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# Multiple northern refugia for Asian sacred lotus, an aquatic plant with characteristics of ice-age endurance

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**Abstract.** The effect of palaeoclimate on the distribution of aquatic plants is little known, although these plants typically have much broader geographical distributions than their terrestrial counterparts. We investigated the structure of genetic variation of chloroplast DNA in the Asian sacred lotus (*Nelumbo nucifera* Gaertn (Nelumbonaceae)) in 15 wild populations across China as well as in four populations from India, Japan and Thailand, to infer the refugia of this ancient plant during Quaternary climatic oscillations. We obtained 37 cpDNA haplotypes in 417 individuals from 19 populations. A moderate to high level of chloroplast genetic differentiation ( $G_{ST}=0.547$ ,  $N_{ST}=0.691$ ) and significant phylogeographic structure ( $N_{ST} > G_{ST}$ ) were observed, suggesting a low level of recurrent seed-mediated gene flow among the populations (29%). The phylogenetic relationships for the recovered haplotypes showed that haplotypes of wild lotus in north-eastern China significantly diverged from those distributed in central and southern regions of China. A high level of haplotype diversity, rather than reduced genetic diversity, in north-eastern China indicated multiple refugia in northern China during the Quaternary glaciations. Lotus plants have the following two characteristics that facilitated survival through Quaternary glaciations: seeds buried in mud remain viable for thousands of years, and self-heating flowers can reproduce in cold temperatures.

#### Introduction

The contemporary distribution of biological diversity has been shaped by the geological history and palaeoclimate of the Earth. The climatic changes of the Quaternary, particularly during the last cycle of glaciation-deglaciation, played an important role in the extinction and changes in the distribution ranges and genetic structure of many animals and plants in the northern hemisphere (Hewitt 1996, 2000, 2004; Comes and Kadereit 1998; Davis and Shaw 2001; Pearson and Dawson 2003). Although the effects of glacial-interglacial cycles of the Quaternary were global, most phylogeographic studies on the genetic structure and range changes of species by using molecular markers have been concerned with Europe and North America (Soltis et al. 1997; Taberlet et al. 1998; Hewitt 2000, 2004; Schönswetter et al. 2005; Beheregaray 2008). In general, the distribution of organisms was characterised by the contraction of ranges to southern regions during the cold glacial periods and northward expansion from the refugia during the following interglacial phases (Bennett et al. 1991; Hewitt 1996; Taberlet et al. 1998).

China has some distinctive biogeographical features and is considered as the centre of biodiversity in the North Temperate Zone (Axelrod *et al.* 1996). Although most parts of China were never covered by ice sheets during glacial periods, the tremendous cooler and drier global climatic changes, particularly during the last glacial maximum (LGM), starting about 18 000 years ago, have forced most plant species to migrate southward according to the fossil records (Yu *et al.* 2000;

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Harrison et al. 2001). On the basis of a phylogeographical analysis of Cunninghamia konishii, Lu et al. (2001) and Hwang et al. (2003) inferred several refugia in southern China. Shen et al. (2005) suggested that the refugia of Ginkgo biloba were located in south-western China. Wang and Ge (2006) inferred four separate glacial refugia for Cathaya argyrophylla in western, eastern, south-eastern and southern China, respectively. However, a recent phylogeographic analysis suggested that a deciduous shrub Ostrvopsis davidiana (Betulaceae) survived in northern China, rather than the species surviving only in the south and, subsequently, colonising northward during the LGM (Tian et al. 2009). Moreover, analyses of the distribution of genetic diversity on Picea jezoensis, which is now disjunctly distributed in north-eastern China (NEC), North Korea and Japan, suggested that this species had an independent glacial refugium in NEC during the ice age and in the postglacial period (Aizawa et al. 2007). Chen et al. (2008) also showed that the refugia of Pinus tabulaeformis in China were located in the Yellow River, nearer to the Yellow Sea, and within the Sichuan Basin, respectively. These putative refugia were located more north than previously recorded in China at LGM (Yu et al. 2000; Harrison et al. 2001). These results support an alternative hypothesis, namely that there were multiple geographically isolated refugia in eastern Asia during the LGM (Qian and Ricklefs 2000).

