced breeding program. The mean genetic identity of the *S. leprosula* and *D. cornutus* mother trees was low (0.471 and 0.557, respectively). Low spatial genetic structure within the population of mother trees was detected in *S. leprosula* and *D. cornutus*. This implies that extensive gene flow occurred in these species within the seed production areas. This is validated in the study for *D. cornutus* where only about 13.3% of alleles detected in saplings seemed to have originated from adult trees outside the forest seed production area (SPA).

Patterns and level of gene flow via pollen and seed dispersal are one of the most critical determinants in the establishment of genetic structure (Hamrick and Nason, 1996). The level of gene flow thus contributes substantially to understanding the genetic structure of plant populations. However, few studies have analyzed pollen and seed movements at local scale. The studies of fine-scale genetic structure in plants have detected significant negative relationships between genetic relatedness and spatial distance which are often explain by limited seed dispersal (Berg and Hamrick, 1995). For example a, *S. leprosula* and *Dipterocarpus cornutus* (Ratnam and Seng, 2003), *Hopea dryobalanoides* and *S. parvifolia.* All of these plant wind dispersed and their flowers pollinated by insects (Takeuchi *et al.*, 2004). However, the correlation between genetic relatedness and spatial distance among adult tree was not significant, indicating an absence of fine-scale genetic structure in *S. acuminata* (Takeuchi *et al.*, 2004) and *Cercidiphyllum japonicum* (Sato *et al.*, 2006).

In flowering plants, pollen transportations could occur by wind and animals. Dipterocarpaceae have bisexual flowers which are pollinated by a variety of animal vectors. Some species growing in swamps or on river banks have fruits with short sepals and may be dispersed by water (Ashton, 1982; Bawa, 1998). In Dipterocarps species, the potential for inbreeding is increased by the fact that self-incompatibility barriers are not strong (Bawa, 1998). The self-incompatibility system in several species is apparently weak for instance, *S. siamensis*, decrease in density due to selective logging caused an increase in selfing rate because pollen flows become restricted (Ghazoul *et al.*, 1998). In *S. megistophylla*, the rates of inbreeding are higher for trees from logged stand than for tree in unlogged stand (Bawa, 1998) and a reduction in population density of *S. megistophylla* following selective logging can significantly elevate the proportion of seeds produced through inbreeding (Murawski *et al.*, 1994).

Fukue *et al.* (2007) studied effect of flowering tree density on the mating system and gene flow in *S. leprosula* in Peninsular Malaysia, which has declined in recent decades and become fragmented due to human activities. The results show that gene diversity and allelic richness were not significantly correlated to the mature tree density. However, the number of rare alleles among the seedlings and the selfing rates of the mother trees were negatively correlated with the density of the adult trees. Furthermore, in a population with high mature tree density pollination distances were

frequently less than 200 m, but in populations with low adult tree density the distances were longer. These findings suggest that the density of flowering trees affects selfing rates, gene flow and, thus, the genetic diversity of *S. leprosula* populations. They also found that an individual *S. leprosula* tree with a unique reproductive system, probably apomictic, mating system.

Apomixis or asexual reproduction through seeds, which is the plant embryos grow from egg cells without being fertilized by pollen. Apomixis is produces progeny that are clones of the mother, a feature allowing the fixation of any favorable genetic combination (Perotti *et al.*, 2006). Apomixis has been reported in many Dipterocarp species such as *S. megistophylla* (Murawski *et al.*, 1994), *S. macroptera*, *S. ovalis* ssp. *sericea* and *Hopea glabra*. Two models have been proposed concerning the role of polyploidy in apomixes; the ploidy regulation model and the genome asynchrony model. The ploidy regulation model postulates that the expression of genes involved in sexual reproduction is dosage dependent. In support of this model, several studies in maize, *Arabiodopsis* and wheat have suggested that the expression of a number of genes is diminished or activated following polyploidization. However, apomixes may result from the hybridization between distinct albeit related species. Asynchronous miss expression of the parent genes in the hybrid could then lead to all the components of apomixes. This model is supported by data demonstrating apomictic components in allopolyploids produced from distinct sexual parents. In addition, some dipterocarp species with apomictic reproductive system are tetraploids, such as *S. ovalis* ssp. *sericea*. In contrast, *S. leprosula* is a diploid species, but hybrid individual are occasionally observed in the forest, which may have been produced apomictically (Perotti *et al.*, 2006; Fukue *et al.*, 2007).

On the basis of controlled pollinations, most Dipterocarps appear to be strongly cross-pollinated. Outcrossing is the usual mode of reproduction in tropical forest trees (Bawa, 1998). Genetic connective in population of plants is determined by gene movements among them. Gene flow in forest tree involves both pollen and seed. Male gametes are dispersed from the paternal to the maternal parent via pollen. Patterns of pollen and seed dispersal greatly influence the genetic structure and effective size of plant population. Habitat fragmentation reduces area of continuous forest to small, separate remnants and may decrease the effective population size and possibly disrupt

the mating system and interrupt gene flow (Bittencourt and Sebbenn, 2007). Long distance of pollen movement has also been reported for many low density and insect-pollinated tropical trees in natural or semi natural habitat. Chase *et al.* (1996) reported a mean pollen dispersal distance of 450 m in an emergent tropical tree *Pithecellobium elegans*, which is pollinated by a know long-distance pollinator hawkmoth. Similar pollen dispersal distance have been found in tropical tree, *Platypodium elegans* (Nason *et al.*, 1998), and *Neobalanocarpus heimii* (Konuma *et al.*, 2000) and *Eurycorymbus cavaleriei* (Wang *et al.*, 2007), with average effective distance of pollen dispersal being 525, 524 and 292.6 m, respectively. Most of these species are pollinated by bee. By using SSRs marker, longer distances of insect pollinations have been reported for several tropical trees (e.g. *Swietenia humilis*, > 4.5 km, White *et al.*, 2002; *Dinizia excels*, mean 1,509 m for fragment landscape, Dick *et al.*, 2003; *Dicorynia guianensis*, >1,000 m, Latouche-Halle *et al.*, 2004). Long-distance dispersal is biologically important for plant because they affect colonization probabilities, the probability of population persistence in a fragmented habitat, and meta - population structure.

Smouse and Sork (2004) have been reviewed the effective number of pollen donor (*Nep*) parameter with species that have wind-vectored and animal-vectored pollinations according to MLTR, Paternity and Twogener model. The results revealed that *Nep* with species that have wind-vectored pollination range from 1 to 100 (e.g. *Larix occidentalis*, *Nep* = 50 - 100, *Picea abies*, *Nep* = 33 - 46, *Pinus echinata*, *Nep* = 3 - 6, *Quercus lobata*, *Nep* = 3 - 4 and *Q. humboldtii*, *Nep* = 1 - 2. Species that have animal-vectored pollination range from 2 to 33 (e.g. *Acacia melaxylon*, *Nep* = 16-33, *Albizia julibrissin*, *Nep* = 3, *Carapa pocera*, *Nep* = 12, *Cornus florida*, *Nep* = 5-11, *Enterolobium cyclocarpum*, *Nep* = 2-4 and *Ficus* spp., *Nep* = 3-11. According to the data, they discern three trends. First, that some wind - pollinated species show much higher values of *Nep* than do animal pollinated species, suggesting that *Nep* is inherently smaller in animal - pollinated than in wind - pollinated species, in spite of the fact that the pollinators can be shown to move considerable distance. While wind - dispersed pollen can move great distance, given open-stand aerodynamic conditions, *Nep* tends to be smaller under closed canopy condition, everything else being equal. In general, *Nep* is inversely correlated with the level of pollen flow across the local landscape, with all of the consequences that usually follow from genetic drift, and the

data suggest that pollen flow is more affected by the landscape context than by the pollination system. Second, *Nep* increase with conspecific density, everything else being equal. With fewer near neighbors, the number of effective contributors is higher, notwithstanding the fact that the most frequent contributors remain the near neighbors. Third, thinning the canopy (of other species) seems to increase the distance of pollination and in some cases increases the effective number of fathers.

4.8 Microsatellite DNA markers

Microsatellite are region within DNA sequence (loci) that contain multiple repeats of short sequence of DNA (nucleotides: adenine - A, thiamine - T, guanine - G, cytosine - C) of 1 - 6 nucleotides found at high frequency in the nuclear genomes of most eukaryote. They are also known as simple sequence repeats (SSRs), variable number tandem repeats (VNTR) and short tandem repeats (STRs) (Selkoe and Toonen, 2006).

A SSRs locus typically varies in length between 5 and 40 repeats, but longer strings of repeats are possible. Dinucleotide, trinucleotide and tetranucleotide repeats are the most common choices for molecular genetic studies. Dinucleotide repeats account for the majority of SSRs for many species. Trinucleotide and hexanucleotide repeats are the most likely repeat classes to appear in coding regions because they do not cause a frame shift. Mononucleotide repeats are less reliable because of problems with amplification. In plant the most common SSRs motifs are AT and CT, while in mammals GT is prevalent (Lagercrantz *et al.*, 1993). Two potential mechanisms can be explaining the high mutation rates of SSRs. The first is recombination between DNA molecules by unequal crossing - over or by gene conversion. The second mechanism involves slipped - strand mispairing during DNA replication (Vendramin *et al.*, 2004).

Originally SSRs marker was used for genetic mapping and as a diagnostic tool to detect human disease. In forest tree, this marker was used in population and ecological applications as genetic mapping, pedigree analysis, and the investigation of the genetic structure of populations and also for the study of mating system and gene flow, effective population size, migration and dispersal processes and parentage and relatedness (Dow and Ashley, 1996; Selkoe and Toonen, 2006). Molecular markers

have allowed us to understand how various evolutionary forces (e.g., drift, selection, recombination, mutation and gene flow) influence the patterns of genetic diversity in natural populations, and therefore help us develop suitable conservation and improvement strategies (Liewlaksaneeyanawin, 2006; Selkoe and Toonen, 2006). Microsatellite or SSRs have emerged as the most popular marker in many studies because of their high variability, ease and reliability of scoring and codominat inheritance (Vendramin *et al.*, 2004).

