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Molecular phylogeography of four endemic *Sagittaria* species (Alismataceae) in the Sino-Japanese Floristic Region of East Asia

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To reveal the role of climate oscillations of the Quaternary in forming the contemporary plant diversity in the temperate Sino-Japanese Floristic Region of mainland China, we assess the phylogeographical patterns of four *Sagittaria* species in the region using sequence data from plastid DNA non-coding regions (*psbA-trnH*, the *rpl16* intron and *trnC-ycf6*) and the internal transcribed spacers of nuclear ribosomal DNA (nrITS). Based on both datasets, the divergence time among the four studied species was estimated to fall in the Late Tertiary (plastid DNA: 7.1–13.7 Mya; ITS: 11.1–16.1 Mya). The ancestral distribution analyses revealed that regions with a great diversity in topography, climate and ecological conditions, e.g. the Hengduan Mountains, Central China and East China, were the areas where the endemics originated. Mismatch distribution analyses revealed that each species had experienced a range expansion in response to Quaternary climatic oscillations. Our findings contradict the hypothesis of Quaternary origins of the endemic *Sagittaria* spp.; we support the view that modern species in the Northern Hemisphere originated mostly during the Tertiary. Range expansion may have profoundly modified the current distribution ranges of *Sagittaria* species in the Sino-Japanese Floristic Region. *Botanical Journal of the Linnean Society*, 2016, **180**, 6–20.

ADDITIONAL KEYWORDS: ancestral distribution – chloroplast DNA – divergence time – nuclear DNA – quaternary climatic oscillations – regional expansion.

INTRODUCTION

The temperate flora of the Sino-Japanese Floristic Region (SJFR) of East Asia is characterized by unique levels of species richness and endemism, comprising *c*. 3000 vascular plant genera of which *c*. 248 are endemics (Wu & Wu, 1996). The SJFR represents one of the most diverse temperate floras of the world and is substantially richer than other temperate climate regions in the Northern Hemisphere, e.g. eastern North America (Qian, 2002; Qian & Ricklefs, 2004). The diversity mostly resides in mainland China, a part of the SJFR (Liu, 1988; Wu & Wu, 1996; Qian & Ricklefs, 2000; Ying, 2001). Addressing the historical origins of the plant richness of the SJFR in mainland China has been of major interest for phylogeographers and palaeo-ecologists (Qiu, Fu & Comes, 2011).

Several major factors have been generally thought to have resulted in such high levels of plant diversity and endemism in the SFJR of mainland China (Qiu *et al*., 2011). One of the key reasons is that during the glacial periods of the Quaternary (< 2 Mya), the climate in most parts of China was less severe than in other parts of the Northern Hemisphere where glaciations were extensive (Shen, 2002; Ehlers & Gibbard, 2007; Chou *et al*., 2011). Many areas in China acted as glacial refugia for plant survival and evolution (Tiffney, 1985; Axelrod, Al-Shehba & Raven, 1996; Ying, 2001; Qiu *et al*., 2011). Currently, the view of modern species in temperate regions in the Northern Hemisphere origi-

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nating mostly in the Tertiary or earlier and surviving through the Quaternary glaciations has been widely accepted by phylogeographers and palaeo-ecologists (Hewitt, 2000; Willis & Niklas, 2004; Rull, 2006, 2008). However, the role of climate oscillations during the Quaternary in forming the contemporary diversity in the SJFR in China remains controversial. Some authors believe that the Quaternary climate oscillations and associated environmental changes may have provided opportunities for speciation through promoting population fragmentation and isolation. Examples include the origin of *Myricaria laxiflora* (Franch.) P.Y.Zhang & Y.J.Zhang (Tamaricaceae; Liu, Wang & Huang, 2009a) and *Kirengeshoma koreana* Nakai (Hydrangeaceae; Qiu *et al*., 2009). Other authors suggest that many temperate plant species were at an incipient stage of allopatric speciation during the Quaternary (Qiu *et al*., 2011). To date, most phylogeographical studies in the SJFR in mainland China have focused on individual species and as yet there are only limited published data examining phylogeographical patterns in groups of closely related taxa, e.g. in the same genus (Qiu *et al*., 2011); there is still much to be discovered about the role of allopatric speciation, especially in locally endemic plant species formation, triggered by the Quaternary climatic oscillations. By combining interspecific and intraspecific phylogeographical analyses, the time and location of origin could be estimated and the question of range expansions in these species in response to climatic oscillations could be answered.

