

Mating system and genetic diversity in natural populations of *Nelumbo nucifera* (Nelumbonaceae) detected by ISSR markers

Yan-Chuang Han · Cai-Zhu Teng ·
Gituru Robert Wahiti · Ming-Quan Zhou ·
Zhong-Li Hu · Yun-Chun Song

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Abstract In this study, outcrossing rates and genetic diversity in natural populations of *Nelumbo nucifera* were investigated. The estimated multilocus outcrossing rate (t_m) based on 28 ISSR loci was over 90%. Analysis of genetic diversity revealed that this index was high at the species level ($H_s = 0.325$, $I = 0.514$), but low within the individual study populations ($H_s = 0.148$, $I = 0.212$). G_{st-B} was 0.547 and N_m was 0.414. The results of AMOVA indicated that 54.6% of the variation was due to the difference between the regions and 45.4% to the variation within the region. Although the populations were predominantly outcrossing, most of the genetic diversity was attributed to geographical effects instead of their habitats because low sexual recruitment and clonal growth deeply reduced the genetic diversity within the populations. On the basis of the high t_m , G_{st-B} and low N_m values, we

recommended that any future conservation plans should include both in situ conservation and germplasm collection.

Keywords Genetic diversity · Genetic structure · ISSR · Outcrossing rate · *Nelumbo nucifera*

Introduction

The Nelumbonaceae also known as the lotus family is a small family of perennial, aquatic angiosperms, which consists of the two species *Nelumbo nucifera* Gaertn. and *Nelumbo lutea* (Willd.) Pers.. The family is characterized by simple, peltate leaves, which lack stipules and are borne on the surface of the water. *Nelumbo nucifera*, also called the Indian or sacred lotus, is found throughout Asia and Australia, whereas *N. lutea*, also known as the American lotus or water chinquapin, occurs in eastern and southern North America. *Nelumbo nucifera* is an important aquatic economic plant, not only as a dainty and ornamental flower but also as a source of herbal medicine with strong anti-pyretic, cooling, astringent, and demulcent properties (Mukherjee et al. 1997; Sinha et al. 2000; Qian 2002). The species is of religious significance in SE Asia (hence the name sacred lotus) and the seeds and leaves are also eaten in this region. Wild lotus populations are an important resource for breeding of cultivated lotus.

As an insect-pollinated, aquatic, herbaceous macrophyte, *N. nucifera* may reproduce by sexual (seeds) and asexual (rhizomes) means. *Nelumbo nucifera* has solitary and bisexual flowers. The pistil matures before the stamens ('protogyny'). The fruit is a nut of about 1.0 g at maturity. It has a hard pericarp that surrounds the seed within (Wang and Zhang 2005). In recent years, local populations of wild lotus in Central Mainland China have been greatly

Y.-C. Han · Z.-L. Hu (✉) · Y.-C. Song
Key Lab of the Ministry of Education for Plant Developmental
Biology, College of Life Science, Wuhan University,
430072 Wuhan, China
e-mail: huzhongli@whu.edu.cn

C.-Z. Teng
College of Life Science, Guangxi Normal University,
541004 Guilin, China

G. R. Wahiti
Botany Department, Jomo Kenyatta University of Agriculture
and Technology, P. O. Box 63000-00200, Nairobi, Kenya

M.-Q. Zhou (✉)
Lotus Center, Wuhan University, 430072 Wuhan, China
e-mail: loyjsx@whu.edu.cn

reduced due to the rapid development of the aquaculture industry. *Nelumbo nucifera* has been included in the list of endangered species in China (Dong and Zheng 2005).