In contrast to the investigations on long-lived tree and shrubs species in China, the effect of past climate events on the current distribution patterns of aquatic species is largely unexplored.

Asian sacred lotus, Nelumbo nucifera Gaertn (Nelumbonaceae), the national flower of India, is a perennial aquatic plant and an old fossil, with the records dating back to the Cretaceous (Snigirevskaya 1964). This species can reproduce sexually (seeds) and asexually (rhizomes). The insect-pollinated hermaphrodite flowers are homeothermic, and thev thermoregulate intrafloral temperature between 30 and 36°C with the carpellary receptacle heating (Seymour and Schultze-Motel 1996, 1998; Li and Huang 2009a, 2009b). The capability of self-heating may facilitate sexual reproduction when this aquatic plant suffers from low temperature. Lotus seed has been recorded to remain viable for up to 1000 years, one of the longest recorded periods for angiosperms (Shen-Miller et al. 1995, 2002), with the two green seed embryos, rather than generally white embryos in other plants, facilitating seed germination even when seeds are buried in meters of mud.

Fossil records have indicated that the genus *Nelumbo* is of Laurasian origin (Borsch and Barthlott 1994). For much of the Tertiary and especially during the Eocene and early–mid-Miocene periods, warm and wet climatic conditions prevailed over northern latitudes (Tiffney 1985; Wen 1999). Aided by land connections and widespread equable climates, the distribution of *Nelumbo* was uninterrupted and extended over almost all parts of Eurasia (Snigirevskaya 1964; Sculthorpe 1967; Borsch and Barthlott 1994). In response to progressive climatic cooling from the start of Oligocene till Quaternary glaciations (2–0 million years ago), this species gradually retreated its range down to Asia (Sculthorpe 1967; Borsch and Barthlott 1994).

Lotus fossils have been found in the deposits of the Cretaceous in the area of the Amur River (Helongjiang River, NEC) (КришТОфОВич 1965), in the late Pleistocene and Eocene beds of Zhejiang and in the Jiangxi Province in southern China (Zhang and Liu 1999; Li and Chen 2002). Nowadays, the wild lotus in China spans a wide geographic range, occurring throughout China from north to south, except north-western areas (Ni 1987; Wang and Zhang 2005; Xue et al. 2005, 2006). NEC contains more wild populations of N. nucifera than does central and southern China. Xue et al. (2006) investigated the genetic diversity of wild lotus with nuclear markers in the Heilongjiang Province, NEC, and found that the accessions of the wild lotus had close genetic relationships and, thus, it was unlikely that wild lotus would have originated many times in the Heilongjiang Province. If the present-day lotus in NEC were derived from a recent range expansion from southern refugia, as suggested for most plants in northern China (Yu et al. 2000), one would expect that there would be reduced genetic diversity within and among the populations of N. nucifera in the north because of recurrent founder effects and genetic drift occurring in the small founding populations as the species migrates northward (Hewitt 2000). However, an alternative hypothesis is that multiple refugia existed for N. nucifera in NEC during the LGM because of its special biological characteristics and ecological requirements mentioned above. If this were correct, high genetic diversity of *N. nucifera* would be detected in these refugia in NEC.

The current wide distribution range of *N. nucifera* in China probably spans the former glaciated regions (e.g. the NEC) and ice-free (the central and southern China) areas. Thus, a phylogeographical study on this aquatic plant allows us to infer the distributional patterns following the Quaternary

climatic oscillation in China. Chloroplast DNA (cpDNA) has been frequently utilised to demonstrate the historical patterns of dispersal in many species (Demesure et al. 1996; El Mousadik and Petit 1996; King and Ferris 1998; Mohanty et al. 2000; Dorken and Barrett 2004), not only because of its non-recombining and maternally inheriting characteristics, but also because of its slow rate of evolution, which allows retention of historical signatures (past migration routes, colonisation dynamics) over longer time periods than do biparentally inherited nuclear markers (Comes and Kadereit 1998). In the present study, we surveyed the variation in the chloroplast DNA (cpDNA) by using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique in wild lotus populations sampled across a broad geographical range in China and neighbouring regions, including Japan, India and Thailand, to address the following specific questions: (1) what is the genetic structure of N. nucifera populations as revealed by cpDNA variation; and (2) where are the potential refugia in China?