The DNA surrounding a SSRs locus is termed the flanking region (Selkoe and Toonen, 2006). Because the sequences of flanking regions are generally conserved (i.e. show low variation within species and/or closely related species) primer can be designed that allow the amplification of the SSRs locus by PCR (Polymerase Chain Reaction) (Selkoe and Toonen, 2006). This method of sequence tagged SSRs is widely used in plant and animal species. Since primer is designed in conserved region, they may also be transferred to other species of the same genus (Gailing *et al.*, 2003).

Nuclear SSRs developed for a particular species can be transferred to closely related species (cross-amplification), usually within the same genus. (Ritland and Ritland, 2000; Finkeldey and Hattemer, 2007) for instance *Shorea* spp., SSRs for *S. curtisii* can be applied to 30 species in Dipterocarpaceae (Ujino *et al.*, 1998), 20 SSRs of *S. leprosula* were developed for this species and were also characterized for applicability in *S. parvifolia* which is 16 loci were successfully cross - amplified and showed high levels of polymorphism (Lee *et al.*, 2004). However, the probability of successful cross - species amplification of SSRs depends on the relatedness between species. Success was achieved in 80 % when transferability was tested within a genus and was considerably reduced to 20 % when tested with species of another genus (Pandey, 2005).

5. Results

5.1 Cytogenetic study

Cytogenetic study, which is the first reported of *Shorea obtusa* revealed that this species has a chromosome number of 2n = 14 (Figure 4.1). Unfortunately, analysis

of the chromosome number of *S. siamensis* and *S. roxburghii* were not success. The result is consistent with previous studies (Bawa, 1998; Missouri Botanical Garden 2007) in other species of the genus *Shorea*, which has a basic chromosome of x = 7. Therefore, *S. obtusa* is a diploid species.

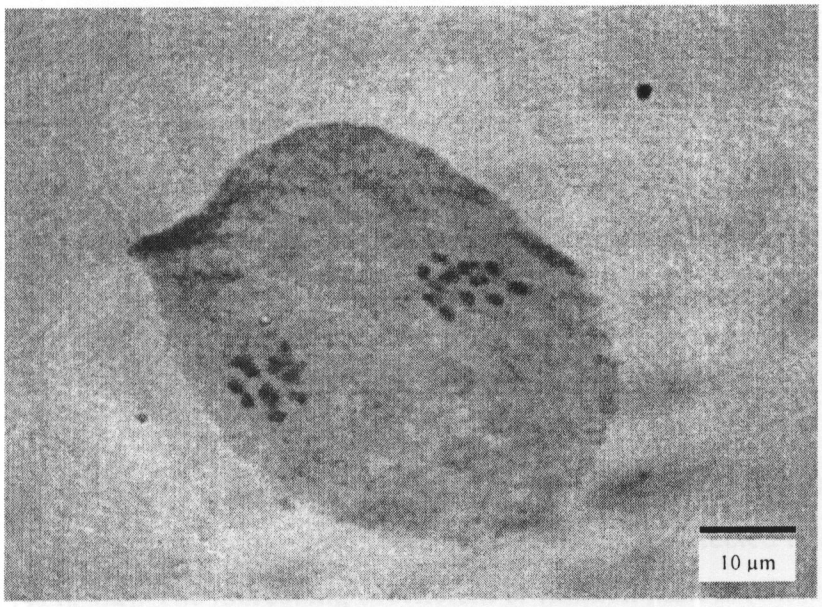

Figure 5.1 Chromosome of pollen mother cell of *Shorea obtusa* at the anaphase I (14:14)

5.2 Genetic diversity and population genetic structure

A total of 146 samples were genotyped for five SSRs loci. Twenty - five alleles were found. All SSRs loci were polymorphic. Allele frequency of five populations is shown in Table 4.3. The number of allele per locus varied from 4 to 7 with an average of five alleles per locus (Table 4.2 and Table 4.3). Chaiyaphum population has the highest allelic diversity with an average of 5.0 alleles per locus and Ubon Ratchathani population has the lowest allelic diversity with an average of 4.4. The effective number of allele per locus (*Ne*) ranged from 2.00 in the Chaiyaphum population to 4.51 in the Chiang Rai population. Expected heterozygosity (*He*) varied among populations, with *He* values ranging from 0.639 in the Ubon Ratchathani population to 0.700 in the Chiang Rai population.

Table 5.1 Geographic location and sample size of *Shorea obtusa*

Population (abbreviation)	Sample size	Latitude	Longitude	Altitude (m)
Utai Thani (UT)	30	15°33.294' N	99°23.741' E	183 - 313
Chiang Rai (CR)	30	20°05.201' N	100°28.609' E	404 - 465
Chaiyaphum (CY)	30	15°51.832' N	101°31.688' E	352 - 440
Maha Sarakham (MK)	33	15°42.336' N	103°13.771' E	123 - 170
Ubon Ratchathani (UB)	23	14°32.336' N	105°00.533' E	188 - 218

Latitude/Longtitude in degree decimal minute (hddd.mm.mmm)

The mean *He* and *Ho* was 0.664 and 0.438, respectively (Table 4.4). Significant departures from Hardy - Weinberg equilibrium were detected in all populations (Table 4.2). The inbreeding coefficient or fixation indices (*Fis*) were positive indicating deficit of the heterozygote. Private alleles were not present in all populations. The linkage disequilibriums for all marker pairs were tested in each population. There was no significant linked locus ($p = 0.741$) indicated independent of SSRs markers in this study.

Table 5.2 Microsatellite loci and conditions of annealing temperatures (Tm), repeat pattern, allele size, number of alleles per locus (*A*), and observed (*Ho*) and expected (*He*) heterozygosity for five SSRs loci in *Shorea obtusa*

Locus	Repeat motif	Primer sequence (5' to 3')	Tm (°C)	Expected allele size (bp)	*A*	*Ho*	*He*
Shc01	$(CT)_8(CA)_{10}CT(CA)_4CTCA$	F: GCT ATT GGC AAG GAT GTT CA	53	152	7	0.254	0.792
		R: CTT ATG AGA TCA ATT TGA CAG					
Shc07	$(CT)_8CA(CT)_5CACCC(CTCA)_3CT(CA)_{10}$	F: ATG TCC ATG TTT GAG TG	55	169	6	0.703	0.771
		R: CAT GGA CAT AAG TGG AG					
Shc09	$(CT)_{12}$	F: TTT CTG TAT CCG TGT GTT G	54.5	197	4	0.400	0.504
		R: GCG ATT AAG CGG ACC TCA G					
Shc11	$(CT)_4TT(CT)_5$	F: ATC TGT TCT TCT ACA AGC C	53.5	166	4	0.282	0.624
		R: TTA GAA CTT GAG TCA GAT AC					
Sle118	$(GA)_{16}$	F: AAA GCG TAC AAA TTC ATC A	55	170	4	0.537	0.677
		R: CTA TTG GTT GGG TCA GAA GG					
All loci					5	0.435	0.674

Table 5.3 Allele frequency for polymorphic microsatellite loci in *Shorea obtusa*

Locus	Allele	Population				
		UT	CR	CY	MK	UB
		N = 22	*N = 25*	*N = 22*	*N = 15*	*N = 14*
Shc01	1	0.295	0.140	0.296	0.266	0.072
	2	-	0.120	0.046	-	-
	3	0.114	0.260	0.318	0.167	0.250
	4	0.136	0.260	0.182	0.400	0.143
	5	0.250	0.080	0.068	0.067	0.464
	6	0.205	0.040	0.045	0.033	0.071
	7	-	0.100	0.045	0.067	-
		N = 28	*N = 26*	*N = 18*	*N = 23*	*N = 18*
Shc07	1	0.036	0.096	0.083	0.109	-
	2	0.071	0.115	0.056	0.109	0.028
	3	0.232	0.115	0.139	0.087	0.083
	4	0.286	0.173	0.417	0.261	0.500
	5	0.250	0.135	0.083	0.130	0.167
	6	0.125	0.366	0.222	0.304	0.222
		N = 28	*N = 26*	*N = 22*	*N = 29*	*N = 19*
Shc09	1	0.286	0.346	0.250	0.155	0.237
	2	0.036	0.115	0.091	0.103	0.026
	3	0.643	0.539	0.636	0.742	0.711
	4	0.035	-	0.023	-	0.026
		N = 30	*N = 21*	*N = 23*	*N = 24*	*N = 19*
Shc11	1	0.266	0.191	0.087	0.313	0.132
	2	0.433	0.643	0.739	0.354	0.342
	3	0.267	0.095	0.130	0.312	0.368
	4	0.033	0.071	0.044	0.021	0.158
		N = 29	*N = 25*	*N = 21*	*N = 21*	*N = 18*
Sle118	1	0.190	0.320	0.357	0.167	0.111
	2	0.138	0.240	0.191	0.095	0.111
	3	0.534	0.420	0.357	0.381	0.583
	4	0.138	0.020	0.095	0.357	0.195

Table 5.4 Averages allele per locus (*Na*), effective number of allele (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity *(He*) and inbreeding coefficient (*Fis*) in five populations of *Shorea obtusa* based on five microsatellite loci

Population	*N*	*Na*	*Ne*	*Ho*	*He*	*Fis*
Utai Thani	30	4.60	3.79	0.467	0.681	0.339
Chang Rai	30	4.80	4.51	0.479	0.700	0.322
Chaiyaphum	30	5.00	2.00	0.462	0.640	0.252
Maha Sarakham	33	4.60	2.85	0.419	0.658	0.393
Ubon Ratchatani	23	4.40	3.23	0.363	0.639	0.464
Over all	146	4.68	3.27	0.438	0.664	0.354

N represents number of individuals analyzed in each population.

Table 5.5 Wright's *F* - staistics based on five microsatellite loci for five populations of *Shorea obtusa*.