Investigation of population genetic diversity and the structure of populations of this species may therefore not only illustrate the evolutionary process and mechanisms but also provide information useful for biological conservation (Schaal et al. 1991; Friedman et al. 2004). Genetic variation in a plant species is determined by its mating system and dispersal ability of pollen and seed, which affect the genetic structure and dynamics of populations within the species (Allard 1975; Tigerstedt 1984; Muona 1990). Outcrossing rates in plant populations have traditionally been estimated from polymorphism data at isozyme loci (Adams 1983). In recent years, however, several dominant markers such as RAPD, AFLP, and ISSR have been successfully developed and can be used to assay mating systems at the DNA level (Fritsch and Rieseberg 1992; Gaiotto et al. 1997; Ge and Sun 1999). Due to their dominant behavior, these markers provide less information per locus than co-dominant markers. This is particularly relevant for applications that require genotype discrimination, as in the case of outcrossing-rate estimation. Through simulation studies, Ritland and Jain (1981) demonstrated, however, that this limitation could be readily overcome by multilocus estimation using a large number of dominant markers with intermediate gene frequencies.

Inter-simple sequence repeat (ISSR) is a technique that uses repeat-anchored primers to amplify DNA sequences between two-inverted SSR (Zietkiewicz et al. 1994). ISSR markers do not require a prior knowledge of the SSR target sequence; furthermore, they are highly reproducible due to their primer length and to the high stringency achieved by the annealing temperature. ISSR markers have been found to provide highly polymorphic fingerprints (Zietkiewicz et al. 1994; Kojima et al. 1998; Bornet et al. 2002). In an earlier study (Han et al. 2007), we successfully employed ISSR to genetic diversity and clonal diversity in *N. nucifera*. Our earlier study of genetic diversity of *N. nucifera* in China was restricted to the Liangzi Lake area in Central Province of Hubei (Han et al. 2007). In the present study, genetic diversity and outcrossing rates in the species were studied in a broader geographic area encompassing the entire natural distribution of the species in Central China.

It is important to have an account of mating processes for a species in order to effectively conserve its genetic resources and to optimize genetic improvement. We investigated the relative frequencies of outcrossing versus selfing and genetic diversity in wild populations of *N. nucifera* with the aim of providing insights into their population genetics in order to facilitate the conservation management of this important natural resource.

Materials and methods

Study species

Nelumbo nucifera is a perennial aquatic plant that produces insect-pollinated flowers with copious quantities of pollen. In the Yangtze River drainage area, the plant flowers from April to September. *Nelumbo nucifera* prefers calm alluvial habitats such as lakes, swamps, pools and rice fields. A single individual may occupy an area of 2–4 m² while the main rhizome may reach to 3–6 m (Wang and Zhang 2005). Dispersal may occur during the gametophyte phase by means of insect-dispersed pollen, while seed dispersal is not likely to be very efficient, given the weight of seeds for the species (approximately 1.0 g effectively excluding wind transport) and their lack of dispersal structures.

Seeds of *N. nucifera* were collected from a natural population (LZB covers about four square kilometers) at Liangzi Lake in Hubei Province of Central China. A total of 240 individuals (a individual refers to a seed) from a random sample of 20 natural families at intervals of 10 m, each of which offered 9–21 seeds, were used for estimation of outcrossing rates. Embryos were removed from seeds and used for genomic DNA extraction.

Plant materials for genetic diversity studies were obtained from ten populations with two populations each from five lake basin regions covering the entire distribution of the species in Central China (Fig. 1). The information about the ten populations is listed in Table 1. In view of the extended length of the main rhizome in *N. nucifera* and the expansive area occupied by a single individual of this species, sample plants for genetic diversity studies were obtained at intervals of 10 m apart, 25 sample plants were obtained from each of the ten study populations. About 5 g of young leaves from each individual plant [the term individual is used here to

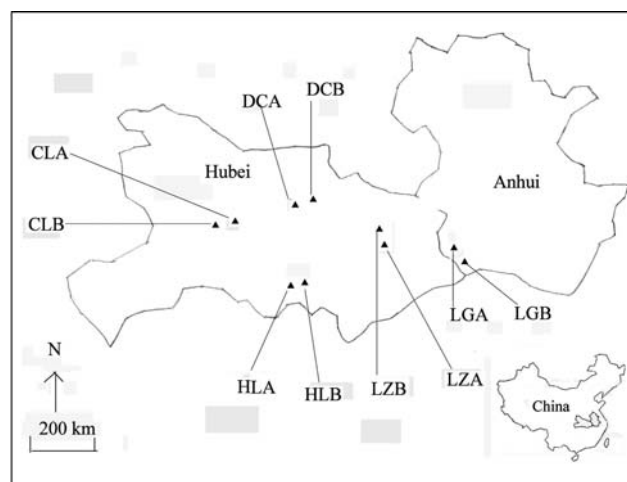


Fig. 1 Sampling locations of the ten study populations of *N. nucifera* from Central China