#### Materials and methods

#### Study species and sampling strategy

Nelumbo nucifera ssp. nucifera Gaertner occurs in Asia, including China, India, Thailand and Japan. Sacred lotus is one of the important ornamental and economic plants in many parts of Asia and has been widely cultivated in water gardens in China (Wang and Zhang 2005). Human activities have extended the range of N. nucifera into the wet tropics close to the equator and into southern hemisphere, such as New Guinea, the Philippines, Singapore and Australia (Borsch and Barthlott 1994; Barthlott et al. 1996). Whereas cultivars usually have enlarged rhizomes or multiplayer-petaled flowers, the wild lotus is characterised with tenuous and fibrous rhizomes, a single whorl of petals and numerous carpels (Ni 1987; Wang and Zhang 2005). To avoid using cultivars, we collected the plants from natural populations without human interference rather than from artificial ponds or water gardens. Wild-population samples were collected from 15 sites in China where wild lotus was recorded (see Wang and Zhang 2005), two sites from Thailand, and one each from India and Japan (Table 1, Fig. 1). Our sampling was concentrated in NEC, which has many more wild populations of N. nucifera than does central and southern China (Wang and Zhang 2005; Xue et al. 2006). Silica gel-dried leaf material was collected from ~20 individuals in each population (Table 1) and the distance between individuals was at least 5 m.

#### DNA extraction, amplification and digestion

Total DNA was extracted from the silica-dried leaf tissue by using a modification of the CTAB extraction procedure of Doyle and Doyle (1990). DNA was quantified by comparison with a knownconcentration lambda DNA following electrophoresis in a 1.5% agarose gel.

Preliminary amplification of cpDNA was carried out with 11 pairs of universal primers (*trnH/trnK*, *trnK/trnK*, *trnC/trnD*, *trnD/trnT*, *psbC/trnS*, *trnS/trnfM*, *psaA/trnS*, *trnS/trnT*, *trnM/* rbcL from Demesure *et al.* 1995; *trnT/trnF* from Taberlet *et al.* 1991; *trnH/psbA* from Hamilton 1999). The amplifications were

 Table 1. Geographic origin, number of investigated individuals (n), number of haplotypes (N<sub>h</sub>), number of unique haplotypes (N<sub>ph</sub>) and the distribution of cpDNA haplotypes of each of the 19 investigated populations

North-eastern China (NEC); Hebei Province, northern China; Shangdong Province, east-central China; Hubei Province, central China; Yunnan Province, south-western China

Population/location	Abbreviation	Latitude, longitude	п	$N_{\rm h}$	$N_{\rm ph}$	Haplotypes
Zhuaji, NEC	ZJ	48°45′N, 134°40′E	25	1	0	22
Guangming, NEC	GM	47°27′N, 132°25′E	22	1	1	31
Shilipao, NEC	SL	47°24′N, 132°19′E	23	2	0	22, 23
Jiamusi, NEC	JMS	46°51′N, 130°15′E	22	7	5	19, 23, 24, 29, 35, 36, 37
Yueyaquan, NEC	YY	45°56'N, 133°33'E	21	10	6	13, 15, 16, 17, 18, 19, 22, 26, 27, 28
Erzhong, NEC	EZ	45°32′N, 131°53′E	26	4	0	16, 22, 23, 30
Hexiangyuan, NEC	HXY	45°30'N, 131°51'E	26	4	0	22, 23, 25, 30
Zhaoyuan, NEC	ZY	45°29′N, 125°21′E	22	3	1	22, 30, 31
Xingkaihu, NEC	XK	45°17′N, 132°4′E	18	4	2	25, 30, 33, 34
Baiyangdian, Hebei	BY	38°53′N, 115°28′E	23	2	0	16, 17
Jining, Shangdong	JN	35°26'N, 116°35'E	23	2	1	4, 5
Weishanhu, Shangdong	WS	34°8′N, 117°12′E	23	3	0	12, 21, 22
Honghu, Hubei	HH	29°48′N, 113°27′E	19	4	2	20, 22, 30, 32
Lantian, Hubei	LT	29°31′N, 113°16′E	18	3	1	3, 4, 14
Caohai, Yunnan	CH	26°55′N, 100°18′E	20	1	1	6
Japan	JP	35°42′N, 139°44′E	22	4	3	9, 10, 11, 21
India	IN	25°58′N, 85°24′E	24	2	1	1, 2
Thailand <sup>A</sup>	$TH^A$	18°46'N, 106°11'E	10	4	0	2, 3, 4, 12
Thailand <sup>B</sup>	$\mathrm{TH}^{\mathrm{B}}$	13°45′N, 105°28′E	30	2	2	7, 8

