Gene flow pattern and mating system in a small population of *Quercus semiserrata* Roxb. (Fagaceae)

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ABSTRACT

Pollen flow from external sources is important for the conservation of tree species in fragmented forests or small populations, because it can be sufficient to prevent differentiation among them, and appears to be able to prevent the loss of their genetic diversity through genetic drift. In this study, we examined the genetic heterogeneity of pollen pools accepted by each *Quercus semiserrata* seed parent at the Khun Wang Royal Agriculture Research Center, Thailand, both within and among two mast fruiting years (2005 and 2007), using paternity analysis and analysis of molecular variance (AMOVA). The mating systems of the trees were also examined using the multilocus mating system model (MLTR), after determining the genotypes at eight microsatellite loci of 26 seed-trees and 435 seeds from 8 seed-trees in the 2 mast fruiting years. The average distance of effective pollen flow within the plot was estimated to be 52.4 m, and 95% of effective pollen was dispersed within 200 m, indicating that effective pollen flow is highly localized and that most effective pollen is contributed by near-neighbor trees. The proportion of effective pollen that immigrated from external sources was estimated to be 26.2%. The AMOVA analysis based on the pollen haplotypes showed that the pollen pools, both total and for each reproductive year, significantly genetically differed among the seed parents. Using a mixed mating model, the estimate of biparental inbreeding for the total population ($t_m - t_s$) was 0.013, indicating that a low proportion of mating occurred among close relatives. The effective number of pollen donors (N_{ep}) was estimated to be 9.987 using the TwoGener model, or 10.989 using the mixed mating model. The effective number of pollen donors of seeds was higher in the mast fruiting year 2005 than in the other examined year, 2007. Consequently the allelic richness and genetic diversity of seeds produced in 2005 were higher than those produced in 2007. Overall, the results show that high outcrossing rates, high levels of gene flow from other populations and heterogeneity in the pollen received by an individual may enhance the ability of populations to maintain effective population sizes. Therefore, these processes may be sufficient to prevent loss of genetic diversity through genetic drift of *Q. semiserrata* at this study site.

1. Introduction

Gene movement among populations (i.e. gene flow) and within populations can significantly affect evolutionary processes such as natural selection and random genetic drift. Consequently it can have important effects on the spatial genetic structure of populations. However, the magnitude of effects of drift and selection on patterns of genetic variation will depend on the reproductive ability of the organism and the rate of gene flow (Gaiotto et al., 2003). In plant species that produce large, immobile seeds (e.g. oaks, hickory and walnut), pollen flow is probably the most important component of gene flow (Dow and Ashley, 1998).

Pollen flow from external sources is important for the conservation of tree species in fragmented forests or small populations, because it can be sufficient to prevent differentiation among them, and appears to be able to prevent the loss of their genetic diversity through genetic drift (Hamrick et al., 1989).

Microsatellites have become the preferred markers for studying the genetic diversity of natural plant populations, quantifying gene flow among them, and identifying populations that should be prioritized for conservation (Dow et al., 1995; Chase et al., 1996; Dayanandan et al., 1997; Streiff et al., 1998; Ueno et al., 2000). Furthermore, microsatellite loci tend to harbor high levels of polymorphism, and thus are useful for identifying pollen parents and characterizing pollen flow at local scales. Several methods have been developed for directly measuring pollen-mediated gene flow and distances of pollen dispersal, most of which rely on paternity exclusion (Smith and Adams, 1983; Devlin and Ellstrand,

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1990; Burczyk and Chybicki, 2004; Marshall et al., 1998) or paternity assignment (Devlin et al., 1988; Chase et al., 1996; Dow and Ashley, 1998; Streiff et al., 1999). CERVUS 2.0 (Marshall et al., 1998) is widely used software for paternity analysis based on the former approach, i.e. determining the parents of offspring by comparing genotypes of candidate fathers and offspring after subtracting the maternal contribution to each offspring.

In wind-pollinated woody plant species, pollen flow often occurs over long distance and generally depends on the distance and direction of pollen parents relative to the seed parent (Streiff et al., 1999; Burczyk and Chybicki, 2004). In addition, since there is often substantial interannual variation in flowering at individual and pollen levels, the genetic composition of the pollen pools accepted by different seed parents is likely to differ both within and among reproductive years (Streiff et al., 1999). Therefore, single-season studies may provide limited indications of mating patterns. The effective pollen donor size per generation is of interest, since annual variations in pollen composition received by an individual are likely to increase genetic variation among progeny (Irwin et al., 2003). Recently, Smouse et al. (2001) developed a new model of molecular variance, dubbed the TwoGener model (parent–offspring), designed to facilitate the characterization of pollination patterns using relatively small samples of offspring. This model provides an indirect method of estimating pollen movement that is a hybrid of traditional paternity analysis and genetic structure analysis, allowing us to quantify heterogeneity among the pollen gene pools of seed samples from individual seed parents scattered across the landscape, in association with dispersion functions. This method does not require knowledge of the genotypes of all seed parent trees in the studied plot. In addition, the TwoGener model allows effective numbers of pollen donors and mean pollination distances to be estimated.