Table 1 Geographic location of the ten studied populations

Population	Geographical origins	Location coordinate	Population size (m ²)	Human disturbance*
CLA	Chang Lake, Hubei Province	112°19.062', 30°24.095'	300	M
CLB	Chang Lake, Hubei Province	112°19.057', 30°24.042'	500	M
DCA	Diaocha Lake, Hubei Province	113°24.031', 30°41.138'	300	M
DCB	Diaocha Lake, Hubei Province	113°44.832' 30°39.801'	300	H
HLA	Hong Lake, Hubei Province	113°24.001', 29°49.268'	500	M
HLB	Hong Lake, Hubei Province	113°24.132', 29°49.371'	300	M
LGA	Longgan Lake, Anhui Province	116°01.095', 29°55.025'	2,000	H
LGB	Longgan Lake, Anhui Province	116°02.061', 29°55.019'	2,000	H
LZA	Langzi Lake, Hubei Province	114°34.506', 30°09.629'	300	M
LZB	Langzi Lake, Hubei Province	114°29.813', 30°16.862'	500	M

* Level of human disturbance (*M* moderate, *H* high)

represent a sampling unit such as a physiologically distinct plant unit (clonal or non-clonal) at a certain distance from each other and does not necessarily denote a separate genetic individual] was obtained and placed in a zip lock plastic bag containing about 50 g of silica gel that speeded up the drying process. The samples were stored at room temperature in the laboratory.

Genomic DNA extraction

Genomic DNA of every individual was isolated according to the protocol of Doyle and Doyle (1987). DNA was quantified by comparison with lambda DNA known concentration following electrophoresis in a 1% agarose gel using image analysis.

Evaluation of primers

Reproducibility is critical for dominant markers such as ISSR. Each primer to be tested was surveyed in duplicate reactions for each individual from the sub-sample. Only primers that yield a reproducible and clear band pattern were used. Primers used in the mating system study were screened using 16 random DNA samples of four individuals, respectively, coming from four families. Five ISSR primers were selected based on the number of polymorphisms amplified, their size, amplification intensity, reproducibility and the presence of polymorphic markers across families. In the genetic variation study, after assessing the effects of Mg²⁺ concentration, template DNA concentration, and temperature during the annealing stage of the amplification, 12 primers (Table 2) that produced clear and reproducible fragments were selected for further analysis.

ISSR amplification

The PCR amplification was performed in a 25 µl reaction volume containing 10 mM Tris-HCl pH 8.0, 50 mM KCl,

Table 2 ISSR primers: their sequence, annealing temperature and the number of bands they produced in the genetic diversity experiments

ISSR primer type	Sequence (5'-3')	Number of polymorphic markers	Annealing temperature (°C)
UBC810	(GA) ₈ T	15	57
UBC811	(GA) ₈ C	12	57
UBC823	(TC) ₈ C	13	55
UBC834	(AG) ₈ YT	14	52
UBC861	(ACC) ₅	17	54
UBC862	(AGC) ₅	19	55
UBC864	(ATG) ₅	15	53
UBC866	(CTC) ₅	12	57
UBC873	(GACA) ₄	13	52
UBC809	(GGAGA) ₃	14	51
UBC889	DBD(AC) ₇	16	56
UBC891	HVH(TG) ₇	14	56

B, C/G/T; D, A/G/T; H, A/C/T; V, A/C/G; Y, C/G

1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.4 µM of primer, 100 µg/ml of BSA, 1 unit of Taq polymerase and 30 ng of genomic DNA. Initial denaturation was for 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at specific annealing temperature, 2 min at 72°C, and a 10-min final extension step at 72°C. The annealing temperature for each primer is given in Table 2. PCR products were analyzed on 2% agarose gels at a constant voltage of 100 V for approximately 4 h, then stained with ethidium bromide, visualized with ultraviolet light and photographed.