<sup>A,B</sup>Different sampling sites in Thailand.

performed in 25 µL of a reaction mixture consisting of 0.25 mM of each of the four dNTPs, 2.5 uL of  $10 \times Taq$  buffer (10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub> and 50 mM KCl), 1 mM of each primer, 2 U *Taq* polymerase (Tian Yuan Biotech, Wuhan, China) and 60 ng of DNA template. The PCR-amplifications were carried out in a PTC-100<sup>TM</sup> thermocycler (MJ Research Inc., Waltham, MA, US), using the following procedure: 1 cycle (1 min at 94°C), 32 cycles (40 s at 94°C, 40 s at annealing temperature, 2 min at 72°C), 1 cycle (10 min at 72°C). Annealing temperatures were 50°C for *trnH/psbA* and 59°C for the other primers.

The PCR products were separated on 1.5% agarose gels running at 80 V in 0.5 × Tris-borc acid-EDTA (TBE) buffer, visualised by staining with ethidium bromide, and photographed under ultraviolet light. Those giving a single, clear band on the gel were collected and digested by three restriction enzymes (Hinfl, PstI and TaqI; TakaRa, Shiga, Japan). The reaction mixtures (20 µL) for HinfI and PstI included 1 µL of enzyme, 2 µL of  $10 \times H$  buffer,  $10 \mu L$  of PCR reaction mixture (~1  $\mu g$  of DNA) and  $7 \,\mu\text{L}$  of nuclease-free water and for TaqI added on  $2 \,\mu\text{L}$  of 0.1% BSA. The reaction mixtures were incubated for 3 h at 65°C for TaqI, and overnight at 37°C for PstI and HinfI. The restricted fragments were resolved on 6% polyacrylamide gels running at 2000 V for 1.5-2 h (time depending on the size of fragment to be resolved) and then silver-stained and scanned. Polymorphisms were scored visually and numbered in the order of increasing molecular weight. The three combinations (trnH/trnK with HinfI, psbC/trnS with HinfI, trnH/psbA with TaqI) revealing polymorphisms were chosen for the complete survey (Table A1, available as an Accessory Publication on the web). Polymorphisms were scored as point mutations or insertions/ deletions (indels), and used to define chloroplast haplotypes.

#### Data analysis

Parameters of population diversity  $(h_{\rm T}, h_{\rm S}, v_{\rm T}, v_{\rm S})$  and differentiation  $(G_{ST}, N_{ST})$  were estimated following the methods described by Pons and Petit (1995, 1996), using the program PERMUT version 1.0 (http://www.pierroton.inra.fr/ genetics/labo/Software/PermutCpSSR/index.html) (accessed on 30 July, 2010). The parameters represent the mean withinpopulation gene diversity  $(h_{\rm S})$ , the total gene diversity  $(h_{\rm T})$ , and the proportion of total diversity, owing to differentiations among the populations ( $G_{ST}$ ). The equivalent parameters ( $v_T$ ,  $v_S$ ,  $N_{ST}$ ) take into account the differences among the haplotypes. A permutation test was designed to compare the difference between these two parameters ( $G_{ST}$  and  $N_{ST}$ ); 1000 random permutations of haplotype identities were made, maintaining the haplotype frequencies and the matrix of pairwise haplotype difference as in the original study (Burban et al. 1999). The distribution of values obtained by permutation was compared with the observed values. A significantly higher value for  $N_{\rm ST}$ than for  $G_{ST}$  indicates the existence of a phylogeographic structure, with closely related haplotypes being found more often in the same area than less closely related haplotypes (Pons and Petit 1996). The numbers of haplotypes and unique haplotypes ( $N_{\rm h}$  and  $N_{\rm ph}$ ) within a population were also estimated to assess genetic diversity.