The genus *Sagittaria* L. (Alismataceae) provides a suitable system for examining the potential influence of Quaternary climatic oscillations on species divergence in the SJFR of mainland China. Alismataceae represent one of the earliest diverging lineages of monocots (Les & Schneider, 1995). *Sagittaria* is an aquatic genus comprising *c*. 40 currently recognized species, which occur naturally in Africa, Asia, Europe and North and South America (Keener, 2005). Of these areas, eastern Asia and North and South America are the most species-rich (Keener, 2005). Five endemic species occur in the SJFR in East Asia (*S. tengtsungensis* H.Li, *S. lichuanensis* J.K.Shen, S.C.Sun & H.Q.Wang, *S. potamogetifolia* Merr., *S. aginashi* Makino and *S. pygmaea* Miq.), with the first three species being endemic to China (Chen *et al.*, 1984; Chen, 1989; Wang, Haynes & Hellquist, 2010). These Chinese endemics are all narrowly distributed: *S. tengtsungensis* is restricted to the south-western Yunnan Plateau; and *S. lichuanensis* and *S. potamogetifolia* are mainly confined to the Wuling Mountains in central and south-eastern China (Chen, 1989). There are only a few populations of *S. aginashi*, a Japanese endemic, in the Japanese archipelago. *Sagittaria pygmaea*, the smallest *Sagittaria* species, is distributed mainly in central and southern China, but its range extends to Japan and Korea (Chen, 1989). In the SJFR of mainland China, *S. pygmaea* is sympatric with the three Chinese endemics. Recent molecular phylogenetic studies, e.g. generic analyses of Alismataceae (Chen *et al*., 2012) and species-level analyses of *Sagittaria* (Keener, 2005), have suggested that *S. pygmaea*, *S. tengtsungensis*, *S. lichuanensis* and *S. potamogetifolia* comprise a monophyletic group.

Based on the world-wide distribution pattern of *Sagittaria* species, Chen (1989) suggested East Asia as one of the centres of high endemic species richness. Given the current distribution ranges, the endemic *Sagittaria* species in mainland China are now confined to putative Pleistocene glacial refugia areas (e.g. the SJFR) and, due to the complex geographical history of these regions, Chen (1989) further proposed that the Chinese endemic *Sagittaria* species may have originated from these regions during the Quaternary. However, due to the lack of convincing evidence, such as molecular phylogenetics and molecular dating and the lack of species-level phylogeographical studies, and despite several recent studies being conducted on within-species phylogeography [e.g. for *S. potamogetifolia* (Tan *et al*., 2008) and *S. lichuanensis* (Liu *et al*., 2010)], the mechanisms of speciation in *Sagittaria* in the SJFR of mainland China remain uncertain.

In this study, we used sequences from three plastid DNA non-coding regions (*psbA-trnH*, the *rpl16* intron and *trnC-ycf6*) and the internal transcribed spacers (ITS) of nuclear ribosomal DNA (nrDNA) to examine phylogeographical patterns in four *Sagittaria* species (*S. pygmaea*, *S. tengtsungensis*, *S. lichuanensis* and *S. potamogetifolia*) from the SJFR of mainland China. Our specific objectives were to infer: (1) the divergence time between these species; (2) the possible geographical areas from where the species originated; and (3) the possible impacts of past geological and climatic oscillations on the evolution and dispersal of these species.