Data analysis

The five ISSR primers amplified a total of 28 dominant markers, which were employed for the estimation of out-crossing rate. In the mating system studies, ISSR markers are typically dominant. Scoring of bands was done

considering only two possible alleles: band presence or band absence. Using MLTR 3.0 (Ritland 2004), we calculated the following mating system parameters: multilocus outcrossing rate (t_m), single locus outcrossing rate (t_s), multilocus correlation of P (pollen and ovule gene population frequencies) estimate (r_{pm}), single locus correlation of P estimate (r_{ps}), correlation of t_m between progeny arrays (r_t), and fixation index of maternal parents (F).

In the genetic variation study, ISSR amplified fragments were scored for band presence (1) or absence (0). Genetic diversity within and among populations was measured by the percentage of polymorphic bands (PPB) and Shannon's Information index (Program POPGENE, version 1.31). Gene diversity was estimated using a Bayesian method implemented with the program HICKORY version 1.0 using all recommended default settings (Holsinger et al. 2002; Holsinger and Lewis 2003; Holsinger and Wallace 2004). HICKORY calculates the Bayesian estimate of gene diversity, H_s (average panmictic heterozygosity), by internally calculating the posterior distribution for allele frequencies at every locus in every population. The Bayesian estimator of genetic diversity is calculated for a free model and a burn-in of 50,000, a thinning sampling runs of 500,000 and factor of 100 iterations were used in each of the three replicate runs.

Hierarchical structuring of genetic variation and pairwise Φ_{ST} distances (analogous to F_{ST} statistics at the molecular level; Excoffier et al. 1992) among the populations were determined by an analysis of molecular variance (AMOVA) with IWINAMOVA version 1.5 (Excoffier et al. 1992). Significance levels of the variance components were based on 1,000 permutations. A pairwise Euclidean distance matrix and all input files needed for the AMOVA were produced using the AMOVA PREP program version 1.01 (Miller 1998). An estimator of F_{ST} under random-effects model of population sampling, θ^B , and G_{ST-B} , a Bayesian analog of Nei's G_{ST} , were obtained for all populations using HICKORY (Nei 1973; Holsinger 1999; Holsinger and Lewis 2003). Cluster analysis was used to describe the genetic relationship among the different populations and the Jaccard similarity coefficients were calculated using the NTSYS-pc version 2.0 computer program package (Rohlf 1992) to generate a dendrogram via the un-weighted pair group method with arithmetic average (UPGMA). Mantel tests (Mantel 1967) were performed to analyze the effects of geographical distance on genetic variation.

Estimation bias can lead to the overestimation of parameters by as much as 5% in the dominant marker data produced by RAPD and ISSR analysis (Lynch and Milligan 1994). To reduce this bias, Lynch and Milligan proposed pruning any locus with a band frequency of higher than

$1 - (3/N)$, where N is the number of individuals sampled. This pruning procedure was implemented with the data set prior to the genetic analyses.

Results

Mating system analysis

The five ISSR primers, including UBC 826 (7), UBC 854 (6), UBC 857 (6), UBC861 (5), and UBC 863 (4), amplified 28 dominant markers. The multilocus (t_m) and single locus (t_s) outcrossing estimates for the population were 0.982 ± 0.041 and 0.907 ± 0.004 , respectively. The difference between the multilocus and single locus ($t_m - t_s = 0.075 \pm 0.039$) was effectively zero. Multilocus correlation of P (pollen and ovule gene population frequencies) estimate (r_{pm}), single locus correlation of P estimate (r_{ps}) and correlation of t_m between progeny arrays (r_t) were 0.086 ± 0.019 , 0.098 ± 0.002 and 0.017 ± 0.011 , respectively. Difference ($r_{ps} - r_{pm}$) of estimate was 0.012 ± 0.017 . The value of the single locus inbreeding coefficient of maternal parents, F , was 0.046 ± 0.021 .