To define groups of populations that maximally differentiate from each other, we divided the 15 wild-lotus populations in China into the following two groups: NEC (Table 1), located in glaciated regions, and all other populations in China (i.e. excluding NEC), situated in ice-free regions. Analyses of molecular variance (AMOVA) assessing the genetic differentiation within and between geographical regions were performed using ARLEQUIN 2.000 (Schneider *et al.* 2002) and



Fig. 1. The localities of populations sampled in the study and geographical distribution of the cpDNA haplotypes detected in each population of *Nelumbo nucifera*. Haplotypes are denoted by colour and their geographic frequency within populations is represented by pie graphs.

significance tests in all cases were conducted using 10000 permutations. Genetic relationships among haplotypes were inferred with Network 4.5.0.1 (Fluxus Technology Ltd, http:// www.fluxus-engineering.com accessed on 30 July, 2010) by using the median-Joining method (Bandelt *et al.* 1999). Point mutations and indels were assumed to evolve with equal

possibility; because the evolutionary rate of cpDNA is slow (Wolfe *et al.* 1987), differences between point and indel mutation rates are unlikely to affect the resolution of intraspecific phylogenetic relationships over short timescales (Gonzales *et al.* 2008; Ronikier *et al.* 2008). Each indel was considered to have originated independently of other indels.

#### Results

### Chloroplast haplotype diversity and population differentiation

We detected a high level of polymorphism in the chloroplast DNA. Nineteen mutations (19 indels and zero point mutations) were detected using the three primer–enzyme combinations (Tables A1, A2, available as an Accessory Publication). After combining information from these 19 mutations, a total of 37 cpDNA haplotypes was distinguished among 417 individuals in the 19 populations investigated (Table A3, available as an Accessory Publication, Fig. 1). Of the 19 populations sampled, most populations were polymorphic, containing two or more haplotypes. Only 3 (GM, ZJ and CH) of the 19 populations were monomorphic for H31, H22 and H6, respectively. Population YY located in Heilongjiang Province, NEC, had the maximum number of haplotypes (10) and unique haplotypes (6) (Table 1, Fig. 1).

Total genetic diversity  $h_{\rm T}$  (0.947) across all populations was higher than the average within-population diversity  $h_{\rm S}$  (0.429), and both  $G_{\rm ST}$  (0.547) and  $N_{\rm ST}$  (0.691) were relatively high (Table 2). The permutation test showed that  $N_{\rm ST}$  was significantly (P < 0.05) higher than  $G_{\rm ST}$ , indicating that groups of related haplotypes were restricted to particular geographical regions. The differentiation of all populations was higher than that of the populations in NEC alone ( $G_{\rm ST} = 0.459$ ). In contrast, within-population diversity was higher in NEC ( $h_{\rm S} = 0.474$ ) than in the other areas ( $h_{\rm S} = 0.388$ ) (Table 2).

The results of the AMOVA among populations showed that most of the total variance (71%) was explained by differences among the populations (Table 3). The comparison of the two regions revealed a significantly high level of variation (12%) between the regions. A large amount of variation (61%) was also found among populations within regions.

## Geographical distribution and phylogenetic relationships of chloroplast haplotypes

The distribution of the 37 cpDNA haplotypes in *N. nucifera* was not random but showed a clear geographical structure (Table 1, Fig. 1). H22 (in 8 of 19 populations), H23 (in 4 of 19 populations) and H30 (in 5 of 19 populations) were the most widespread haplotypes (Table A3, available as an Accessory Publication). Some haplotypes were shared by distinct regions, such as NEC and central China (H16, H17, H22, H30), central China and Thailand (H4, H12), central China and Japan (H21) (Table 1, Fig. 1). Except for the shared haplotypes distributed across multiple regions or populations, most haplotypes were private and fixed in particular regions. The composition of haplotypes in populations located in southern China, India and Thailand was different from that in NEC (Table 1, Fig. 1).