MATERIAL AND METHODS

Plant sampling

In total, 161 samples from 31 populations were analysed in this study, representing four endemic *Sagittaria* species in the SJFR. For each of the three Chinese endemic species, all extant populations were sampled. To account for the genetic divergence and speciation of endemics in the SJFR of mainland China, for sampling in East Asia endemic *S. pygmaea*, emphasis was placed on sampling the populations sympatric or close to the three Chinese endemics. Sampling sites of *S. pygmaea* covered most parts of

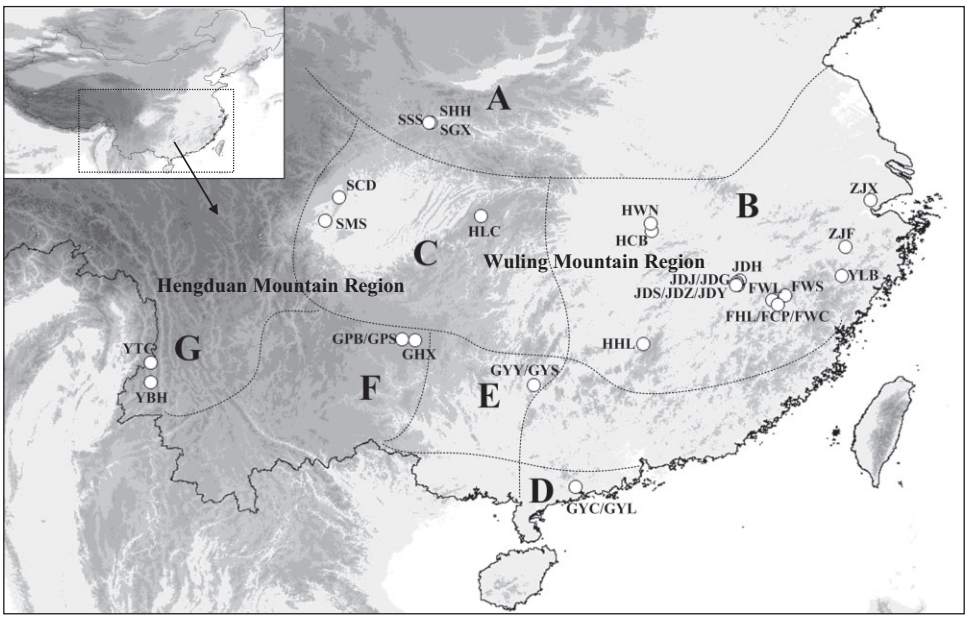

Figure 1. Collection localities (population codes as in Table 1) of four *Sagittaria* species in China. Seven geographical regions (A–G) in China defined by Wu (1979) used for BBM analyses in this study are also shown. A, Loess plateau sub-region; B, East China region; C, Central China region; D, South China Sea region; E, Yunnan, Guizhou, Guangxi region; F, Yunnan Plateau region; G, Hengduan Mountains region.

its distribution range in the SJFR of mainland China (Fig. 1). The Japanese endemic *S. aginashi* was not included in this study. The latitude and longitude of each sampling site were recorded by a global positioning system (GPS). Vouchers of all sampled populations are deposited in the Herbarium of Wuhan Botanical Garden, Wuhan, China (WBG). Details of collection sites and vouchers are given in Table 1. *Sagittaria guayanensis* was selected as the outgroup based on recent phylogenetic studies (Keener, 2005; Chen *et al*., 2012). About 5 g of fresh leaves was harvested from each plant and immediately dried in a ziplock plastic bag containing about 70 g of silica gel. The samples were stored at room temperature until DNA was isolated in the laboratory.