The mating system is a key determinant of the spatial genetic structure within and among populations, and strongly influences both the extent of inbreeding and genetic differentiation among populations. Knowledge of mating systems is helpful for forest conservation, tree breeding, and targeted seed collection for environmental reforestation strategies (Bittencourt and Sebbenn, 2007). The mixed mating system model, as implemented in MLTR software (Ritland, 2002, 2004) is widely used to examine the mating systems of plant species. In this model, the mating system of plants can be summarized by estimates of the outcrossing rate, mating with relatives, and the probability of two progeny having the same mother and father (Ritland and Jain, 1981). In addition, information about the probability that two progenies share the same father can reveal the extent of diversity in pollen donor pools, and provide indications of the effective number of pollen donors for a given maternal plant (Ritland, 1989). Simultaneous use of paternity analysis, the TwoGener model (AMOVA) and the mixed mating system model can provide complementary information on the temporal and spatial genetic heterogeneity of pollen flow, variations in pollen pools at local scales and pollen dispersal distances.

Quercus semiserrata Roxb. (Fagaceae) is a large, late successional, evergreen tree with a dense crown and straight stem, reaching heights up to 30 m and diameters at breast height of up to 100 cm. It occurs in scattered locations in mixed evergreen/deciduous and deciduous evergreen/pine forests, at elevations of 850–1400 m, in northern Thailand, and various other wet, tropical hill forests in India, Myanmar and Indo-China (FORRU, 2000). It is being planted in northern Thailand as a "framework species" (one of 20–30 native tree species being planted in mixtures to provide a framework for re-establishing biodiversity) following nursery and field trials at the Forest Restoration Research Unit of Chiang Mai University, Thailand (Elliott et al., 2003). Flowering occurs in spring, in the hot, dry season before the onset of monsoon rains,

and fruiting from November to March. As a rule, the acorns drop in January simultaneously with partial leaf shedding. We previously examined the genetic diversity and differentiation of Q. semiserrata within and between ten populations in northern Thailand using nuclear and chloroplast microsatellite markers (Pakkad et al., 2008). The results suggest that four populations (located at the Khun Wang Royal Agricultural Research Center, Obluang National Park, Doi Suthep National Park and Doi Inthanon National Park) had the highest genetic diversity and high numbers of chloroplast haplotypes, and thus should be given the highest priority for conservation of this species. However, in the previous study we did not examine the species' pollen dispersal and pollen pool patterns, or mating system, although knowledge of these features is essential for understanding the reproductive processes and developing efficient strategies to conserve viable populations. Therefore, the objectives of this study were to investigate the genetic heterogeneity of pollen pools accepted by individual seed parents both within and among reproductive years, and to evaluate the mating system of Q. semiserrata, using paternity analysis, analysis of molecular variance (AMOVA) and multilocus mating system analysis (MLTR).

2. Materials and methods

2.1. Study site

The study site was located in the grounds of the Khun Wang Royal Agriculture Research Center (approximately 18°37′52.02″N, 98°29′49.68″E, Chiang Mai province, Thailand), a primary objective of which is develop alternatives to replace opium poppy cultivation and improve conditions for people living in villages in the surrounding hills. It is situated at 1280 m above sea level along a ridge top and is the highest establishment in the Mae Wang sub-watershed of the Mae Khan watershed. The study site was cleared of evergreen forest that had previously covered it approximately 50 years ago, to provide land for cultivating cabbages, corn, potatoes and other cash crops. There are now sparsely scattered tree across the site, and it is surrounded by a remnant forest. The average temperature and annual rainfall are 19.5 °C and 2137 mm, respectively.

All 26 mature trees (density, ca. 2.4 trees/ha) at the study site were mapped, and young leaves were sampled from all of them for DNA extraction. In addition, acorns were collected from eight seed parents in early April in both 2005 and 2007 (mast fruiting years). One hundred acorns per seed parent per year were collected and sown in modular germination trays. The first true leaves produced by all germinated seeds from each seed parent were harvested, stored at −80 °C and used at a later date for DNA extraction. Total genomic DNA was extracted from the leaves of each sampled tree using the modified CTAB method described by Murray and Thompson (1980).

2.2. Microsatellite markers and genotyping

Eight nuclear microsatellite markers were selected for genotyping Q. semiserrata: Qm50-3M developed for Quercus myrsinifolia (Isagi and Suhandono, 1997), CA15 developed for Q. salicina (Kawahara, personal communication), ssrQpZag9 and ssrQpZag46 developed for Q. petraea (Steinkellner et al., 1997), quru-GA-1C08 and quru-GA-0C19 developed for Q. rubra (Preston et al., 2002), and bcqm07 and bcqm96 developed for Q. magnolica var. crispula (Mishima et al., 2006). The sequences of forward and reverse primers for the locus CA15 were CGGTAAGACGTTTGGTGTAG and TTGTACGGACGCCATTGAAA, respectively.

PCR amplification was performed in 10 μl reaction mixtures containing 10 ng of template DNA, 1× PCR buffer (20 mM Tris–HCl

pH 8.3, 50 mM KCl), 200 μM of each dNTP, 1.5 mM MgCl$_2$, 0.2 μM of each primer and 0.25 units of *Taq* polymerase. The PCR conditions were: an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, annealing temperature for 30 s, 72 °C for 30 s, then a final extension at 72 °C for 7 min. PCR products were separated using a 3100 Avant Genetic Analyzer with GeneScan software (Applied biosystems).