The network illustrated the relationships among the 37 haplotypes (Fig. 2). All haplotypes were distinguished from each other by one or two mutational steps. The haplotypes harboured by populations in central China had close relationships with those distributed in southern regions, such as India and Thailand, whereas, most haplotypes located in NEC were significantly diverged from those in other places. All tip haplotypes, except H2 and H31, in the network were unique to a particular population, indicating that these haplotypes were formed recently (Table A3, available as an Accessory Publication).

#### Discussion

Our survey of cpDNA variation throughout the geographical distribution of *N. nucifera* in Asia, including China and some more southern regions, showed that the haplotype diversity of *N. nucifera* was relatively high in the formerly glaciated NEC, especially in Population YY. The high-level population

 Table 2. Gene-diversity  $(h_S, h_T, v_S, v_T)$  and differentiation  $(G_{ST}, N_{ST})$  parameters within (1) north-eastern China (NEC), (2) all populations in China, excluding NEC, and (3) all populations of *Nelumbo nucifera* combined

Values are means, with s.d. in parenthesis.  $h_{\rm S}$  = diversity within populations,  $h_{\rm T}$  = the total gene diversity,  $G_{\rm ST}$  = level of population subdivision of diversity ( $v_{\rm S}, v_{\rm T}$  and  $N_{\rm ST}$  are parameters equivalent of  $h_{\rm S}$ ,  $h_{\rm T}$  and  $G_{\rm ST}$ ). \*Indicates that  $N_{\rm ST}$  is significantly different from  $G_{\rm ST}$  (0.01 < P < 0.05)

Region	$h_{\rm S}$	$h_{\mathrm{T}}$	$G_{\rm ST}$	vs	$v_{\mathrm{T}}$	$N_{\rm ST}$
(1) NEC	0.474 (0.113)	0.877 (0.076)	0.459 (0.114)	0.326 (0.089)	0.808 (0.086)	0.596* (0.096)
(2) All areas, excluding NEC	0.388 (0.070)	0.976 (0.014)	0.602 (0.077)	0.248 (0.060)	0.915 (0.044)	0.729* (0.072)
(3) All areas combined, including NEC	0.429 (0.064)	0.947 (0.028)	0.547 (0.068)	0.285 (0.052)	0.923 (0.044)	0.691* (0.058)

 Table 3.
 Analysis of molecular variance (AMOVA) of *Nelumbo nucifera* genetic variation for the whole distribution range and for the two regions, north-eastern China and the other regions (d.f. = degrees of freedom)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P-value
The whole area					
Among populations	18	311.141	0.774	70.83	< 0.001
Within populations	398	126.936	0.319	29.17	< 0.001
Total	416	438.077	1.093		
Divided into two regions					
Between regions	1	44.232	0.134	11.59	< 0.001
Among populations within regions	17	266.909	0.703	60.83	< 0.001
Within populations	398	126.936	0.319	27.57	< 0.001
Total	416		1.157		



Fig. 2. A phylogenetic reconstruction of the relationships among the 37 haplotypes of *Nelumbo nucifera*. The area of each circle is proportional to the mean frequency of the haplotype across all populations. Filled portion of circles represents the proportion of haplotypes harboured by north-eastern China, whereas the open portion represents the proportion of haplotypes harboured by the other areas (i.e. areas not including north-eastern China).

divergence for cpDNA variation in the wild lotus indicated low levels of seed-based gene flow and a significant phylogeographical structure. The phylogenetic relationships of the recovered haplotypes showed that the haplotypes of wild lotus in NEC were significantly diverged from those distributed in central China and southern regions. These findings are informative of the demographic history of the species, suggesting multiple northern refugia in NEC for *N. nucifera* during the LGM.