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Total genomic DNA was extracted from silica-dried leaves following a modified cetyltrimethylammonium bromide (CTAB) extraction procedure (Doyle & Doyle, 1987). PCR was performed to amplify three non-coding regions of plastid DNA (*psbA-trnH*, the *rpl16* intron and *trnC-ycf6*) and the ITS region (including ITS1, 5.8S and ITS2). The amplification primers for the four regions were as follows: (1) *psbA-trnH* (5′-GTTATG CATGAACGTAATGCTC-3′ and 5′-CGCGCATGGTG GATTCACAATC-3′) (Sang, Crawford & Stuessy, 1997); (2) the *rpl16* intron (5′-GCTATGCTTAGTGTGTGA CTCGTTG-3′ and 5′-CCCTTCATTCTTCTATGTTG-3′) (Small *et al*., 1998); (3) *trnC-ycf6* (5′-CCAGTTCRA ATCYGGGTG-3′ and 5′-GCCCAAGCRAGACTTACT ATATCCAT-3′) (Demesure, Sodzi & Petit, 1995); and (4) ITS (5′-TCCTCCGCTTATTGATATGC-3′ and 5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White *et al*., 1990). PCRs were carried out in a volume of 50 μL containing 0.25 mM each dNTP, 5 μL 10× Taq buffer [10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 50 mM KCl], 1 mM each primer, 2 U Taq polymerase (TransGen Biotech) and 60 ng template DNA. Amplification of genomic DNA was performed using an Eppendorf AG 22331 Hamburg thermocycler (Eppendorf). The PCR profile for the three plastid DNA non-coding regions was programmed with an initial denaturation of 4 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 2 min annealing at 53–60 °C (depending on the primers) and 2 min extension at 72 °C, with a final extension cycle of 7 min at 72 °C. For the nuclear ITS region, the PCR cycling parameters were set as follows: 5 min at 95 °C, followed by 35 cycles of 20 s at 94 °C, 30 s annealing at 50 °C and 40 s extension at 72 °C, and a final extension cycle of 10 min at 72 °C.

The size of each PCR product was determined electrophoretically on a 1.5% (w/v) agarose gel run at 100 V in 0.5× TBE (Tris-boric acid-EDTA) and

Table 1. Collection details of accessions of four *Sagittaria* species from China; plastid DNA haplotypes for each species are also shown

Species	Locality	Population code	Voucher	Coordinates (E/N)	Haplotype(s)
S. potamogetifolia	Huli, Hunan	HHL	SAGIT001	113°40′/26°16′	H12, H14
	Dongxiang, Jiangxi	JDZ	SAGIT002	116°33′/28°06′	H14, H17, H18
	Dongxiang, Jiangxi	JDS	SAGIT003	116°31′/28°04′	H13, H14, H15, H16
	Yanshan, Guangxi	GYY	SAGIT004	110°19′/25°01′	H12, H14
	Dongxiang, Jiangxi	JDY	SAGIT005	116°31′/28°05′	H11
S. lichuanensis	Dongxiang, Jiangxi	JDG	SAGIT006	116°34′/28°05′	H26, H27
	Pingba, Guizhou	GPB	SAGIT007	106°16′/26°25′	H24, H25
	Wuyishan, Fujian	FWL	SAGIT008	117°37′/27°37′	H26, H27
	Dongxiang, Jiangxi	JDJ	SAGIT009	116°32′/28°10′	H26
	Wuyishan, Fujian	FCP	SAGIT010	117°48′/27°28′	H19, H22, H26, H27
	Lichuan, Hubei	HLC	SAGIT011	108°42′/30°11′	H20, H21, H23
S. tengtsungensis	Tengchong, Yunnan	YBH	SAGIT012	98°33′/25°07′	H30
	Tengchong, Yunnan	YTC	SAGIT013	98°34′/25°43′	H29, H30
S. pygmaea	Pingba, Guizhou	GPS	SAGIT014	106°16′/26°25′	H1
	Jinghua, Zhejiang	ZJF	SAGIT015	119°53′/29°14′	H2
	Yanshan, Guangxi	GYS	SAGIT016	110°19′/25°01′	H5, H6
	Dongxiang, Jiangxi	JDZ	SAGIT017	116°33′/28°06′	H3
	Dongxiang, Jiangxi	JDH	SAGIT018	116°37′/28°13′	H3, H8, H10
	Yangchun, Guangdong	GYL	SAGIT019	111°36′/21°55′	H4
	Wuyishan, Fujian	FWS	SAGIT020	118°01′/27°46′	H3, H8
	Wuyishan, Fujian	FWL	SAGIT021	117°37′/27°37′	H3
	Lishui, Zhejiang	YLB	SAGIT022	119°46′/28°21′	H4
	Jiaxing, Zhejiang	ZJX	SAGIT023	120°38′/30°40′	H7
	Chibi, Hubei	HCB	SAGIT024	113°56′/29°43′	H3
	Huaxi, Guizhou	GHX	SAGIT025	106°40′/26°24′	H4
	Meishan, Sichuan	SMS	SAGIT026	103°54′/30°03′	H1
	Chengdu, Sichuan	SCD	SAGIT027	104°22′/30°44′	H1
	Hanzhong, Shaanxi	SHH	SAGIT028	107°08′/33°02′	H6
	Hanzhong, Shaanxi	SGX	SAGIT029	107°08′/33°02′	H6
	Hanzhong, Shaanxi	SSS	SAGIT030	107°06′/33°02′	H6
	Wuhan, Hubei	HWN	SAGIT031	113°53′/29°58′	H9