2.3. Statistical data analysis

The genetic diversity at the eight nSSR loci of adult trees and seed populations was quantified in terms of the number of alleles per locus (*A*), allelic richness (Ar) (El-Mousadik and Petit, 1996), the mean observed heterozygosity (*H*$_O$), and gene diversity (*H*) (Nei, 1987) using FSTAT 2.9.3 software (Goudet, 1995) for each locus and averaged over all loci. Deviations from Hardy–Weinberg expectations and inbreeding coefficients (*F*$_{IS}$, Wier and Cockerham, 1984) were determined using Genepop web Version 3.4 (Raymond and Rousset, 1995; http://genepop.curtin.edu.au/genepop) and their significance (*F*$_{IS} \neq 0$) was tested by 1000 permutations. The paternity exclusion probability for second parents was estimated using the program CERVUS Version 2.0 (Marshall et al., 1998).

In addition, paternity analysis was performed using CERVUS Version 2.0 (Marshall et al., 1998) to assign paternity to tested seedlings, at a threshold 95% as strict and 80% as relaxed confidence level as suggested by Marshall et al. (1998), using log-likelihood ratios (LOD score) based on the observed multilocus genotypes of all adults and offspring. Confidence levels were determined through simulation and defined by the statistic delta (Δ); the difference between the LOD scores of the two most likely candidates. The simulation parameters were as follows: 10,000 cycles, 26 individuals as candidate parents, 100% as the proportion of candidate parents sampled, 100% as the proportion of loci typed, 1% as the rate of scoring or typing errors. The error rate per locus was also estimated by determining the frequency of offspring that were incompatible with the seed parent at a given locus using the CERVUS program. Pollen dispersal distances were calculated based on the locations of seed parents and putative pollen donors within the plot.

The following *mating system parameters*—multilocus outcrossing rate (*t*$_m$), single-locus outcrossing rate (*t*$_s$), outcrossing rates among relative trees (*t*$_m$ − *t*$_s$) and multilocus paternity correlation coefficient (*r*$_p$) were estimated for each reproductive year and over both reproductive years. These parameters were analyzed using the maximum likelihood procedures of Ritland and Jain (1981) as implemented in the multilocus mating system program MLTR (Ritland, 2004). The expectation–maximization procedure that bound outcrossing rates between 0 and 1 was used for the iterations, and default settings 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 populations. Parental genotypes were estimated from the original dataset (not bootstrap means).

2.3.1. TwoGener analysis

The pollen pool structure was analyzed using the TwoGener approach (Smouse et al., 2001), in which the genetic structure of pollen pools sampled by individual trees is compared to the global pollen pool. The procedures were as follows: the parental contribution of the seeds was deduced by subtracting the female gamete contribution from the diploid genotype of each seed, locus by locus. The population of deduced haplotypes was then analyzed using AMOVA (Excoffier et al., 1992) based on the pairwise squared Euclidean distances between the pollen profiles. *Φ* statistics (analogs of Wright's *F*$_{ST}$ values) were calculated for both the spatial genetic variation of the pollen pools among seed parents for overall reproductive years (*Φ*$_{ft}$) and genetic variation of pollen pools among reproductive years for all seed parents (*Φ*$_{yf}$). The significance of the *Φ*$_{ft}$ values was tested by 1000 randomizations (Excoffier et al., 1992). Pairwise *Φ*$_{ft}$ values, as genetic distances between the pollen pools of the seed parents were also obtained, and their significance was tested by 1000 randomizations after Bonferroni correction. Two-Gener analysis was performed using POLDISP 1.0b (Robledo-Arnuncio et al., 2007) under the exponential model. The estimates of *Φ*$_{ft}$ were used to calculate estimates of the average distance of pollination (*δ*), the effective number of pollinators (*N*$_{ep}$) and the effective pollination neighborhood (*A*$_{ep}$) (Smouse et al., 2001).

3. Results

3.1. Characteristics of the eight polymorphic microsatellite loci

The eight microsatellite loci were found to be highly polymorphic, numbers of alleles detected ranging from 6 to 14, at QM50-3M and bcqm07, respectively (data not shown). In total 82 alleles were identified among the 26 adult trees and 435 seeds, with a mean of 10.25 per locus. The expected heterozygosity (*H*$_E$) for each locus over all parental and offspring populations ranged from 0.533 (Qm50-3M) to 0.859 (quru-GA-1C08), with a mean of 0.703 over all loci (Table 1). Fixation indices (*F*$_{IS}$, Weir and Cockerham) were negative and did not deviate significantly from zero for all loci, indicating an excess of heterozygotes and that null alleles at these loci are rare.