Genetic variation analysis

For a locus to be pruned according to Lynch and Milligan (1994) in the ISSR data ($N = 250$), the frequency of the band had to be 0.988 or higher. A total of 173 reproducible bands were revealed across all 250 individuals from the 10 populations. ISSR fragments from 250 to 1,600 bp were counted, with an average of 14.42 bands per applied primer.

Within populations, ISSR diversity was considerably lower than at the species level (Table 3). Bayesian gene diversity (H_s) ranged from 0.116 to 0.233, with an average of 0.148 within the populations level and 0.325 at the species level. The Shannon indices (I) ranged from 0.147 to 0.336, with an average of 0.212 within population level and 0.514 at the species level. Among the ten populations, population HLB exhibited the highest level of variability (PPB = 58.9%, $H_s = 0.233$, $I = 0.336$) and population LGB, the lowest (PPB = 25.1%, $H_s = 0.116$, $I = 0.147$). For populations in the same lake, the genetic diversity of the populations displayed a moderate increase with increase in latitude (Tables 1, 3).

Among the ten populations, a Bayesian analog of Nei's G_{ST} (G_{ST-B}) was 0.547, indicating that 45.3% of the genetic diversity was within the populations. Based on G_{ST-B} value, the mean estimated number of gene flow (N_m) between the populations was 0.414.

Based on Jaccard similarity coefficient, a UPGMA dendrogram among the ten populations of *N. nucifera* was

Table 3 Statistical analysis of genetic variation in the studied populations of *N. nucifera*

Population	PPB (%)	<i>H_s</i>	<i>I</i>
CLA	37.7	0.143 (0.013)	0.217 (0.294)
CLB	32.6	0.123 (0.012)	0.176 (0.270)
DLA	36.6	0.139 (0.014)	0.203 (0.286)
DLB	32.0	0.133 (0.014)	0.187 (0.285)
HLA	35.4	0.144 (0.015)	0.214 (0.295)
HLB	58.9	0.233 (0.009)	0.336 (0.304)
LGA	28.6	0.116 (0.014)	0.155 (0.260)
LGB	25.1	0.118 (0.013)	0.147 (0.260)
LZA	34.9	0.141 (0.016)	0.208 (0.291)
LZB	46.3	0.188 (0.013)	0.274 (0.307)
Mean	36.8	0.148 (0.015)	0.212 (0.313)
Species level		0.325 (0.145)	0.514 (0.161)

PPB, Percentage of polymorphic band; *H_s*, Bayesian gene diversity; *I*, Shannon's information index

built (Fig. 2). In the dendrogram, all the populations in each region clustered together. The populations from Diaocha Lake (DLA and DLB) and Liangzi Lake (LZA and LZB) showed the closest genetic similarity between any of the study populations. The populations from Chang Lake had less genetic similarity to those from the other four lakes. The Mantel test revealed that there was no statistically significant correlation between pairwise genetic

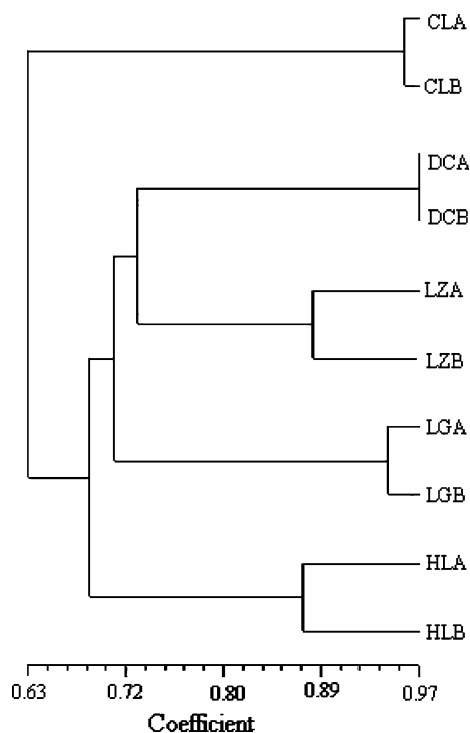


Fig. 2 The unweighted pair group method with arithmetic mean (UPGMA) dendrogram for the ten populations of *N. nucifera* based on Jaccard similarity coefficient from ISSR markers

distance and the corresponding geographic distance among the populations ($r = 0.526$, $P = 1.000$).