Locus	*Fis*	*Fit*	*Fst*
Shc01	0.668	0.679	0.035
Shc07	0.100	0.121	0.023
Shc09	0.216	0.218	0.003
Shc11	0.522	0.547	0.052
Sle118	0.209	0.233	0.030
over all loci	0.351	0.371	0.030*

*$p < 0.05$

The coefficient of population differentiation (*Fst*) among populations ranged between 0.003 (shc09) and 0.052 (shc01) with a global value of 0.030 ($p < 0.05$). The pairwise *Fst* between populations were shown in Table 4.6. Pairwise *Fst* were low but there were significant genetic differentiations in all population comparisons. Exceptions to these ware comparisons between Chiang Rai and Chaiyaphum populations which have high number of shared alleles (24 out of 25) and between Uthai Thani and Ubon Ratchathani populations. The Mantel's test of isolation by distance was not significant

($r = 0.03$, $p = 0.78$) indicating that the level of genetic differentiation between populations was not associated with geographic distances.

Table 5.6 Matrix of the *Fst* values according to Weir and Cockerham (1984) (above diagonal) and the geographic distances (km) (below diagonal) between populations

Population	UT	CR	CY	MK	UB
Utait Thani (UT)		0.0284***	0.0284**	0.0182**	0.0093^{NS}
Chiang Rai (CR)	514		0.0002^{NS}	0.0321***	0.0560***
Chaiyaphum (CY)	480	230		0.0384**	0.0525***
Maha Sarakham (MK)	566	412	185		0.0336**
Ubon Ratchatani (UB)	781	614	230	226	

NS, not significant; ***$p < 0.0001$ and **$p < 0.001$

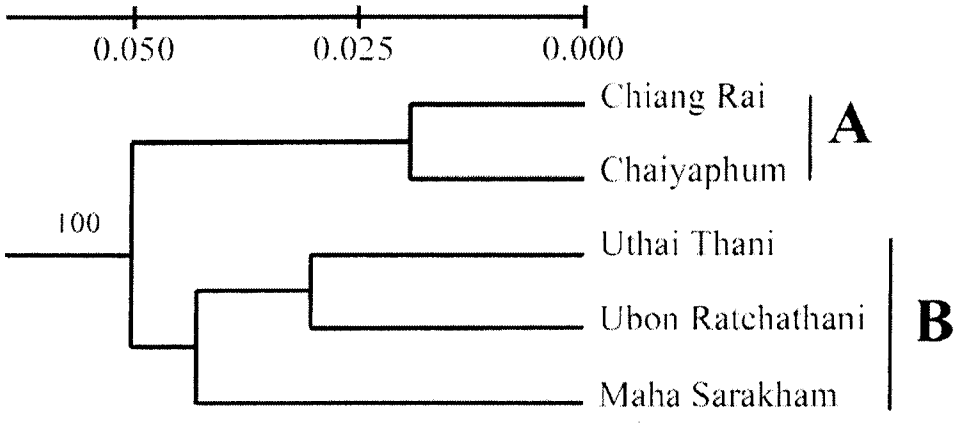

Figure 5.2 Dendrogram from UPGMA cluster analysis based on Nei (1978) unbiased minimum distance among the five populations of *Shorea obtusa* in Thailand. The bootstrap values based on 1,000 replications

The Nei's genetic distances (D_A) (Nei, 1972, 1978) were used to construct the UPGMA dendrogram. An UPGMA tree (Figure 4.2) separated the populations into 2 groups (A and B). Separation of group A (Chiang Rai population from the north and Chaiyaphum population from the northeast) from group B (Uthai Thani from the west, Ubon Ratchathani and Maha Sarakham population from the northeast) was supported by a strong bootstrap value (100).

5.3 Genetic relatedness, paternity analysis and mating system of *Shorea obtusa* in WRBRI, Maha Sarakham

4.3.1 Genetic diversity of the parental and offspring populations

Diversity parameters for the five polymorphic SSRs loci in the parent and offspring populations are presented in Table 4.8. Numbers of alleles for each primer range from 3 (shc09) to 6 (shc01 and shc07). In the parent, the total number of alleles (*A*) was 23, with a mean of 4.6, the average *Ho*, *He* and *Fis* values across loci were 0.424, 0.674 and 0.383, respectively. The *Fis* value showed significant positive, indicating deficit of the heterozygote. A significant departure from Hardy - Weinberg equilibrium was detected on four loci (shc01, shc09, shc11 and sle118). The deviations might be a due to the present of null alleles leading to an apparent excess of homozygote (Marshall *et al.*, 1998). For offspring population, average over all loci, the number of allele detected was 24 with mean of 4.8. The average *Ho*, *He* and *Fis* values across loci were 0.406, 0.547 and - 0.152, respectively. In contrast, in offspring population, *Fis* value was significant negative, indicating an excess of heterozygote (*Fis* < 0).

Table 5.7 Genetic diversity of the five *Shorea obtusa* seed maternal examined in WRBRI, Maha Sarakham province

Seed maternal	*N*	*A*	*Ar*	*Ho*	*He*
MK8	26	5	2.470	0.531	0.423
MK14	24	5	2.111	0.398	0.371
MK22	41	5	1.896	0.439	0.337
MK25	54	5	2.376	0.494	0.425
MK32	34	5	1.746	0.284	0.265

N, Number of seed analyzed; *A*, Number of alleles detected;
Ar, Allelic richness; *Ho*, observed heterozygosity; *He*, expected heterozygosity

Genetic diversity of seed maternal tree (MK8, MK114, MK22, MK25 and MK32) was shown in Table 4.7. The allelic richness (*Ar*) ranges from 1.746 to 2.470. The observed heterozygosity (*Ho*) ranges from 0.284 to 0.531 and expected heterozygosity (*He*) ranges from 0.265 to 0.425. Genetic diversity in the fine scale population of *S. obtusa* (0.674) is similar to previous reports using microsatellite for the dipterocarp plant. For examples, the mean *He* of *S. leprosula*, *D. cornutus* , *S. parvifolia*, *S. acuminate* and *Hopea dryobalanoides* were 0.640 , 0.464, 0.678, 0.590 and 0.630, respectively (Ratnam and Seng, 2003; Takeuchi *et al.*, 2004).

5.3.2 Genetic relatedness and spatial distance

The relationship between relatedness and spatial distance is shown in Figure 4.3. The genetic structure of potentially flowering trees of *S. obtusa* in ca. 100-ha plot in WRBRI was significant, with a negative correlation between relatedness and spatial distance (r = - 0.129, $p < 0.05$; Mantel test). Therefore, these results indicate that the spatial genetic structure among the 29 trees of *S. obtusa*, in WRBRI was low.

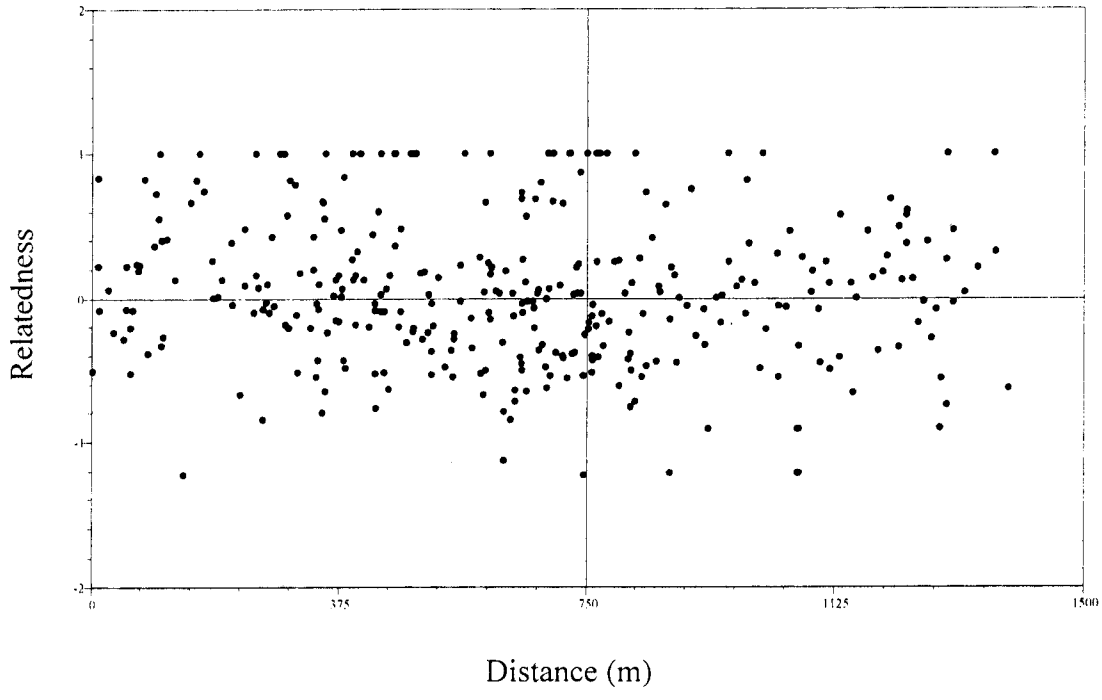

Figure 5.3 Diagram shows the correlation between genetic relatedness and spatial distance among 29 samples of *Shorea obtusa*. The correlation is significantly negative (r = - 0.129, $p < 0.05$) based on 5,000 permutations test

Table 5.8 Characteristics of the five microsatellite loci of *Shorea obtusa* in WRBRI, Maha Sarakham province

Locus	Offspring					Parents					
	N	*A*	*Ho*	*He*	*Fis*	*N*	*A*	*Ho*	*He*	*Fis*	*Pe*
Shc01	92	5	0.164	0.222	- 0.123^{NS}	15	6	0.200	0.756	0.742***	0.495
Shc07	164	6	0.622	0.742	- 0.179^{NS}	23	6	0.739	0.809	0.088^{NS}	0.405
Shc09	165	5	0.436	0.577	- 0.257***	29	3	0.241	0.420	0.430*	0.788
Shc11	169	4	0.349	0.608	- 0.124^{NS}	24	4	0.417	0.691	0.402*	0.609
Sle118	172	4	0.459	0.587	- 0.063^{NS}	21	4	0.524	0.696	0.252*	0.584
Mean		**4.8**	**0.406**	**0.547**	**- 0.152*** **		**4.6**	**0.424**	**0.674**	**0.383*****	**0.576**