3.2. Genetic diversity of the parental and offspring populations

Diversity parameters for the eight polymorphic microsatellite loci in the seed parent and offspring populations are presented in

Table 1
Characteristics of the eight microsatellite loci

Locus	Seed parent					Offspring				
	N	*A*	*H*$_O$	*H*$_E$	*F*$_{IS}$	*N*	*A*	*H*$_O$	*H*$_E$	*F*$_{IS}$
ssrQpZag46	8	4	0.875	0.750	−0.181	431	8	0.722	0.679	−0.063
CA15	8	5	0.625	0.725	0.146	434	14	0.721	0.679	−0.062
QM50-3M	8	2	0.250	0.500	0.517	434	6	0.594	0.528	−0.126
quru-GA-1C08	8	6	1.000	0.842	−0.204	433	12	0.912	0.857	−0.065
bcqm96	8	6	0.750	0.833	0.106	433	12	0.824	0.798	−0.034
bcqm07	8	5	0.875	0.767	−0.153	433	13	0.751	0.756	0.007
quru-GA-0C19	8	5	0.875	0.750	−0.181	432	7	0.706	0.713	0.010
ssrQpZag9	8	3	0.750	0.608	−0.254	431	8	0.571	0.592	0.035
Average		4.5	0.750	0.722	−0.042		10	0.725	0.700	−0.037
Total	36					80				

N, number of samples analyzed; *A*, number of alleles detected; *H*$_O$, observed heterozygosity; *H*$_E$, expected heterozygosity; and *F*$_{IS}$, fixation index.

Table 2
Genetic diversity of pollen pools accepted by each of the eight *Quercus semiserrata* seed parents examined in the paternity analysis in each reproductive year and over both reproductive years

Seed parent	Reproductive year	N	A	Ar	H
66	2005	21	6.1	5.501	0.667
	2007	40	6.1	4.943	0.639
	Total	61	7.3	5.232	0.649
70	2005	15	4.9	4.806	0.620
	2007	39	4.8	3.726	0.567
	Total	54	5.6	4.094	0.685
71	2005	18	5.3	4.885	0.620
	2007	33	4.8	3.916	0.601
	Total	51	5.9	4.283	0.607
73	2005	22	5	4.439	0.599
	2007	22	4.5	3.996	0.555
	Total	44	5.4	4.164	0.581
74	2005	28	6	4.964	0.638
	2007	41	5.8	4.310	0.581
	Total	69	7	4.542	0.604
75	2005	14	5.6	5.625	0.604
	2007	23	4.6	4.110	0.580
	Total	37	6.4	4.649	0.589
76	2005	35	6.5	5.414	0.668
	2007	47	8	5.638	0.655
	Total	82	8.3	5.549	0.662
79	2005	21	5.8	5.217	0.681
	2007	16	5	4.868	0.678
	Total	37	6.1	5.180	0.682

N, number of seeds analyzed; A, number of alleles detected; Ar, allelic richness; H, gene diversity.

Table 1. In the 8 seed parents, the total number of alleles (A) detected was 36, with a mean of 4.5 across loci, while the average expected heterozygosity, gene diversity and F_{IS} values across loci were 0.750, 0.722 and −0.042, respectively. For offspring populations, averaged over all loci, the number of alleles detected per locus in the pollen pool in each reproductive year for each seed parent ranged from 4.5 to 8.0. The corresponding average and total numbers over all reproductive years for each seed parent ranged from 4.8 to 7.3, and from 5.4 to 8.3, respectively. The allelic richness (Ar) in the pollen pool in each reproductive year for each seed parent, calculated for 14 gene copies, ranged from 3.73 in 2007 for seed parent no. 70, to 5.64 in 2007 for seed parent no. 76. Gene diversity in the pollen pool in each reproductive year for individual seed parents ranged from 0.56 in seed parent no. 73 in 2007 to 0.68 in seed parent no. 79 in 2007 (Table 2). The mean and total values of allelic richness over both reproductive years for each examined seed parent ranged from 4.22 to 5.53, and from 4.09 to 5.55, respectively, and the corresponding values for gene diversity ranged from 0.58 to 0.68, and 0.58 to 0.69, respectively. In most cases, the number of alleles detected, allelic richness and gene diversity in the pollen pool across loci were higher for 2005 than the corresponding values for 2007, for all seed parents, except seed parent no. 76. Comparison of diversity in the parental and offspring populations across loci showed that the mean number of alleles per locus was greater in the offspring population than in the parental population. In contrast, observed heterozygosity and gene diversity across loci were greater in the parental population than in the offspring population.

3.3. Paternity assignment

The total paternity exclusion probability for the second parents over the eight loci was 0.998, and the error rate per locus,

estimated from the frequency of mismatches between seeds and their putative seed parents at a given locus, was 0.0068. The per seed error rate was $1 − (1 − 0.0068)^8$ (0.053). The numbers of seeds analyzed from each of the seed parents used in the paternity analysis ranged from 37 to 82, in total, over the two study years and from 14 to 47 in either one of the years, providing a grand total of 435 seeds, of which 321 (73.8%) could be assigned fathers in the study plot based on 95% as strict and 80% as relaxed confidence level. Nine seeds (2.1%), four and five from 2005 and 2007, respectively, resulted from self-pollination. The distance between seed parents and pollen donors within the study plot ranged from 0 (self-pollination) to 570 m, with an average of 60.47 m (±82.03 m S.D.) for 2005, 47.27 m (±61.17 m S.D.) for 2007 and 52.4 m (±70.2 m S.D.) overall. About 95% of the assigned fathers of seeds were less than 200 m from their respective mother-trees. The remaining 114 (26.2%) seeds, 49 and 65 seeds for 2005 and 2007, respectively, could not be assigned fathers, presumably because the true fathers were located outside the study plot. The probability of cryptic gene flow, calculated from the allele frequencies (Westneat and Webster, 1994) was $1 − 0.998^{26}$ (0.051). Therefore, the number of seed that matched unrelated by chance was likely to be *ca.* $321 × 0.051$ (16.4), and equivalent to 3.77% of the total number of 435 seeds.