visualized with ethidium bromide. All PCR products were purified from agarose gels using the TIANquick Midi Purification Kit following the protocols provided by the manufacturer (Tiangen). The purified PCR products were sequenced in both directions using the ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) and performed on an ABI 3730 automated sequencer (Applied Biosystems). The primers used for sequencing were same as those used for PCR. The LASERGENE program (DNASTAR) was used to assemble the two sequences of each individual.

Plastid DNA diversity and population structure

The sequences of the three plastid DNA regions were combined and aligned with CLUSTAL X (Thompson, Higgins & Gibson, 1994) and then adjusted manually with BioEdit (Hall, 1999). Insertions/deletions (indels) were treated as point mutations and equally weighted with other mutations. Plastid DNA haplotypes were determined from nucleotide substitutions and indels of the aligned sequences. Haplotype diversity (h) and nucleotide diversity per population (π) of each species (Nei & Tajima, 1983) were calculated using the program ARLEQUIN ver. 3.1 (Excoffier, Laval & Schneider, 2006).

Analysis of molecular variance (AMOVA) of *Sagittaria* populations was partitioned by two levels: species and regional. At the species level, variance was apportioned among individuals within populations, among populations within species and among species. At the regional level, variance was apportioned among individuals within populations, among populations within regions and among regions. Seven

regions of the distribution range of the four endemic *Sagittaria* species from China were determined according to Wu (1979) (Fig. 1). All AMOVAs were performed in ARLEQUIN.

To assess the significance of isolation by distance between populations, pairwise population F_{ST} values were regressed against the logarithm of geographical distances between localities for all pairs of populations. Pairwise F_{ST} values between populations were estimated using ARLEQUIN, geographical distances between populations were calculated with PASSAGE v. 1.0 (Rosenberg & Anderson, 2011) and a Mantel test was used to evaluate significance with 1000 random permutations using ARLEQUIN.

To infer the demographic history of the four *Sagittaria* species in China, we used two approaches. First, we calculated Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997) for each entire species and groups of populations using ARLEQUIN. The significance of the *D* and *Fs* values was tested using 1000 randomization replicates. Second, we tested the null hypotheses of a spatial expansion and of a pure demographic expansion in ARLEQUIN by revealing the frequency distribution of several pairwise differences in the sequences among all individuals of each species (the mismatch distribution). For each expansion model, goodness-of-fit was tested using the sum of squared differences (SSD) and Harpending's raggedness index (*HRag*) (Harpending, 1994). A parametric bootstrap approach (Schneider & Excoffier, 1999) with 1000 replicates was used to test the observed fit of the mismatch distribution to the sudden expansion model and to test the significance of *HRag*.