N, number of sample analyzed; *A*, number of alleles; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *Fis*, inbreeding coefficient and *Pe*, probability of paternity exclusion

***$p < 0.0001$; *$p < 0.05$; NS, non significant

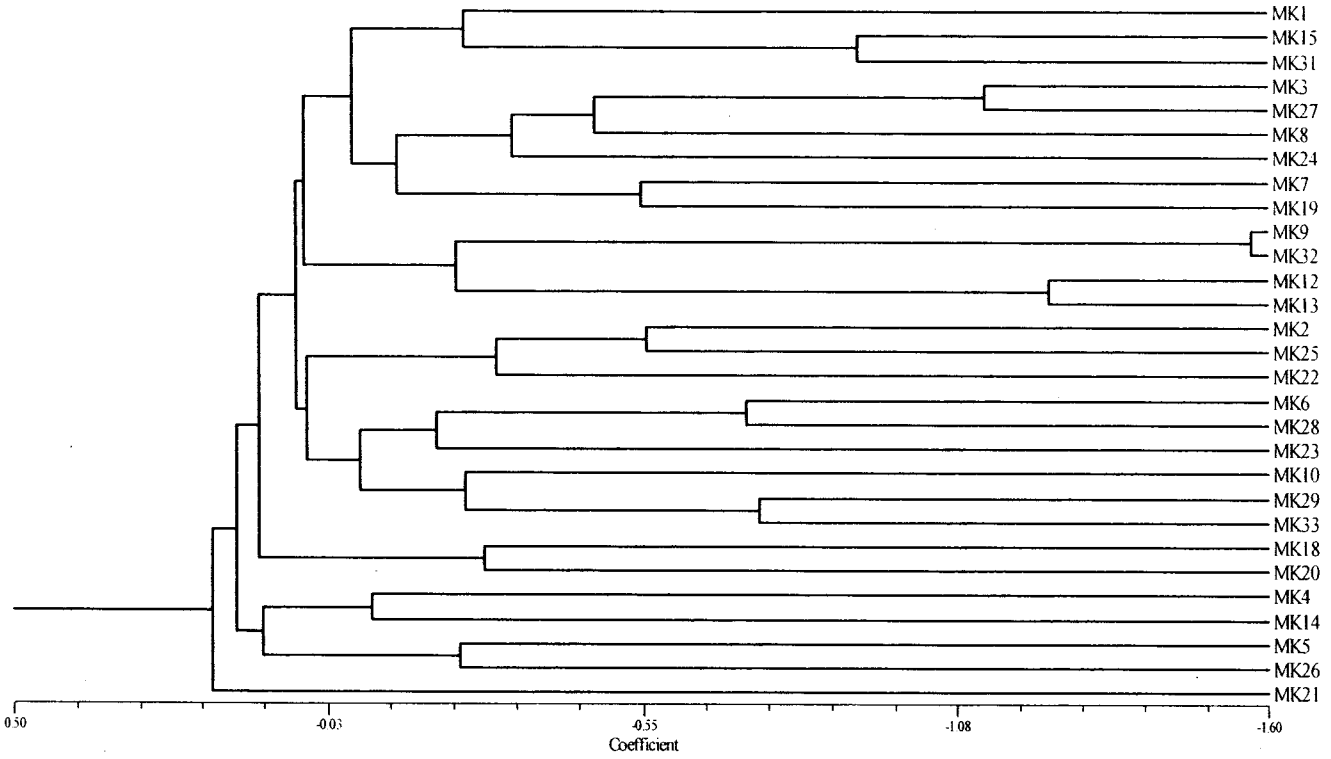

Figure 5.4 An UPGMA cluster analysis based on genetic relatedness values of 29 trees of *Shorea obtusa* in WBRI calculated from five SSRs ($r = 0.024$, $p = 0.054$)

An UPGMA based on genetic relatedness (Queller and Goodnight, 1989) among 29 tree of *S. obtusa* revealed four main clusters. Cluster 1:MK21; Cluster 2: MK4, MK5, MK14, MK26; Cluster 3: MK18 and MK20 and Cluster4: MK1,MK15, MK31,MK3, MK27,MK8, MK24,MK7, MK19,MK9, MK32,MK12, MK19,MK9, MK32 and MK12 (Figure 4.4). Individual MK21 was distinctly divergence from the other individuals within cluster 1. Based on UPGMA tree one of the five mother trees, MK14, was not closely related and may be used for seed production.

Relatedness value based on Queller and Goodnight (1989) of 29 trees of *S. obtusa* was low and not significant ($r = 0.023$, $p = 0.054$). This implies that extensive gene flow occurred in this species within the WRBRI forest.

5.3.3 Paternity analysis

The total paternity exclusion probability over the five loci for *S. obtusa* was 0.576 (Table 4.7). The frequency of null alleles detected in parent tree was 25 % with locus shc01, shc09, shc11 and sle118. Paternity analysis based on the paternity exclusion method found that 57.6 % of probability of paternity exclusion (*Pe* = 0.576).

Table 5.9 Parentage analysis for *Shorea obtusa* in WRBRI, Maha Sarakham province

Locus	Observed alleles	
	Parent	Offspring
Shc01	A, C, D, E, F,G	*B*, C, D, E, F
Shc07	A, B, C, D, E, F	A, B, C, D, E, F
Shc09	A, B, C	A, B, C, *D*, *F*
Shc11	A, B, C, D	A, B, C, D
Sle118	A, B, C, D	A, B, C, D

Underlineed alleles probably originated from parent trees within the WRBRI forest. *Italicized alleles* probably originated from parent trees outside the WRBRI forest.

Table 5.10 Parentage allele contribution of *Shorea obtusa* in WRBRI, Maha Sarakham province

Locus	Parentage allele contribution (%)	
	Within WRBRI	Outside WRBRI
Shc01	85.7	14.3
Shc07	100.0	0.0
Shc09	60	40
Shc11	100.0	0.0
Sle118	100.0	0.0
Mean	89.1	10.9

The effective pollination distances between the mother and the definite father tree (inter - mate distance) were calculated when one potential father was assigned. For each maternal tree, the distance to its closest male tree (*d1*) and the average distance to its closest male tree (*d2*) were also measured (Table 4.11). In addition, the frequency of the definite paternal donors was determined for 100 m distance categories from the maternal tree, which was calculated as the number of definite pollination events against the total number of definite pollination events against the total number of progeny of maternal tree. The data were plotted per maternal tree, based on the cumulative frequencies per distance class for each maternal tree, together with the frequency of male trees within each distance category, generating distance - frequency histogram of direct pollen flow to each maternal tree (Figure 4.5).

Table 5.11 Estimates of pollen dispersal parameters of *Shorea obtusa* in WRBRI, Maha Sarakham province

M	*N*	*d1*	*d2*	*Nep*	δ
MK8	26	32.8	742.7	7	659.6
MK14	24	52.0	760.5	4	444.2
MK22	41	202.3	579.3	3	552.5
MK25	54	77.0	667.6	12	678.5
MK32	34	139.3	831.2	4	798.8
Average	35.8	100.7	716.3	6	626.7

M maternal tree, *N* number of offspring (seeds) analyzed per maternal tree, *d1* distance from the maternal tree to the closest male tree (m), *d2* average distance from the maternal tree to all the male tree (m), *Nep* effective number of pollen donor, δ effective dispersal distance (m) for each maternal tree.

The exact distance from maternal tree to their closest pollen-donor tree (*d1*) ranges from 32.8 to 202.3 m. The average distance from maternal trees to all potential pollen-donor trees (*d2*) varied from 579.3 to 831.2 m, with a mean distance of 716.3 m (Table 4.11). The longest - distance pollination occurred between MK8 and MK33, suggesting the maximum pollen dispersal distance of 1,254 m. The average effective distance of pollen dispersal (δ) of maternal tree ranged between 444.2 m for MK14 and 798.5 m for MK32, with a mean value of 626.7 m. The effective number of pollen donor (*Nep*) for each maternal tree ranges from 3 to 12, with an average *Nep* of 6. The distance - frequency histogram of pollen flow to each maternal tree was shown in Figure 4.4. Most of maternal trees showed a high frequency of pollen flow in the 1,201 - 1,300m, 300 - 400 m and 501 - 600 m distance with male trees indicating long distance pollination in the population.