3.4. Genetic heterogeneity among the pollen pools

The AMOVA analysis based on the pollen haplotypes of 435 seeds showed that the total pollen pools genetically differed significantly among the seed parents (Φ_{ft} = 0.047, $P < 0.001$). Furthermore, the pollen pools of the seed parents also differed significantly between the two reproductive years, and the total pollen pool differentiation was higher for 2007 than 2005 (Φ_{ft} = 0.065 and 0.030, respectively, $P < 0.001$ in both cases). The estimates of Φ_{ft} were further used to calculate, for 2005, 2007 and overall: the average distance of pollination (δ: 109.2, 68.3 and 81.4 m, respectively), the effective number of pollen donors (N_{ep}: 17.925, 7.031 and 9.987, respectively) and the effective neighborhood pollination area (A_{ep}: 7.489, 2.930 and 4.161 ha, respectively). The average distance of pollination obtained from the TwoGener model was higher than those obtained from paternity analysis, because the distances travelled by pollen from outside the study plot that sired seeds were included in the calculations.

The total pollen pool's differentiation for each seed parent between reproductive years (Φ_{yf}), ranged from −0.001 to 0.04 (for seed parent nos. 71 and 70, respectively). For seed parent nos. 70 and 79, genetic differentiation of pollen pools was significant among reproductive years nested within each seed parent (Φ_{YF} = 0.04 and 0.034, $P < 0.05$, respectively). For seed parent nos. 66, 73, 74, 75 and 76, there was no significant genetic differentiation of pollen pools between the two reproductive years examined.

3.5. Mating system

The multilocus outcrossing rate (t_m) was estimated to be 0.992 and 1.000 for 2005 and 2007, respectively, and 0.995 overall. All t_m values were not significantly less than 1.0. The single-locus outcrossing rate for 2005 was 0.998, and not significantly less than 1.0, but it was significantly less than 1.0 for 2007, and overall (Table 3). Consequently, the estimate of biparental inbreeding ($t_m − t_s$) was not significantly greater than zero for 2005, but was significantly greater than zero for 2007, and overall, indicating that a small amount of biparental inbreeding occurred in 2007. The multilocus paternity correlation coefficients (r_p) were significantly different from zero for 2005, 2007 and overall, but they were low. These results suggest that most seeds within families had different fathers (i.e. they were half-sibs). The multilocus paternity

Table 3
Results from the mixed mating system and TwoGener pollen structure model analysis

Model	Parameter	Reproductive year		Total
		2005	2007	
Mixed mating model (MLTR)	Parental inbreeding, F	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)
	Multilocus outcrossing rate, t_m	0.992 (0.007)	1.000 (0.005)	0.995 (0.005)
	Single-locus outcrossing rate, t_s	0.998 (0.007)	0.964 (0.012)	0.982 (0.005)
	Biparental inbreeding, $t_m - t_s$	0.004 (0.008)	0.035 (0.001)	0.013 (0.007)
	Multilocus paternity correlation, r_p	0.084 (0.028)	0.113 (0.048)	0.091 (0.031)
	Effective number of pollen donors, N_{ep}	11.904	8.850	10.989
	Effective neighborhood pollination area, A_{ep} (ha)	4.960	3.688	4.579
TwoGener analysis	Differentiation in pollen gene pool among seed-parents, Φ_{ft}	0.030 ($P < 0.001$)	0.065 ($P < 0.001$)	0.047 ($P < 0.001$)
	Effective number of pollen donors, N_{ep}	17.925	7.031	9.987
	Average distance of pollinations, δ (m)	109.2	68.3	81.4
	Effective neighborhood pollination area, A_{ep} (ha)	7.489	2.930	4.161

correlation coefficients were used to calculate effective numbers of pollen donors (N_{ep}) and effective neighborhood pollination areas (A_{ep}), both of which were higher for 2005 than for 2007 (Table 3).

4. Discussion

4.1. Gene movement into and within the study site

Microsatellite markers were used for paternity analysis of seeds from 26 Q. semiserrata parental trees located in the grounds of the Khun Wang Royal Agricultural Research Center. The eight microsatellite markers used in this study were highly variable and appeared to be powerful tools for tracing pollen flow in this context. The occurrence of null alleles, mutation and cryptic gene flow can cause the exclusion of actual pollen parents within a study plot. However, the error rate per locus at a given locus and the error rate per seed were very low (0.0068 and 0.053, respectively). Furthermore, the probability of cryptic gene flow, calculated from allele frequencies for the total number of 435 seeds examined, was low (3.77%), and the fixation index values for each locus were negative, indicating that the probability of null alleles occurring at these loci in the studied population was also low. The mutation rate of microsatellites sequences has been estimated to be 10^{-5} to 10^{-4} mutations per locus per generation (Edwards et al., 1992; Ellegren, 1992; Schlötterer and Tautz, 1992). Therefore, false exclusion resulting from null alleles, mutation and cryptic gene flow is unlikely to have seriously biased the results of this study.