Analysis of molecular variance indicated that more than half of the total variations (54.6%) could be accounted for by differentiation among the five lakes, with a further 9.0% being accounted for by variation between the populations within a lake. The remainder (36.4%) was produced from individual variations within a population (Table 4). All the components of molecular variance were significant ($P < 0.001$).

Discussion

Dominant RAPD, AFLP, and ISSR markers were previously used to estimate the outcrossing rate in populations of flowering plants (Fritsch and Rieseberg 1992; Gaiotto et al. 1997; Ge and Sun 1999). Although ISSR technology supplies dominant markers with lower information content than traditional co-dominant isozymes, it is adequate for the study of the mating system in populations of *N. nucifera*. An empirical analysis by Gaiotto et al. (1997) suggested that 18 was the minimum number of dominant marker loci necessary to achieve robust estimates of *tm* (multilocus outcrossing rate). In our study, 28 ISSR markers loci were easily obtained with only five primers. However, this study also points to the fact that when dominant markers were applied for outcrossing rate estimation it is imperative that an adequate screening of primers should be done so as to maximize the probability of amplifying a large number of polymorphic markers in the progeny arrays.

Both multilocus ($tm = 0.982 \pm 0.041$) and mean single locus ($ts = 0.907 \pm 0.004$) outcrossing rates were relatively high, indicating that *N. nucifera* was predominantly an allogamous species. The 'protogynous' phenomenon (Wang and Zhang 2005) could reasonably account for the high degree of outcrossing in this species. This is to the best of our knowledge, the first published report on the outcrossing rate in *N. nucifera* and should contribute to optimizing genetic resources conservation and formulating breeding strategies for this species.

In the present study, the lack of any difference between the multilocus (*tm*) and single locus (*ts*) estimates indicates that there is no 'biparental inbreeding' in the population. In this study, the absence of 'biparental inbreeding' strengthens the somewhat tentative conclusion that the studied populations were predominantly outcrossing.

No differences in the outcrossing rates among the mother plants were observed [*rt* (correlation of *tm* between progeny arrays) was low]. The *rp* (correlation of outcrossed paternity within progeny arrays) values suggested that most of the individuals within the progeny arrays were

Table 4 Summary of nested analysis of molecular variance (AMOVA) based on ISSR genotypes of *N. nucifera*

Source of variation	<i>df</i>	SSD	Variance component	Percentage	<i>P</i> value
Among regions	4	1916.90	15.71	54.62	<0.001
Among populations within regions	5	228.79	2.89	9.00	<0.001
Within populations	241	2542.31	10.46	36.38	<0.001
Total	250	4688.00	29.05		

df Degree of freedom; *SSD* sum of square deviation; *P* value, probability of null distribution

Levels of significance are based on 1, 000 iteration steps

outcrosses. The *rps* [single locus correlation of *P* (pollen and ovule gene population frequencies) estimate] was greater than *rpm* (multilocus correlation of *P* estimate), which indicated that there was an effect of population substructure on the male similarity between the outcrosses. *F* (fixation index of maternal parents) was near zero suggesting no Hardy–Weinberg deviations in the populations, which indicated that individuals within the populations mate at random. *F* in the progeny estimated was higher than that expected based on the estimate of *tm*. Taking *tm* = 0.982, the expected fixation index was [$F = (1 - t)/(1 + t)$] = 0.009, while the estimated *F* was 0.046. A higher *F* than the expected one suggested an excess of inbreeding and less heterozygotes than that expected in the progeny population analyzed. However, the analysis of progeny genotypes revealed that they were largely the products of outcrossing [*rp* (correlation of outcrossed paternity within progeny arrays) was very low]. This indicated that selfed offspring of *N. nucifera* did not mostly survive to reproductive maturity. The most probable mechanism causing differential survival of selfed and outcrossed progeny was partial self-incompatibility.