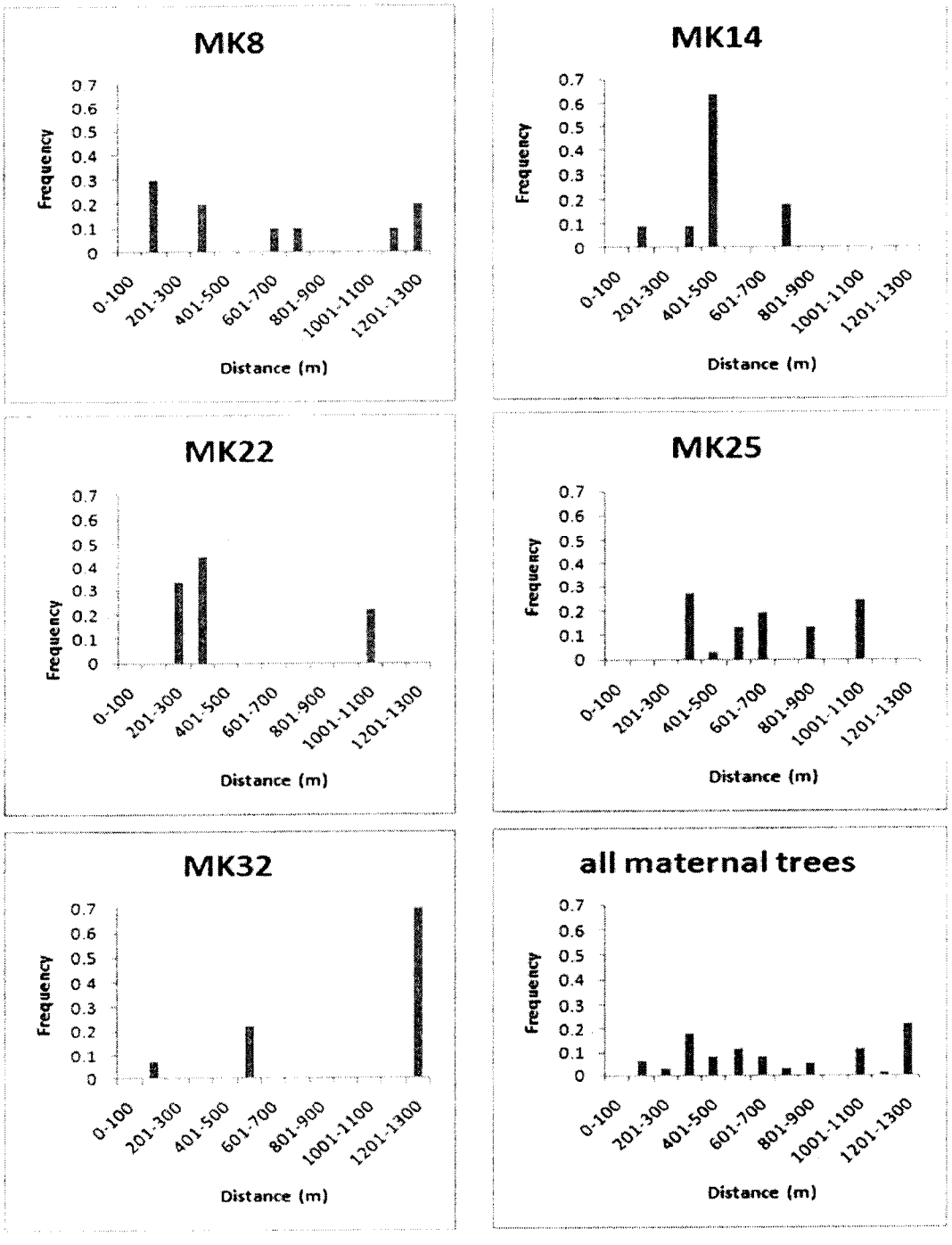

Figure 5.5 Distance-frequency histogram of direct pollen flow determined by paternity analysis to the five *Shorea obtusa* maternal trees (MK8, MK14, MK22, MK25 and MK32) and the entire studies maternal tree (all maternal trees) in WRBRI, Maha Sarakham province

5.4.4 Mating system of *Shorea obtusa*

The mating system parameter; parental inbreeding, multilocus outcrossing rate (t_m), single locus outcrossing rate (t_s), biparental inbreeding (t_m - t_s) and multilocus paternity correlation coefficient (r_p) were estimated for *S. obtusa* (Table 4.12). These parameters were analyzed using in the multilocus mating system program MLTR (Ritland, 2008). The expectation-maximization procedure that bound outcrossing rates between 0 and 1 was used for the iteration, and default setting were used with initial values of outcrossing rate, $t = 0.90$, parental inbreeding, $F = 0.1$ and paternity correlation, $r_p = 0.1$. Standard errors for t_s, t_m and r_p were calculated from 500 bootstrap replicates with resampling among maternal plants within population. The effective number of pollen donors (*Nep*) was estimated following $1/r_p$ formula (Ritland, 1989).

The outcrosssing rate was significantly lower than 1 for *S. obtusa* population in WRBRI. The population of *S. obtusa* was found to be a lower outcrossing rate (t_m = 0.569) with about 56.9 % of the mating attributable to outcrossing and 43.1 attributable to self - fertilization. The values of the single-locus outcrossing rates (t_s = 0.531) were also significantly and were lower than t_m.

The positive differences between t_m and t_s were significantly different from zero in *S. obtusa* (t_m - t_s = 0.037) indicating that biparental inbreeding contributed to the apparent selfing rate in population. Inbreeding coefficients of maternal parents (F) were not significantly different from zero (F = -0.095). The estimates for correlation of paternity (r_p = 0.340) were significantly from zero. The r_p values indicated that less than four fathers contributed to individual progeny arrays in the *S. obtusa* population. In addition, the value of r_p was used to calculated effective number of pollen donor (*Nep*). The number of effective donors (*Nep)* was 2.94.

Table 5.12 The mating system parameters of *Shorea obtusa*. Standard errors are shown in parentheses

Parameter	Results
Parental inbreeding, F	0.095 ±0.113
Multilocus outcrossing rate, t_m	0.569 ±0.191
Single-locus outcrossing rate, t_s	0.531 ±0.185
Biparental inbreeding, $t_m - t_s$	0.037 ±0.033
Multilocus paternity correlation, r_p	0.340 ±0.192
Effective number of pollen donor, *Nep*	2.94

6. Discussions and Conclusions

6.1 Cytogenetic study of *Shorea obtusa*

Chromosomes of *Shorea obtusa* were very well stained with propionocarmine and were distinctively paired as bivalent at diakinesis which separated to 14:14 at anaphase I. Therefore the chromosome number of *S. obtusa* was 2n = 14. This indicated that *S. obtusa* is a diploid species (2n = 14), since the chromosome could be matched together to form seven homologous chromosome. The results indicated that *Shorea* has basic chromosome number x = 7. As seen the previous reported in *S. acuminata* Dyer, *S. agami* Ashton, *S. argentifolia* Symington, *S. gardneri* Ashton, *S. leprosula* Miq., *S. macrophylla* Ashton, *S. macropter* Dyer, *S. multiflora* (Burck) Symington, *S. pauciflora* King, *S. pinanga* Scheff, *S. platyclados* Slooten, *S. robusta* Gaertn., *S. splendida* (De Vries) Ashton, *S. stenoptera* Burck and *S. trapezifolia* Ashton (Bawa, 1998; Missouri Botanical Garden, 2010).

6.2 Population genetic structure and diversity of *Shorea obtusa* in Thailand

The level of genetic diversity of *S. obtusa* was high (0.664) and comparable to those of other dipterocarps species, for example, *S. leprosula* (*He* = 0.70) (Ng *et al.*, 2004). *S. macrophylla* (*He* = 0.745), *S. beccariana* (*He* = 0.685), *Dryobalanops lanceolata* (*He* = 0.603), and *D. aromatica* (*He* = 0.461) (Konishi *et al.*, 2004). High genetic diversity suggests long - term stability of the populations. However, in the last few decades a large area of DDF in the Thailand has been clear for agricultural and other purposes. These anthropogenic impacts decreased the *S. obtusa* population size by both number of the trees and fragmented the populations into a smaller forest. Therefore, high genetic diversity in *S. obtusa* possibly represents a relic of the historical populations. During the Pleistocene glaciations, it has been suggested that DDF expanded because of the dry climatic condition (Lechon and Curtet, 1998) and covered most of the Thailand and Southeast Asian mainland (Pooma and Newman, 2001). This large population could have increased the genetic diversity of this species. The recent decrease in population size probably has not yet affected the level of genetic diversity.

Departures from the Hardy - Weinberg equilibrium (HWE) have been detected in all populations. This could be due to several factors, including selection on the genetic markers under study, mixing of genetically distinct lineages (i.e. Wahlund effect), inbreeding, and presence of null alleles. Given that microsatellite loci were considered as selectively neutral markers this hypothesis could be exclude. The Wahlund effect is unlikely because levels of genetic differentiation between populations are low (*F*st rages between 0.003 (shc09) and 0.052 (shc11) with a mean of 0.030). The inbreeding can also be excluded because this would be expected to affect all loci. Tests of the HWE revealed that only two loci (shc01 and shc11) showed strong departure from HWE. Therefore, departure from HWE is most likely due to the presence of a null allele.

The overall degree of population differentiation was low for *S. obtusa* in Thailand. Despite being low, there were significant genetic differentiation in all populations, except the comparison between Chiang Rai and Chaiyaphum and Ubon Ratchathani and Uthai Thani populations. The non - significant result of the Mantel's test indicated that the level of genetic differentiation was not related to the geographic distance between populations, being genetically closely related and the sharing of

several alleles between populations such as Chiang Rai and Chaiyaphum indicating gene flow between these populations. However, given the large geographic isolation, ongoing gene flow is unlikely. A possible explanation is the sharing of recent population history of this species through recent population expansion which occur during the last glaciations (see above). Recent population expansions have also been reported in other species in Thailand such as mosquitoes (Walton *et al.*, 2001) and black fly (Pramual *et al.*, 2005).

An UPGMA tree separated the populations into the two groups. The difference of altitudinal zonation in *S. obtusa* may be supported by genetic isolation: group A (Chiang Rai and Chaiyaphum) were located up to 350 m from sea level and group B (Ubon Ratchathani, Uthai Thani and Maha Sarakham) were located lower than 350 m from sea level (Table3.1). Asian dipterocarps are limited altitudinally by climatic conjunction of altitude and other natural barriers such as large rivers and mountains. In the Malaysia Penninsular, the altitudinal zonation of the main habitat types range from 0 - 300 m (low - undulating dipterocarp forest) to 300 - 750 m (hill dipterocarp forest). This altitudinal zonation of Asian dipterocarp species might then explain the pattern of genetic structure in *S. obtusa* in Thailand. Further study is necessary to test this hypothesis.

6.3 Genetic relatednss, paternity analysis and mating system of *Shorea obtusa* inWRBRI, Maha Sarakham province

There was no genetic structure among the 29 potentially flowering trees of *S. obtusa* within WRBRI population ($r = 0.023$, $p = 0.054$). This implies that there was extensive gene flow in previous generation. Although the correlation between genetic relatedness and spatial distance among 29 individuals was significantly negative ($r = -0.129$, $p < 0.05$). A similar result was also detected in the degree of genetic structure in Dipterocarp plant for instance, *S. parvifolia* ($r = -0.149$, $p < 0.05$), *Hopea dryobalanoides* ($r = -0.301$, $p < 0.01$) (Takeuchi *et al.*, 2004) and *Neobalanocarpus heimii* ($r = -0.228$, $p < 0.01$) in Penninsula Malaysia (Konuma *et al.*, 2000). Several studies of fine - scale genetic structure in plants have detected significant negative relationship between genetic relatedness and spatial distance, which often explained by

limited seed dispersal (Berg and Hamrick, 1995). A population is genetically structured and inbreeding or outbreeding depression occurs, as indicated by the genetic relatedness of adult tree, some kind of selection on pollen grain could have occurred (Isagi *et al.*, 2000). In addition, Konuma *et al.* (2000) stated that genetic structure is induced when gene flow by pollen and seed dispersal is limited. This result suggested that long-distance gene flow and seed migration are responsible for the poorly develop genetic structure in *S. obtusa* in WRBRI.