The paternity analysis, based on the paternity exclusion method, found that 97.2% of seeds resulted from outbreeding, in accordance with expectations of high outcrossing rates and levels of gene flow in wind-pollinated trees species, particularly oak species (Hamrick and Godt, 1996; Schwarzmann and Gerhold, 1991; Fernandez-Manjarres et al., 2006). Of the 435 analyzed seeds, 114 were sired from pollen originating from trees located outside the study site, and more alleles were detected in the seed population than in the seed parent population. The total immigration rate of Q. semiserrata pollen was found in this study to be 26.2%, increasing to 30.0% after adjusting for the probability of cryptic gene flow. In addition, the rate of pollination by immigrating pollen appears to have been particularly high for seed parent no. 76, which may have been partly attributable to the prevailing wind direction and the fact that this tree is situated near the edge of the study site (in accordance with general expectations that seed parents located near the borders of study sites will capture more immigrating pollen than seed parents located near the centers of the sites). The rate of immigration of Q. semiserrata pollen found in this study is lower than reported values for several other oak species, e.g. 57% in Q. macrocarpa (Dow and Ashley, 1998); 52.2% in Q. salicina (Nakanishi et al., 2004), 38% in Q. petraea, 34% in Q. pyrenaica

(Valbuena-Carabana et al., 2005), 65% in Q. robur and 69% in Q. petreae (Ducousso et al., 1999). The relatively low rate of pollen immigration was probably due to the Q. semiserrata population at the study site being a fragmented population, surrounded by agricultural land and isolated far from other conspecific populations. The results indicate that gene flow via immigrating pollen into the Q. semiserrata population examined was limited by geographical distances to neighboring populations.

Patterns of pollen dispersal within a study site can be analyzed by identifying individual pollen donors within a site (using paternity analysis) and measuring distances of pollen movement within it (Dow and Ashley, 1998). Father trees were assigned to 321 seeds, and the average distance of pollen flow within the study site calculated from the positions of the respective pollen donors and seed-trees was 52.4 m. Since the proportion of effective pollen flow from outside the plot (and thus further than 500 m) was 26.2%, the average distance of actual pollen flow was greater than this. These distances are similar to those reported for other wind-pollinated species, such as other oaks (Dow and Ashley, 1996; Streiff et al., 1999). Approximately 72.8% and 95% of seeds were sired by pollen from pollen donors within 50 and 200 m of the seed-trees, respectively (Fig. 2), indicating that the effective pollen flow is highly localized and most effective pollen is contributed by near-neighbor trees. Significant excesses of near-neighbor mating have also been detected in Q. macrocarpa by Dow and Ashley (1998) and both Q. petraea and Q. robur by Streiff et al. (1999). Streiff et al. (1999) also found indications of prevalent mating with neighboring trees and a negative exponential pollen dispersion curve, together with large rates of pollen immigration, in the material they examined, indicating that both local dispersion and long-distance dispersion may make substantial contributions to effective pollination in oaks.