#### Genetic diversity and population differentiation

In the present study, we detected a high level of genetic variation in the chloroplast genome of *N. nucifera*, using a PCR–RFLP approach across the 19 field populations. We observed 37 haplotypes in 417 *N. nucifera* individuals, which was higher than the number of haplotypes in other plants detected by using similar molecular markers, e.g. eight haplotypes in 158 *Ginkgo biloba* individuals (Shen *et al.* 2005), 11 haplotypes in 399 *Fagus sylvatica* individuals (Demesure *et al.* 1996), 13 haplotypes in 217 *Alnus glutinosa* individuals (King and Ferris 1998), 23 haplotypes in 1412 white oaks (Dumolin-Lapegue *et al.* 1997) and eight haplotypes in 608 *Sagittaria latifolia* individuals (Dorken and Barrett 2004). The higher level of total diversity recorded in lotus might reflect a longer evolutionary history and the wide distribution of this species.

A relatively high level of haplotype differentiation among populations of N. *nucifera* was detected in our study. The high population subdivision can be caused by the mode of reproduction, seed dispersal and geographical isolation (Schnabel and Hamrick 1990; Hampe et al. 2003; Zhang et al. 2007). Lotus seeds are transported mainly by water, because the seeds quickly drop into water as they mature, with the flat-topped receptacle turning downwards. Gene flow among the populations may also be limited by separation by rivers or lakes, despite occasional dispersal by birds that may disperse seeds over long distances (Xue et al. 2006), resulting in population differentiation. We found lower population differentiation in NEC than in the other regions in China. This could be attributed to the local river system, where three major rivers, Songhua River, Heilong River and Wusuli River, that cross most habitats of N. nucifera in NEC, may enhance the gene flow among populations. However, the river systems crossing China, such as Yellow River and Yangtze River, flow from west to east and there is no river system connecting northern and southern China. This would reduce connectivity and increase differentiation between northern and southern regions. Lower differentiation in NEC may also be due to denser sampling of populations in this area.

#### Refugia of Asian sacred lotus

The pattern of haplotype divergence in NEC is complex. Some populations, e.g. JMS in the west and YY in the east, were highly variable, with large numbers of haplotypes, many of them unique. Other populations had much lower haplotype diversity, and some were fixed for specific haplotypes. Moreover, haplotypes of *N. nucifera* in NEC were diverged from those in central China and more southern regions (India, Thailand). This pattern is not

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consistent with the hypothesis of lotus in NEC being extirpated during the Quaternary glaciations, with subsequent colonisation. Such a scenario would lead to low genetic diversity and uniform composition of haplotypes in these populations because of founder effects and genetic drift (Hewitt 2000). Moreover, if this were the case, the present distribution range in NEC would have been created by post-glacial range expansion, and haplotypes existing in the southern refugial populations would also be detected in the entire distribution range.

An alternative hypothesis is that some of the extant populations in NEC may have served as refugia during the Quaternary glacial periods. Populations YY and XK located in Heilongjiang Province, near Lake Xingkai, are highly genetically variable with a large number of haplotypes and are likely to have been refugia for Asian lotus during the Pleistocene glaciations. High genetic variation was also found in Population JMS, located in the Songhua River valley, with seven haplotypes and five unique haplotypes. The diversity of haplotypes in JMS indicated that it had also persisted through glacial cycles and, thus, represented another refugium. The low diversity in other populations (e.g. ZJ, GM and SL) between these two refugial areas most likely indicates a more recent colonisation.

Similar patterns were also present in central China where some populations had high diversity, whereas others had lower diversity. In our study, central China (Hubei Province) could be a potential refugium for N. nucifera because relatively high genetic diversities were detected, including seven haplotypes and three unique haplotypes. This is quite possible, given that most parts of central China were never covered by ice sheets during glacial periods (Axelrod et al. 1996). In addition, more southern regions were also potential refugia for N. nucifera, such as Thailand, which harboured six haplotypes and two unique haplotypes. Although most haplotypes were specific to these regions of NEC, central China or Thailand, some were shared among regions. Shared haplotypes can occur through colonisation from other regions or may be a result of incomplete lineage sorting. Haplotypes H3 and H4 that occurred in both central China and Thailand were tip haplotypes and are likely to have arisen from colonisation, most likely from Thailand to central China. In contrast, Haplotypes H12 (occurring in Thailand and central China) and H22 (occurring in central China and NEC) were internal haplotypes and are most likely to represent ancestral haplotypes retained in populations in different regions through time.