Phylogenetic analyses (nuclear and plastid DNA)

Pairwise differences between plastid DNA haplotypes were calculated using ARLEQUIN. A statistical parsimony haplotype network, based on the matrix of pairwise differences between plastid DNA haplotypes, was obtained with TCS 1.06 (Clement, Posada & Crandall, 2000) using a 95% connection probability limit and treating gaps as single evolutionary events.

Relationships among plastid DNA haplotypes were analysed with maximum-parsimony (MP) and neighbour-joining (NJ) using MEGA 6 (Tamura *et al*., 2013) (Supporting Information Figs S1, S2). The MP analyses involved a heuristic search strategy with 1000 replicates of ten additional sequences, in combination with tree bisection-reconnection (TBR) branch-swapping on all the resulting trees. All characters were unordered and had equal weight. All sites (including gaps/missing) were used. Branch support was assessed by bootstrap analysis with 1000 replicates. The NJ tree was reconstructed according to Kimura's (1980) two-parameter model. Confidence in the nodes was tested by performing 1000 bootstrap replicates (Felsenstein, 1985). Gaps/missing data were treated as 'pairwise deletion'. The plastid DNA haplotype cladogram was rooted using the haplotype from *S. guayanensis*.

The MP and NJ analyses were also performed on the nuclear ITS region sequences of *Sagittaria* plants sampled in China using the program MEGA 6 (Figs S3, S4). The analysis methods using MEGA 6 for ITS sequence data were the same as for the plastid DNA dataset described above.

Estimate of divergence time

The divergence times between four endemic *Sagittaria* species were estimated via a Bayesian approach implemented in BEAST v 1.8.1 (Drummond *et al*., 2012). A strict molecular clock assumption was rejected for both plastid DNA and nrDNA haplotype sequence datasets because of significant rate heterogeneity among lineages; in addition, no fossils are available for *Sagittaria* in China. Thus, we constructed two alternative datasets, first to estimate the nucleotide mutation rates of plastid DNA and nrDNA sequences for *Sagittaria* (Figs S5, S6) and, based on these mutation rates, secondly to estimate the possible divergence timescales of the main plastid DNA lineages across the four sampled species in China. Two datasets were constructed for plastid DNA sequences. The first comprised samples from seven genera of Alismataceae: seven *Sagittaria* species (two haplotypes for each species except *S. guayanensis*, for which there was only one haplotype), two species of *Caldesia* Parl., and one species each of *Hydrocleys* Rich., *Limnocharis* Bonpl., *Alisma* L., *Ranalisma* Stapf and *Echinodorus* Rich. & Engelm. ex A.Gray (Table S1). The other dataset comprised samples from *Sagittaria* only: four endemics of *Sagittaria* in the JFR (all haplotypes revealed in this study were included) and *S. guayanensis* as outgroup. Two more datasets were constructed for the nrDNA haplotype sequence data files. The first comprised the same seven genera of Alismataceae as selected for the plastid DNA datasets (Table S1). Another dataset comprised all haplotypes revealed in the four endemic *Sagittaria* species in this study and *S. guayanensis* as the outgroup. The indels in each dataset were removed and only nucleotide mutations were considered.

When running the BEAST program, each of the constructed plastid DNA and nrDNA haplotype sequence data files was specified as follows: GTR + Γ + G model with six Gamma categories and the starting tree was randomly generated. The best-fit models (GTR + Γ + G) for Bayesian analyses were selected using MrModeltest 2.3 (Nylander, 2004) in conjunction with PAUP 4.0 (Swofford, 1998) to gener-