4.2. Genetic differentiation of pollen pools

Limitations in pollen dispersion and correlated mating can generate genetic heterogeneities in pollen pools among seed parents (Bittencourt and Sebbenn, 2007). Estimates of genetic differentiation (Φ_{ft}) of the pollen pools for 2005 and 2007, obtained by TwoGener analysis, were 0.030 and 0.065, respectively ($P < 0.001$ in both cases), indicating that there were significant differences in the contributions of potential father trees to the pollen pools accepted by the seed-trees in both years. However, significant differentiation in the pollen pools accepted by each seed parent between the two reproductive years was only detected for seed parent nos. 70 and 79, and not for any of the other seed parents. The results suggest that there was not much genetic difference between the pollen pools accepted by most seed parents in 2005 and 2007, and the exceptions may be due to their locations,
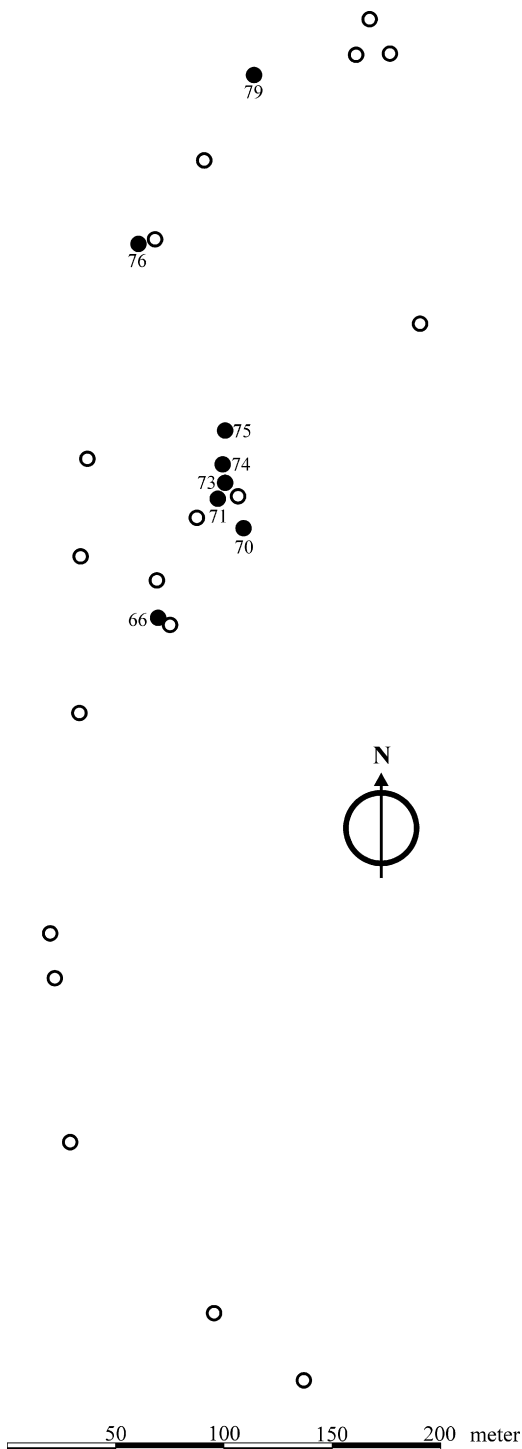
Fig. 1. Spatial location of the 26 *Q. semiserrata* individuals in the Khun Wang Royal Agriculture Research Center.
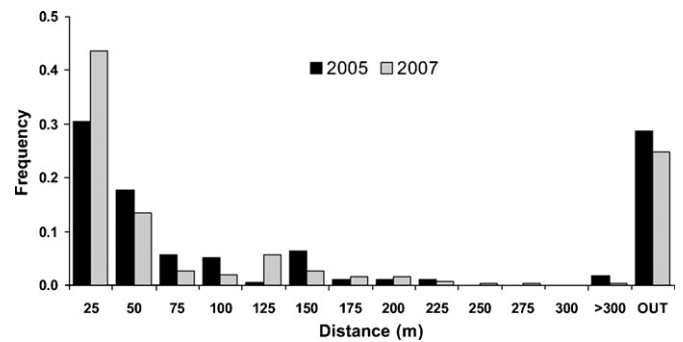

Fig. 2. Frequency distribution of assigned seeds versus distances between seed parents and pollen donors (paternity analysis).

phenology of individual trees and the wind direction during the mating season. High variations in flowering phenology at the individual, population, and annual levels have been found in several oak species, including *Q. robur* and *Q. petraea* (Bacilieri et al., 1995) and *Q. alba* (Sharp and Chisman, 1961), thus significant variations in flowering timing are also likely to occur in *Q. semiserrata*. Variations in wind direction may also have contributed to genetic differences in the pollen pool among seed parents and between years. Unfortunately, however, information on wind parameters was not obtained in this study.

The total pollen pool differentiation (Φ_{ft}) among seed parents was estimated to be 0.047, which is similar to reported values for other oak species, for instance 0.061 in *Q. alba* (Smouse et al., 2001); 0.076 in *Q. velutina* (Fernandez-Manjarres et al., 2006); 0.082 in *Q. humboldtii* (Fernandez-Manjarres and Sork, 2005) and 0.136 in *Q. lobata* (Sork et al., 2002). The high estimate of Φ_{ft} obtained in this study contrasts with much lower reported values for *Pinus sylvestris* (−0.006–0.007; Robledo-Arnuncio et al., 2004) and *Picea abies* (0.011–0.015; Finkeldey, 1995). However, the low cited levels of Φ_{ft} for *Pinus sylvestris* and *Picea abies* were obtained for populations with high densities (80–315 trees/ha) and populations surrounded by extensive forests mostly dominated by conspecifics, respectively. In contrast, comparatively low population densities and highly degrees of canopy closure in mixed forests probably lead to small genetic neighborhoods and, hence, heterogeneous pollen pools (Finkeldey, 1995; Dyer and Sork, 2001).

4.3. Mating system

The results of the MLTR analysis suggest that the population of *Q. semiserrata* at this study site has a very high outcrossing rate ($t_m = 99.5\%$), in accordance with the results of the paternity analysis. In addition, the estimate of biparental inbreeding for the total population ($t_m - t_s$) was only 0.013, indicating the occurrence of a low proportion of mating among relatives, mainly in 2007. 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 (Barrett et al., 1993; Raijmann et al., 1994; Young et al., 1996) – similar to levels found in large, natural populations of other oaks, e.g. ($t_m - t_s$) values of 0.051, in mixed stands of hybridizing *Q. robur* and *Q. petrea* (Bacillieri et al., 1996) and 0.022 for *Q. velutina* (Fernandez-Manjarres et al., 2006). The results of this study are consistent with findings by Fernandez-Manjarres and Sork (2005) that levels of biparental inbreeding are generally low in fragmented populations of *Q. humboldtii*, probably because the mating system of these trees appears to be resilient to reductions in population size, and support the hypothesis that oaks are virtually complete outcrossers

since seed-tree nos. 79 and 70 are located far from the other seed parents, and near the edge of a cluster of seed parents, respectively (Fig. 1). The between-year variation in the genetic composition of pollen pools accepted by each seed parent contributed to the total genetic diversity of their seeds. Consequently, the overall genetic diversity of seeds from seed parent nos. 70 and 79 was higher than that of seeds from the other seed parents. Several factors may have influenced pollination and led to genetic heterogeneities in pollen among both the seed parents and the reproductive years examined in this study, including (*inter alia*) variations in the flowering

(Schwarzmann and Gerhold, 1991). Both the low estimate of biparental inbreeding and the finding that at least 30.0% of the pollen accepted by trees in the population we examined originated from trees outside the study plot provide evidence of such resilience in *Q. semiserrata* at our study site.