In the present study, the genetic diversity index [*Hs* (Bayesian gene diversity) = 0.325, *I* (the Shannon indices) = 0.514] revealed that the genetic diversity of *N. nucifera* was very high at the species level, but low at the within-population level (*Hs* = 0.148, *I* = 0.212). The results indicated pronounced genetic variation among the populations of the species. Similar results were found in earlier studies (Xue et al. 2006; Han et al. 2007). Geographical isolation may contribute to the high genetic diversity at the species level. The low levels of genetic diversity harbored within the study populations may be due to the following four possible reasons. Firstly, the efficient sexual recruitment was inhibited because of the unique fruit structures, which resulted in lower genetic diversity within the populations. Secondly, they might be due to low inherent variability of the ancestral species. Thirdly, the repeated decrease and increase of temperature in climatic oscillations during the Pleistocene might have caused the repeated enlarging and decreasing of populations, hence causing founder effects. This might partly account for the

lower levels of variation within the populations of *N. nucifera*. Finally, the explosive increase in the human population and destructive utilization had caused a dramatic decline of the species. Although the populations in Longgan Lake were large in size, they had the lowest diversity owing to the destructive utilization by man.

The genetic structure of plant populations reflects the interactions of various factors, including the long-term evolutionary history of the species (shifts in distribution, habitat fragmentation and population isolation), genetic drift, mating system, gene flow and selection (Schaal et al. 1998). The founding number, probability of common origin, kin structure, and inbreeding within populations all have significant effects on genetic differentiation among populations (Whitlock and McCauley 1990). Differentiation or speciation has mainly occurred during periods when habitats were fragmented (Bridle et al. 2004). A high *Gst-B* (a Bayesian analog of Nei's *G_{ST}*) value (0.547) indicated pronounced genetic differentiation among the ten studied populations. AMOVA revealed that there was a considerable geographical-effected genetic variance (54.6%, *P* < 0.001) in *N. nucifera*. This was probably due to the differences in the geographic fragmentation of populations of the species. Generally, the mating system of flowering plant species greatly affects population genetic differentiation (Hamrick and Godt 1989). Estimates of genetic differentiation between the populations for outcrossing species based on AMOVA, derived by analyzing RAPD markers, have usually been <28%. For inbred species, estimates of interpopulation genetic variation have usually been >70% (Nybom and Bartish 2000). The genetic differentiation between the populations of *N. nucifera* (54.6%) were neither in the range of outcrossing species nor in that of inbred species. This was mainly because *N. nucifera* is both a predominant outcrossing and a clonal aquatic plant.

The fact that estimates of *Nm* (gene flow) were <1 for the species suggested that gene flow between the populations was insufficient to counter the effects of random drift (Real 1994). In this study, the relatively high genetic differentiation and low levels of gene flow detected (*Nm* = 0.414) strongly indicated that genetic drift had

greatly affected the genetic composition of individual populations. The lack of correlation between genetic distances and geographic distances in *N. nucifera* suggested that there was low genetic flux between populations of this species, and that stochastic differentiation due to genetic drift had occurred. Gene flow between populations appeared to be mostly via pollen movement (Richards et al. 1999). Between-population gene flow was limited by pollen and seed dispersal. Being an insect-pollinated plant, pollen dispersal was limited by the short flight ranges of the insects. Moreover, seed dispersal is not likely to be very efficient, given the weight of seeds for the species (approximately 1.0 g effectively excluding wind transport) and their lack of dispersal structures. The limited seed dispersal contributes to the restricted gene flow and increases the probability that individuals in close physical proximity mated with one another. Both effects would promote inter-population differentiation.

Knowledge of genetic diversity within and among the populations is crucial for conservation purposes, when interpreted within a broader ecological and organismic context. Considering the high level of genetic differentiation among populations, preservation of any one population would not protect all the variation in the species. Therefore several populations throughout the entire range should be considered for conservation. If possible, all populations studied here should be conserved. But it is difficult to carry out in situ protection because of the dispersive distribution of wild populations and local continuous destruction. The high outcrossing rate and low gene flow among the populations indicated that ex situ plantings would result in possible contamination of the different populations. Moreover, seed longevity is very long and seeds are easily preserved. Thus, germplasm collection is a better plan for conservation.

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