ate model scores. Three tree prior models (coalescent: constant size, exponential growth; and speciation: Yule process) and three prior distribution models (uniform, normal and lognormal) were implemented in BEAST. The models (tree prior model: Yule process; prior distribution model: lognormal distribution) that yielded the highest posterior probability estimates were used to perform a final analysis. Two separate Monte Carlo Markov chain analyses were run for 10 000 000 generations with sampling at every 1000 generations to ensure that all the effective sample size (ESS) values were > 200. Tracer v1.5 (Rambaut & Drummond, 2007) was used to check the parameters and the first 10% of generations were discarded as burn-in. TreeAnnotator 1.6.0 (Rambaut & Drummond, 2009) was used to combine and annotate the Bayesian trees. In the analyses of nucleotide mutation rates of plastid DNA and nrDNA sequences of *Sagittaria*, three calibration points were used (Figs S5, S6). (1) *Caldesia* first appeared in the Oligocene of Eurasia (Haggard & Tiffney, 1997) and the split between *Caldesia* and *Echinodorus* was set to be not later than 29.0 Mya (offset = 29.0, mean = 0.7, SD = 0.7). According to the divergence times between the genera of Alismataceae estimated by Chen *et al*. (2012), two other calibration points were set: (2) the split between *Limnocharis* and *Hydeocleys* was set to be not later than 38.29 Mya (offset = 38.29, mean = 1.0, SD = 1.0); and (3) the split between clade A (including *Ranalisma*, *Caldesia* and *Echinodorus*) and clade B (including seven *Sagittaria* species) was set to be not later than 59.29 Mya (offset = 59.29, mean = 1.0, SD =1.0). According to the analyses, a mutation rate of plastid DNA [1.7×10^{-9} substitutions per site per year (s/s/y)] and a mutation rate of nrDNA (1.5×10^{-9} s/s/y) were obtained for *Sagittaria* (Figs S5, S6). These mutation rates were used to calibrate the divergence times of the four endemic *Sagittaria* species separately.

Biogeographical analyses

Ancestral distributions of the endemic *Sagittaria* species from China were reconstructed, using the Bayesian binary method (BBM) (Ronquist & Hulsenbeck, 2003) implemented in RASP (Yu, Harris & He, 2011). Condensed tree, default options and the Jukes-Cantor model were chosen for the analysis. Posterior probabilities for ancestral distributions were calculated using 1000 trees sampled from the trees (excluding burn-in) obtained in the MP analysis based on plastid DNA datasets. The haplotype of the outgroup (*S. guayanensis*) was excluded from the analysis. The distribution of four *Sagittaria* species in China was partitioned among seven area categories according to the definition of Wu (1979): A, Loess plateau sub-region; B, east China region; C, central China region; D, south China Sea region; E, Yunnan, Guizhou, Guangxi region; F, Yunnan Plateau region; and G, Hengduan Mountains region. Analyses that were implemented in the MP tree resulted from the plastid DNA haplotype sequences.

RESULTS

Sequence variation and population structure

The aligned sequences of *psbA-trnH*, the *rpl16* intron and *trnC-ycf6* were 376, 1069 and 539 bp in length, respectively. The total length of combined alignments was 1984 bp. A total of 103 polymorphic sites in the combined three plastid DNA non-coding regions resulted in the resolution of 29 haplotypes (Hap1–Hap27, Hap 29, Hap30) across the 161 individuals (31 populations) of *Sagittaria* plants sampled (Table 1). The identified haplotypes have been submitted to GenBank (accession numbers: KC285063–KC285089, KC285091 and KC285092). Five of the 31 sampled populations contain three or more haplotypes and most of the populations (17) are fixed in one haplotype. Ten haplotypes occurred in more than one sampling location and the most widespread haplotype H3 occurred in five sampling sites. Among the seven defined geographical regions, more than half of the haplotypes (19) occurred in region B (east China region) and the genetic diversity in this region was much higher than in others (Table 2). Haplotype diversity varied across regions, ranging from 0.00 (A, D) to 0.90 (B). Nucleotide diversity was estimated within regions, ranging from 0.0000 (A, D) to 0.0178 (B) (Table 2). Sequence variation demonstrated non-significant deviation from expectations of neutrality with Tajima's criterion and Fu's F_S tests (Table 3).