The low potential of the paternity analysis (*Pe* = 0.576) could be due to the present of null allele. Microsatellite with high null allele frequency can lead to false parentage exclusion (0.05 or more), thus reduce the rate of parentage assignment (Dakin and Avise, 2004). Pemberton *et al.* (1998) reported that null alleles are responsible for mismatches between parent-offspring pairs such as the offspring do not amplify an allele that is present in the parents. Null allele is a common cause of apparent deviation from HWE at SSR loci (Pemberton *et al.*, 1995). In this study, the frequency of null allele detected in parent tree of *S. obtusa* was 25 %. Callen *et al.* (1993) found that in a survey of $(AC)_n$ SSR marker in human, null alleles were found in 7 % to 30 % of the 23 markers surveyed. Roa *et al.* (2000) study on cross-species amplification of *Manihot* species using 10 SSR loci, found that the presence of null alleles in *Manihot* species was on average 11 %. In addition, with SSR loci, a null allele most often occurs because of mutations in one or both primer binding sites, sufficient to prevent effective amplification of the SSR allele. This problem is particularly common when the SSR locus developed in one species and typed in a different species using the same SSR primers.

The parentage analysis revealed that about 10.9 % of the alleles detected in *S. obtusa* offspring (Table 4.9 and 4.10). It is suggested that this result could be probably originated from adult tree outside the WRBRI. The similar situations have been reported in other species. For examples, Ratnam and Seng (2003) found that about 13 % of the possible parent of *Dipterocarpus cornutus* saplings were from the outside study plot in Labis Forest Reserve, Segamat (SPA), Johor. Isagi *et al.* (2000) found that about 57 % of the possible parent of *Magnolia obovata* saplings were from the outside study plot in Ogawa Forest Reserve revealed by eight SSRs loci. In contrast, all of the saplings of *S.*

leprosula shared the same alleles with mother trees inside the *S. leprosula* SPA at each locus, suggested that shared are probably their parent.

The paternity analysis revealed long distance pollination in the *S. obtusa* of the WRBRI population. An average effective distance of pollen dispersal (δ) was estimated as 626.7 m and a maximum realized pollen distance of 1,254 m, which was also maximum observed geographic distance between maternal trees and the potential paternal trees. Similar results were reported in other tree species. For examples, Kunuma *et al.* (2000) reported that average effective distance of pollen dispersal was 524 m in *Neobalanocarpus heimii*. The long distance pollen dispersal of *S. obtusa* in this study may be related to pollinator composition and their flight behavior and flowering tree density. In WRBRI, *S. obtusa* may be visited by a broad spectrum of pollinators such as bees and other insects. These insects are usually discriminated as long-distance pollinators (Dayanandan *et al.*, 1990). By using hypervariable microsatellite marker, longer distance of insect-mediated pollination have been reported in several tropical tree species such as *Swietenia humilis*, with the distance of more than 4.5 km (White *et al.*, 2002); *Dinizia excels* have a distance of pollination with mean of 1,509 m in the fragment landscape (Dick *et al.*, 2004) and *Dicorynia guianensis* with the distance of more than 1,000 m (Latouche - Halle *et al.*, 2004). Similar pattern of pollen movement were found in fragmented populations of the tropical tree *S. humilis*, in which increased pollen dispersal distance was observed in a single isolated tree (White *et al.*, 2002). These results supported the hypothesis of increasing spatial isolation promote the long - distance pollen movement (White *et al.*, 2002).

The effective number of pollen donors (*Nep*) of *S. obtusa* population was estimated to be 6 according to the paternity analysis and 2.96 according to the MLTR model. Although the results of these analyses are different but both suggest that the effective number of pollen donor was low. Comparably low values of *Nep* have been observed in savanna populations of *Quercus lobata* (*Nep* = 3 - 4), forest fragment of *Q. humboldtii* (*Nep* = 1 - 2), mixed conifer - deciduous forest of *Pinus echinata* (*Nep* = 3 - 6) and scattered clumps of *Albizia julibrissin* (*Nep* = 3) (Smouse and Sork , 2004). However, these values are lower than effective number of pollen donor reported for other tropical tree species, for instance *Q. semiserata* (*Nep* = 9.987 - 10.989) (Pakkad *et al.*, 2008), *Acacia melaxylon* (*Nep* = 16 - 33) and *Carapa pocera* (*Nep* = 12) (Smouse

and Sork , 2004). In general, *Nep* is inversely correlated to the level of pollen flow across the local landscape. Previous reported suggest that landscape context play more important role on the pollen flow than the pollination system. *Nep* tends to be higher in open conditions than the closed canopies. (Smouse and Sork , 2004). The low number of effective donors observed in *S. obtusa* indicating that most pollination is much localized. This is possibly because of the low densities of tree in the surrounding area. However, this very low estimated might also be reflecting a bias in MLTR program and paternity analysis.

The outcrossing rates of *S. obtusa* in WRBRI population (t_m = 0.569) were lower than those of *Shorea* and other Dipterocarp species including *S. congestiflora, S. megistophylla, S. leprosula, S. trapezifolia* and *S. siamensis* were range from 0.617 to 0.918 (Murawski *et al.*, 1994; Lee *et al.*, 2000; Chaisurisri *et al.*, 2005). *Dryobalanops aromatica* were 0.794 - 0.856 and 0.923, respectively (Kitamura *et al.*, 1994; Lee, 2000) and *Stemonoporus oblongifolius* was 0.898. In addition, most tropical trees species, which are predominantly outcross, such as *Pterocarpus macrocarpus* (t_m = 0.719 - 0.959; Leiengsiri *et al.*, 1998), *Senna multijuga* (t_m = 0.540 - 0.838; Ribeiro and Lovato 2004) and *Quercus semiserrata* (t_m = 0.995; Pakkad *et al.*, 2008). However, the mating system of *S. obtusa* in WRBRI is dynamic, with variable outcrossing among individual. In addition, the estimate of biparental inbreeding for the total population (t_m - t_s) was 0.037, indicating the occurrence of a low proportion of mating among relatives. This proportion was lower than expected since several studies have suggested that levels of self-pollination and/or biparental inbreeding are likely to be higher in fragmented or small populations than in continuous and/or larger populations (Pakkad *et al.*, 2008). The results of this study are consistent with finding by that levels of biparental inbreeding are generally low in fragmented populations of *Quercus humboldtii* and *Q. velutina*, probably the mating system of these species is resilient to reductions in population size (Fernandez - Manjarres and Sork , 2005; Fernandez - Manijarres *et al.*, 2006).

Mating system and pollen structure analyses of *S. obtusa* at the WRBRI has revealed interesting results. Despite a low outcrossing rate, the biparental inbreeding is low indicated that those individual siring the offspring are weakly related to the

maternal plant, the low number of effective number of pollen donor and the long distance of pollen movement. The finding of this study may be relatively to lower density of flowerings trees and changes in the foraging behavior of pollinators.

6.4 Conclusions

S. obtusa is a keystone species in major forest types (i.e. dry deciduous dipterocarp forest) in Thailand. Population genetic structure, genetic diversity, genetic relatedness, paternity analysis and mating system of the *S. obtusa* were examined based on microsatellite marker. This is the first report on the population genetic structure and diversity of *S. obtusa* in Thailand. The information obtained in this study will be valuable not only for the theoretical advancement of knowledge on plant genetic diversity, but also for designing genetically sound conservation and management programs. These results indicate that high levels of genetic variation remain in populations of *S. obtusa*. However, given the recent rates of deforestation it is necessary to protect the high diversity and genetically isolated populations. The Chaiyaphum, Chiang Rai, and Uthai Thani populations posses the highest genetic diversity and are also the most geographically isolated, thus conservation priority should be considered for these populations. Interestingly, the *Fis* values observed of *S. obtusa* in WRBRI, Maha Sarakham shown difference values between parent and offspring population. The *Fis* value of parent was positive indicating deficit of the heterozygote. In contrast, in offspring population, *Fis* were significant negative, indicating an excess of heterozygote. This is because; *S. obtusa* is possibility mostly insect - pollination and seed dispersal by wind. The density of *S. obtusa* in this forest and long distance gene flow may be responsible for pattern of genetic structure of this species.

Further studies by more extensive sampling should be conducted to test the hypothesis of altitudinal zonation on pattern of genetic differentiation. SSRs markers developed for *S. curtisii* and *S. leprosula* were successfully cross - species amplifications in *S. obtusa*. However, the results indicated that there are problems of null allele in some loci. This problem relevant to the SSRs locus developed in one species and typed in a different species using the same SSRs primers. Thus, development of SSRs marker in *S. obtusa* is important for the further genetic variation analysis of this species. Finally, understanding the pattern of gene movement within

keystone species is of great importance for designing appropriate conservation program and insights on the ex - situ conservation design and management.

7. References

Ashton PS (1982) Dipterocarpaceae. *Flora Malesiana Ser.I* 9, 237 - 552.

Austerlitz F, Dick CW, Dutech C, Klein EK, Muratorion SO, Smouse PE, Sork V (2004) Using genetic markers to estimate the pollen dispersal curve. *Molecular Ecology*, 13, 937 - 954.

Bawa KS (1998) Conservation of genetic resource in the Dipterocarpaceae. In: Appanah S, Turnbull JM (eds.) *A review of Dipterocarps: taxonomy, ecology and silviculture.* Bogor Indonesia: Center for International Forestry Research, pp 45 - 55.

Barrett SCH and Harder LD (1996) Ecology and evolution of plant mating. *Tree* , 11(2), 73 - 78.

Bayer C, Fay MF, de Bruijn AY, Savolainen V, Morton CM, Kubitzki K, Alverson WS, Chase MW (1999) Support for an Expanded Family Concept of Malvaceae within a Recircumscribed Order Malvales: A Combined Analysis of Plastid atpB and rbcL DNA Sequences. *Journal of the Linnean Society Botany*, 129, 267 - 303.