4.4. The effective number of pollen donors (N_{ep})

The effective number of pollen donors (N_{ep}) was estimated to be 9.987 according to the TwoGener model, 10.989 according to the MLTR model, and smaller (in both cases) than the total number of pollen donors obtained from the paternity analysis (11.625). Estimates of the effective number of pollen donors obtained using the TwoGener model were also lower than estimates of the total number of pollen donors obtained through paternity analysis in studies of *Q. macrocarpa* by Dow and Ashley (1996) and *Q. robur* and *Q. petraea* by Streiff et al. (1998). The differences between the two approaches have been reviewed by Smouse et al. (2001). The effective number of pollen donors of seeds from 2005 was higher than those from 2007, and thus both allelic richness and genetic diversity were higher in seeds from 2005. High levels of variation in the composition of the pollen pool received by an individual are likely to increase genetic variation among progeny, and may enhance the ability of populations to maintain effective population sizes that can withstand stochastic demographic and genetic changes (Schemske et al., 1994). The analyses have different bases, but they both suggest that the effective number of pollen donors was, but similar to N_{ep} estimates obtained for other oak species, for instance: 8.2 (TwoGener) in *Q. alba* (Smouse et al., 2001), 7.58 (TwoGener) and 2.39 (MLTR) in *Q. velutina* (Fernandez-Manjarres et al., 2006), 5–7 (MLTR) in *Q. lobata* (Sork et al., 2002) and 5.4 (MLTR) and 6.1 (TwoGener) in *Q. humboldtii* (Fernandez-Manjarres and Sork, 2005). However, these values are lower than effective numbers of pollen donors reported for other wind-pollinated species, e.g. 50–100 for *Larix occidentalis* (El-Kassaby and Jaquish, 1996), >70 for *Pinus sylvestris* (Robledo-Arnuncio et al., 2004), and 33–46 for *Picea abies* (Finkeldey, 1995). The site examined in the present study is in an open landscape, without a closed canopy, in which scattered trees provide approximately 20% forest canopy cover, which should theoretically promote the free movement of pollen (Okubo and Levin, 1989). N_{ep} also tends to be higher in open conditions than under closed canopies, other factors being equal, according to Smouse and Sork (2004). However, the low number of effective donors observed in *Q. semiserrata* compared with *Larix occidentalis* and *Picea abies* indicates that most pollination is very localized in the *Q. semiserrata* population, possibly because of the low densities of trees in the surrounding area.

4.5. Estimates of the mean effective pollination distance (δ)

Estimates of the mean effective pollination distance (δ) were derived from the estimated value of Φ_{ft} and the density of adults (d) at the study site (Austerlitz and Smouse, 2001; Smouse et al., 2001; Smouse and Sork, 2004). Therefore, values of effective pollination distance are expected to be high in low-density forests, and this expectation was met in this study, since the low population density (2.4 trees/ha) was accompanied by high mean effective pollination distances (81.4 m overall, and 109.2 and 68.3 m for 2005 and 2007, respectively). The pollination distances obtained from the paternity analysis were lower because the distances travelled by pollen from outside the study plot that sired seeds were not included in the calculations. The mean effective pollination distance of a savanna population of *Q. lobata* Sork et al., 2002), with a stand density of 1.19 trees/ha, has been found to be similar to these

distances (65 m), while that of *Q. alba* in a closed-canopy, mixed conifer-deciduous forest, with a stand density of 93 trees/ha, was reportedly substantially lower (~17 m; Smouse et al., 2001).

5. Conclusion

In fragmented, small or residual populations of plants their breeding systems can be severely disrupted, resulting in increases in inbreeding and population differentiation, accompanied by the erosion of genetic variability within populations. However, wind-pollinated species (especially oak species) are expected to be resilient to effects of habitat fragmentation and small populations due to ample gene flow from surrounding populations. Pakkad et al. (2008) studied the genetic diversity and differentiation of *Q. semiserrata* in northern Thailand, and found high levels of genetic variation and low levels of genetic differentiation between populations connected by gene flow, which was deemed probably to be important for maintaining their genetic diversity and minimizing genetic drift, as confirmed by this study. In conclusion, this study provides evidence that *Q. semiserrata* at this study site has a high outcrossing rate and high level of gene flow from outside populations. In addition, the variation in pollen composition received by individual trees is likely to increase genetic variation among their progeny, and may enhance the ability of populations to maintain effective population sizes. Therefore, these processes may be sufficient to prevent losses of genetic diversity of this species at this study site through genetic drift.

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