Overall, our results (high diversity, divergent haplotypes and differentiation among populations) indicate multiple refugia throughout the distribution of the species in NEC, central China and Thailand. Populations with low diversity of common haplotypes may have been colonised from the refugial populations later, particularly after the LGM. These patterns indicative of multiple refugia have been found in other species recently and demonstrate that the temperate northern hemisphere model is not necessarily applicable to other biomes (Byrne 2008).

#### Why a northern refugium for Asian sacred lotus?

The location of lotus refugia in NEC is much further north from that of the other ancient plants in China (Ying *et al.* 1993; Wang and Liu 1994; Lu *et al.* 2001; Hwang *et al.* 2003; Li *et al.* 2005;

Shen *et al.* 2005; Su *et al.* 2005; Zhang *et al.* 2005; Wang and Ge 2006; Gao *et al.* 2007; Yang *et al.* 2008). Given that low temperature is a key factor shaping the distribution of lotus, one may expect that lotus needed to be sheltered in the southern area. Below, we discuss several possible reasons why JMS, XK and YY could be refugia.

First, lotus fossils were found in the deposits of the Cretaceous in the Amur River (КришТОфОВич 1965), which is near Songhua River where Population JMS was located. Second, Lake Xingkai was of tectonic origin in the Cretaceous, whereas tectonic movements were weak during the late Pleistocene, thus enabling the formation of a relatively stable freshwater lake, surrounded by mountains, in the Quaternary period (Qiu et al. 1988). Rivers and lakes could have offered the necessary moist habitat for lotus during the ice ages, when other parts of China were cooler and drier, with the environment being hostile for aquatic plants (Axelrod et al. 1996). Third, specialised characteristics of the lotus could have contributed to its survival in situ in the north. Fluctuations of temperature in aquatic habitats are generally less violent than in the aerial environments (Sculthorpe 1967). The damage resulting from low temperatures on aquatic plants can be reduced if seeds are buried in deep water and in the mud. Old lotus seeds buried in the mud over thousands of years could have germinated and reproduced successfully when microclimate became more favourable during the interglacial period (Shen-Miller et al. 2002). Green seed embryos of lotus seeds might have facilitated germination under deep mud, enabling the germinants to resurge when microclimate became favourable during the ice age. Finally, lotus flowers have the ability to thermoregulate intrafloral temperature between 30 and 36°C with the carpellary receptacle heating when air temperature fluctuates between 10 and 45°C (Seymour and Schultze-Motel 1996, 1998). The self-heating flowers could have provided heat reward to pollinators in cool conditions and facilitated sexual reproduction even when the plant was suffering under low temperatures (Seymour and Schultze-Motel 1996; Li and Huang 2009*a*).

Our understanding of the effect of Quaternary climatic changes on plant distribution comes basically from terrestrial plants, particularly from trees (Comes and Kadereit 1998). Little is known about the effects of palaeoclimate on the distribution of aquatic plants, although these plants typically have much broader geographical distributions than do their terrestrial counterparts (Santamaría 2002; Dorken and Barrett 2004). The extensive distribution of aquatic flowering plants has been attributed to aquatic habitats, clonal growth, long-distance dispersal or relicts of continental drift (see Les et al. 2003). Indeed, the genus *Nelumbo* traditionally comprises two extant species, namely the Asian N. nucifera and the North American Nelumbo lutea (Willd.) Person. Because there is no distinctive morphological differentiation between the two taxa, Borsch and Barthlott (1994) treated N. lutea as a subspecies of N. nucifera. Whereas other phylogeographical studies have documented that central and south-western China were valley areas of sheltered topography that protected numerous terrestrial plants from extinction during the ice ages, our investigation documented multiple northern refugia of this ancient plant on the basis of analyses of cpDNA markers and fossil records. Our study presents an example from aquatic plants that may respond to historical climate changes differently from many terrestrial species.

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