Berg EE and Hamrick JL (1995) Fine-scale genetic structure of a Turkey oak forest. *Evolution*, 49, 110 - 120.

Bisby FA (1995) Characterization of biodiversity. In: Heywood VH (ed.) *Global Biodiversity assessment.* Cambridge University Press, pp 21 - 106.

Bittencourt JVM and Sebbenn AM (2007) Pattern of pollen and seed dispersal in a small fragmented population of the wind-pollinated tree *Araucaria angustifolia* in southern Brazil. *Heredity*, 99, 580 - 591.

Boys J, Cherry M, Dayanandan (2005) Microsatellite analysis reveals genetically distinct population of red pine (*Pinus resinosa*, Pinaceae). *American Journal of Botany*, 92(5): 833 - 841.

Callen DF, Thompson AD, Shen Y, Phillip HA, Richards RI, Mulley JC and

Sutherland GR (1993) Incidence and origin of "null" alleles in the (AC) n microsatellite markers. *American Journal Human Genetic*, 52, 922 - 927.

Cao CP (2006) *Genetic variation of the genus Shorea (Dipterocarpaceae) in Indonesia.* Ph.D. thesis. Georg-August University of Göttingen.

Cao CP, Finkeldey R, Siregar IZ, Siregar UJ and Gailing O (2006) Genetic diversity within and among population of *Shorea leprosula* Miq. and *Shorea parvifolia* Dyer (Dipterocarpaceae) in Indonesia detected by AFLPs. *Tree Genetics and Genomes*, 2, 225 - 239.

Chaisurisri K (2005) *Application of isoenzyme electrophoresis to assess genetic diversity in conservation of tropical forest genetic resources: Theory and practice.* Bangkok, Forest and Plant Conservation Research Office.

Chalermpong A, Tapyai C and Ramanwong K [2007] *Diseases and Microorganisms of Dipterocarp Forest Trees.* [Online]. Available from: http://www.forest.go.th/Research/English/abstracts_pathology/diseases4.htm. [Cited 10 November 2007].

Changtragoon S (2001) Evaluation of genetic diversity of *Dipterocarp alatus* genetic resources in Thailand using isozyme gene marker. In: *In Situ and Ex Situ conservation of Commercial Tropical Trees: Proceeding of the Contribution of Genetic Resources Conservation to Tree Breeding, Biotechnology, and Future Commercial Plantation Program . 11 - 13 June 2001*, Yogyakarta, Indonesia. pp 349 - 354.

Chase MR, Moller C, Kesseli R and Bawa KS (1996) Distant gene flow in tropical trees. *Nature* , 383, 398 - 399.

Costin BJ, Morgan JW and Young AG (2001) Reproductive success does not decline in fragmented population of *Leucochrysum albicans* subsp. *Albicans* var. *tricolor* (Asteraceae). *Biological Conservation*, 98, 273 - 284.

Craft KJ (2005) *Gene flow dynamics and population genetic structure in White oak species of Northeastern Illinois.* Ph.D. thesis University of Illinois at Chicago, Illinois.

Dakin EE and Avise JC 2004) Microsatellite null alleles in parentage analysis. *Heredity*, 93, 504 - 509.

Dick CW, Etchelecu G, Austerlitz F (2003) Pollen dispersal of tropical trees (*Dinizia*

excels: Fabaceae) by native insects and African honeybees in pristine and f ragmented Amazonian rainforest. *Molecular Ecology*, 12, 753 - 764.

Dow BD and Ashley MV 1996) Microsatellite analysis of seed dispersal and parentage of saplings in bur oak, *Quercus macrocarpa*. *Molecular Ecology*, 5, 615 - 627.

Doyle JJ and Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bullelitin*, 19, 11 - 15.

Fernandez-Manjarres JF, Idol J, Sork VL (2006) Mating pattern of black oak *Quercus velutina* (Fagaceae) in a Missouri oak-Hickory forest. *Journal of Heredity*, 97(5), 451 - 455.

Fernandez-Manjarres and Sork VL (2005) Mating pattern of a subdivided population of the Andean oak (*Quercus humboldtii* Bonpl., Fagaceae). *Journal of Heredity*, 96, 635 - 643.

Finkeldey R and Hattemer HH (2007) *Tropical Forest Genetics*. Berlind Heidelberg, Springer-Verlag.

Fukue Y, Kado T, Lee SL, Ng KKS, Muhammad N, Tsumura Y (2007) Effect of flowering tree density on the mating system and gene flow in *Shorea leprosula* (Dipterocarpaceae) in Peninsular Malaysia. *Journal of Plant Resource*, 120, 413 - 420.

Gailing O, Leinemann L, Finkeldey R (2003) Molecular Tool for the conservation of forest genetic resource. Göttingen, Georg-August University of Göttingen.

Ghazoul J, Liston KA, Boyle TJB (1998) Disturbance-induced density-dependent seed set in *Shorea siamensis* (Dipterocarpaceae), a tropical forest tree. *Journal of Ecology* , 86, 462 - 473.

Goodnight KF [2009] Relatedness version 5.0.8 , [Online]. Available from: www.gsoftnet.us/Gsoft.html [Cited 15 February 2010].

Goudet J (1995) *FSTAT: A program to estimated and test gene diversities and fixation indices, version 2.9.3*. [Online]. Available from: www/unil.ch/izea/software/fstat [Cited 13 March 2010].

Hamrick JL (1982) Plant population genetics and evolution. *American Journal of Botany*, 69(10), 1685 - 1693.

Hamrick JL and Murawski (1991) Level of allozyme diversity in population of

uncommon Neotropical tree species. *Journal of Tropical Ecology*, 7, 395 - 399.

Ingleby K, Munro RC, Noor M, Mason, PA , Clearwater MJ (1998) Ectomycorrhiza population and growth of *Shorea parvifolia* (Dipterocarpaceae) seeding regenerating under three different forest canopies following logging. *Forest Ecology and Management*, 111, 171 - 179.

Isagi Y, Kanazashi T, Suzuki W, Tanaka H, Abe T (2000) Microsatellite analysis of the regeneration process of *Magnolia obovata* Thunb. *Heredity*, 84, 143 - 151.

Kalinowski, ST, Taper ML, Marshall TC (2007) Revising how the computer Program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* , 16, 1099 - 1006.

Kitamura K, Rahman MYBA, Ochai Y, Yoshimaru H (1994) Estimation of the outcrossing rate on *Dryobalanops aromatic* Gaertn. F. in primary and secondary forest in Brunei, Borneo, Southeast Asia. *Plant Species Biology*, 9, 37 - 41.

Kimura M and Crow JF (1964) The number of allele that can be maintained in the finite population. *Genetics*, 49, 725 - 738.

Konishi T, Harada K, Chong L, Chai E, Kendawang JJ, Lee HS, Yamakura T, Itoh A, Sakura K, Ogino K (2004) Comparative study of AFLP and microsatellite variation in four Dipterocarp species from natural and artificial populations in Sarawak, Malaysia. *TROPICS*, 14(1), 75 - 86.

Konuma A, Tsumura Y, Lee CT, Lee SL, Okuda T (2000) Estimation of gene flow in the tropical - rainforest tree *Neobalanocarpus heimii* (Dipterocarpaceae), inferred from paternity analysis. *Molecular Ecology*, 9, 1843 - 1852.

Lagercrantz U, Ellegren H , Anderson L (1992) The abundance of various polymorphic microsatellite motif differs between plants and vertebrates. *Nucleic Acid Research*, 21, 111 - 1115.

Latouche - Halle C, Ramboier A, Bandou E, Caron H, Kremer A (2004) Long – distance pollen flow and tolerance to selfing in a Neotropical tree species. *Molecular Ecology*, 13, 1055 - 1226.

Lee SL (2000) Mating system parameters of *Dryobalanops aromatic* Gaertn.f

(Dipterocarpaceae) in three different forest types and a seed orchard. *Heredity*, 85, 338 - 345.

Lee SL, Wickneswari R, Mahani M, Zakri AH (2000a) Genetic diversity of a tropical tree species, *Shorea leprosula* Miq. (Dipterocarpaceae), in Malaysia: Implications for conservation of genetic resources and tree improvement. *Biotropica*, 32(2), 213 - 224.

Lee SL, Wickneswari R, Mahani M, Zakri AH (2000b) Mating System Parameters in a Tropical Tree Species,, *Shorea leprosula* Miq. (Dipterocarpaceae), from Malaysia: Lowland Dipterocarp Forest. *Biotropica*, 32(4a), 693 - 702.

Lee SL, Tani N, Ng KKS, Tsumura Y (2004) Isolation and characterization of 20 Microsatellite loci for an important tropical tree *Shorea leprosula* (Dipterocarpaceae) and their applicability to *S. parvifolia*. *Molecular Ecology Notes*, 4, 222 - 225.

Liengsiri C (1999) *Genetic variation studies in Pterocarpus macrocarpus* Kurz *as revealed by isozyme, morphological and physiological trait*. PhD thesis. University of Alberta Edmonton, Alberta.

Liewlaksaneeyanawin C (2006) *Genetic evaluation of natural and domesticated logepole pine populations using molecular marker*. Ph.D. thesis University of British Columbia, British Columbia.

Lim S, Wickneswari R, Lee SL, Latiffa A (2001) Genetic structure of natural population of *Dryobalanops aromatic* Gaertn.f (Dipterocarpaceae) in Peninsular Malaysia using microsatellite DNA markers. In: *In Situ and Ex Situ conservation of Commercial Tropical Trees: Proceeding of the Contribution of Genetic Resources Conservation to Tree Breeding, Biotechnology, and Future Commercial Plantation Program . 11 - 13 June 2001*, Yogyakarta, Indonesia. pp 309 - 324.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, 27, 209 - 220.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, 7, 639 - 655.

Maury-Lechon L and Curted L (1998) Biogeography and evolution systematices of

Dipterocarpaceae. In: Appanah S, Turnbull JM (eds.) *A review of Dipterocarps: taxonomy, ecology and silviculture.* Bogor Indonesia: Center for International Forestry Research, pp 5 - 44.

Miller MP (1997) *Tools for population genetic analyses (TFPGA) version 1.3: A window program for the analysis of allozyme and molecular population genetic data* [Online]. Available from: www.marksgeneticsoftware.net/tfpga.htm [Cited 25 March 2010].

Missouri Botanical Garden (2007) *Index of Plant Chromosome Number.* [Online]. Available from: http://www.mobot.mobot.org/ [Cited 5 November 2007]

Murawski DA and Bawa KS (1994) Genetic structure and mating system of *Stemonoporus oblonglifolius* (Dipterocarpaceae) in Sri Lanka. *American Journal of Botany*, 81, 155 - 160.

Murawski DA and Hamrick JL (1992a) The mating system of *Cavanillesia platanifolia* under extremes of flowering tree density: a test of predictions. *Biotropica*, 24, 99 - 101.

Murawski DA and Hamrick JL (1992b) Mating system and phenology of *Ceiba pentandra* (Bombacaceae) in Central Panama. *Heredity*, 83, 401 - 404.

Murawski DA, Dayanandan B, Bawa KS (1994) Outcrossing rates of two endemic *Shorea* sp. from Sri Lankan tropical rain forests. *Biotropica*, 26, 23 - 29.

Murawski DA, Gunatilleke IAUN, Bawa KS (1994) The effect of selective logging on inbreeding in *Shorea megistophylla* (Dipterocarpaceae) from Sri Lanka. *Conservation Biology*, 8(4), 997 - 1002.

Myers M, Mittermeier RA, Mittermeier CG, da Fonseca GAB , Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature*, 403, 853 - 858.

Nason JD, Herre EA, Hamrick JL (1998) The breeding structure of a tropical keystone plant resource. *Nature*, 391, 685 - 687.

Nei M (1972) Genetic distance between populations. *American Naturalist*, 106, 283 - 292.

Nei M (1973) Analysis of gene diversity in subdivided population. *Proceeding of the Natural Academic of Science of the USA*, 70, 3321 - 3323.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small

number of individual. *Genetics*, 89, 583 - 590.

Nei (1987) Molecular evolution and genetics. Columbia University Press, New York .

Ng KKS, Lee SL, Saw LG, Plotkin JB, Koh CL (2004) Spatial structure and genetic diversity of two tropical tree species with contrasting breeding system and different ploidy level. *Molecular Ecology*, 13, 657 - 669.

Ouborg NJ, Piquot Y, van Groenendale (1999) Population Genetics, Molecular markers and the Study of Dispersal in Plants. *The Journal of Ecology*, 87(4), 551 - 568.

Pakkad G, Ueno S, Yoshimaru H (2008) Gene flow pattern and mating system in a small population of *Quercus semiserrata* Roxb. (Fagaceae). *Forest Ecology and Management*, 255, 3819 - 3826.

Pandey M (2005) *Development of microsatellite in sycamore maple (Acer pseudoplatanus L.) and their application in population genetics.* Ph.D. thesis Georg-August University of Göttingen.

Pemberton JM, Slate J, Bancroft DR, Barrett JA (1995) Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. *Molecular Ecology*, 4, 249 - 252.

Perera L, Russell JR, Provan J, Powell W (2003) Studying genetic relationships among coconut varieties/populations using microsatellite markers. *Euphytica* , 132, 121 - 128.

Perotti E, Grimanelli D, John P, Hoisington D, Leblance O (2006) Why is transferring apomixes to crops still a dream? [Online]. Available from: http://www.cropscience.org.au/icsc2004/poster/3/2/1/1367_perottie.htmlprint=1 [Cited 7 January 2011].

Pooma R (2003) *Dipterocarpaceae in Thailand:Taxonomic and Biogeographical analysis.* [Online]. Available from: http://www.dnp.go.th/Botany/publication_online/RP_thesis. [Cited 10 November 2007].

Pooma R and Newman M (2001) Checklist of Dipterocarpaceae inThailand. *Thai Forest Bulletin (Botany)*, 29, 110 - 187.

Pramual P, Kurangkadilok C, Baimai V, Walton C (2005) Phylogeography of the

black fly *Simulium tani* (Diptera: Simuliidae) from Thailand as inferred from mtDNA sequence. *Molecular Ecology*, 14, 3989 - 4001.

Queller DC and Goodnight KF (1989) Estimating relatedness using genetic marker. *Evolution*, 43, 258 - 275.

Ratnam W and Seng HW (2003) Determination of genetic relatedness of selected individual trees of *Shorea leprosula* Miq. and *Dipterocarpus cornunus* Dyer in forest seed production areas. *Tropics* , 13(2),139 - 149.

Raymond M and Rousset F (1995) *Genepop Web Version 3.4.0* [online]. Available from: www.genepop.curtin.edu.au/genepop [Cited 20 March 2010].

Ribeiro RA and Lovato MB (2004) Mating system in a neotropical tree species. *Senna multijuga* (Fabaceae). Genetics and Molecular Biology, 27 (3), 418 - 424.

Ritland K (1989) Correlated - mating in the partial selfer, Mimulus guttatus. *Evolution*, 43, 848 - 859.

Ritland K (2002) Extensions of models for the estimation of mating system using n independent loci. *Heredity*, 88, 221 - 228.

Ritland K (2008) MLTR: Multilocus mating system program version 3.2. [Online]. Available from: http://genetics.forestry.ubc.ca/ritland/programs [Cited 22 December 2010].

Ritland C and Riltand K (2000) DNA - Fragment Marker in Plants. In: Allan J. Baker (ed). *Molecular methods in ecology*. Blackwell Science Ltd., pp 208 - 234.

Roa AC, Chavarriaga - Aguirre P, Duque CM, Maya MM, Bonierbale MW, Iglesias C, Tohme J (2000) Cross - species amplification of cassava (*Manihot esculenta*) (Euphorbiaceae) microsatellite: allelic polymorphism and degree of relationship of relationship. *American Journal of Botany*, 87 (11), 1647 - 1655.

Rohlf FJ (2000) *NTSYS-pc. Numerical Taxonomy and Multivariate Analysis System version 2.1*. Applied Biostattistic Inc., New York.

Rousset F (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, 145, 1219 - 1228.

Sato T, Isagi Y, Sakio H, Osumi K, Goto S (2006) Efeect of gene flow on spatial genetic structure in the riparial canopy tree *Cercidiphyllum japonicum* revealed by microsatellite analysis. *Heredity*, 96, 79 - 84.

Selkoe KA and Toonen RS (2006) Microsatellites for ecologist: practical guide to

using and evaluating microsatellite marker. *Ecology Letter*, 9, 615 - 629.

Shiva MP and Jantan I (1998) Non - Timber Forest Product from Dipterocarps,. In: Appanah S, Turnbull JM (eds.) *A review of Dipterocarps: taxonomy, ecology and silviculture*. Bogor Indonesia: Center for International Forestry Research, pp 187 - 198.

Slatkin M (1985) Rare alleles as indicators of gene flow. *Evolution*, 39, 53 - 63.

Smouse PE and Sork VI (2004). Measuring pollen flow in forest trees an exposition of alternative approaches. *Forest Ecology and Management*, 197, 21 - 38.

Sork VL, Nason J. Campbell DR, Fernandez JF (1999) Landscape approaches to historical and contemporary gene flow in plant. *Tree*, 14(6), 219 - 223.

Soontornchainaksaeng P, Chantaranothai P, Senakun C (2003) Genetic Diversity of *Croton* L. (Euphorbiaceae) in Thailand. *Cytologia*, 68(4), 379 - 382.

Takeuchi Y, Ichikawa S, Konuma A, Tomaru N, Niiyama K, Lee SL, Muhammad N, Tsumura Y (2004) Comparison of the fine-scale genetic structure of three dipterocarp species . *Heredity*, 92, 323 - 328.

Tennakoon MMD, Gunatilleke IAUN, Hafeel KM, Seneviratne G, Gunatilleke, CVS, Ashton PMS (2005) Ectomycorrhizal colonization and seeding growth of *Shorea* (Dipterocarpaceae) species in simulated shade environment of Sri Lankan rain forest. *Forest Ecology and Management*, 208, 399 - 405.

The Forest Herbarium Royal Forest Department (BKF) (2001) *Thai Plant Names Tem Smitinand revised 2001*, The Forest Herbarium Royal Forest Department.

The World Conservation Union (IUCN) [2007] *Red List of Threatened Species*. [Online]. Available from: www.iucnredlist.org [Cited 29 October 2007].

Vendramin GG, Scotti I, Ziegenhagen B (2004) Microsatellite in Forest tree Species Characteristics, Identification and Application. In Kumar S and Fladung M (eds.) *Molecular Genetic and breeding of forest trees*. New York: Food Products Press, pp 359 - 386.

Walton C, Handley JM, Collins FH, Baimai V, Harbach RE, Deesin V, Butlin RK (2001) Genetic population structure and introgression in *Anopheles dirus* mosquitoes in South-east Asia. *Molecular Ecology*, 10, 569 - 580.

Wang J, Qigang Y, Kang M (2007) Novel polymorphic microsatellite loci and

patterns of pollen-mediated gene flow in an ex situ population of *Eurycorymbus cavaleriei* (Sapindaceae) as revealed by categorical paternity analysis. *Conservation Genetic* DOI 10.1007/s10592-007-9369-0.

Weir BS and Cockerham CC (1984) Estimating F - statistics for the analysis of population structure. *Evolution*, 38, 1358 - 1370.

White GM, Boshier DH, Powell W (2002) Increase pollen flow counteracts fragmentation in a tropical dry forest; an example from *Swietenia humilis* Zuccarini. *Proceeding of the Natural Academic of Science of the USA*, 99, 2038 - 2204.

White TL, Adams WT, Neale DB (2007) *Forest Genetics*. UK, CAB International.

Ujino T, Kawahara T, Tsumura Y, Nagamitsu T, Wickneswari R , Yoshimaru H (1998) Development and polymorphism of simple sequence repeat DNA markers for *Shorea curtisii* and other Dipterocarpaceae species. *Heredity*, 81, 422 - 428.