The aligned sequences of the ITS region were 480 bp in length. Seventy-eight polymorphic sites resulted in the solution of 11 haplotypes (GenBank accession numbers: KC285047, KC285053–KC285062). Only a few haplotypes were found in most of the studied species and thus the sequence data were only used to reconstruct a species-level phylogenetic tree and to estimate the divergence time between species.

From the plastid DNA sequence dataset, based on grouping of species, hierarchical AMOVA showed that a great amount of variation (85.78%) occurred among species and only 12.87% revealed differences among populations within species and 1.35% of the variation was within populations ($F_{ST} = 0.8578$). Based on regional grouping, hierarchical AMOVA showed that most variation (93.06%) occurred among populations within species and only 5.08% revealed differences among regions and 1.86% of the variation was within populations ($F_{ST} = 0.0508$) (Table 4). Mantel tests did not reveal a significant association ($r^2 = 0.962$,

Table 2. Plastid DNA genetic diversity within and across seven defined geographical regions (N_{POP}, number of populations; N_{IND}, number of individuals; N_{HT}, number of haplotypes; π, nucleotide diversity; *h*, haplotype diversity)

Region	N_{POP}	N_{IND}	N_{HT}	π (mean ± SD)	*h* (mean ± SD)
A	3	12	1	0.0000 ± 0.0000	0.00 ± 0.0000
B	17	92	19	0.0178 ± 0.0087	0.90 ± 0.0180
C	3	16	4	0.01219 ± 0.063	0.53 ± 0.1368
D	1	5	1	0.0000 ± 0.0000	0.00 ± 0.0000
E	2	11	4	0.0160 ± 0.0085	0.76 ± 0.0833
F	3	22	4	0.0112 ± 0.0057	0.69 ± 0.0739
G	2	11	2	0.0001 ± 0.0001	0.22 ± 0.2880
Total	31	161	29	0.0170 ± 0.0082	0.93 ± 0.0072

Table 3. Results of Tajima's *D* and Fu's F_S tests, and mismatch distribution analysis for four *Sagittaria* species used in this study

	Tajima's *D* test		Fu's F_S test		Mismatch distribution				
Species	*D*	*P*	F_S	*P*	τ	θ_0	θ_1	*P* (SSD)	*HRag* (*P*)
S. potamogetifolia	0.842	0.871	7.590	0.980	2.800	0.001	1.676	0.510	0.038 (0.480)
S. lichuanensis	−0.219	0.46	1.619	0.781	10.387	0.000	2.892	0.550	0.048 (0.780)
S. tengtsungensis	−1.088	0.202	−0.263	0.161	0.218	0.051	0.516	0.430	0.358 (0.650)
S. pygmaea	0.567	0.741	−0.289	0.483	2.855	0.000	17.242	0.210	0.082 (0.360)

Note: τ = units of mutational time: $\theta_0 = \theta$ before population growth; $\theta_1 = \theta$ after population growth; *P* (SSD) = probability of the sum of the square deviations; *HRag* (*P*) = raggedness statistic (probability of raggedness statistic) ($P < 0.05$, $P < 0.01$).

Table 4. AMOVA of *Sagittaria* populations, partitioned by species and regions

Partitioning	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
By species	Among species	3	1924.201	18.801	85.78
	Among populations	29	388.386	2.821	12.87
	Within populations	122	36.083	0.295	1.35
	Total	154	2348.671	21.918	
By regions	Among regions	6	518.949	0.808	5.08
	Among populations	26	1793.639	14.815	93.06
	Within populations	122	36.083	0.295	1.86
	Total	154	2348.671	15.919	

$P > 0.01$) between genetic differentiation and geographical distances between populations over the distribution of all the species. Mismatch distribution analyses indicated that the observed variance (SSD) and *HRag* were not significantly different from that expected under the population expansion model for each species ($P > 0.1$) (Table 3).

Phylogenetic relationships of haplotypes

The topology of the MP tree based on the 29 plastid DNA haplotypes for *Sagittaria* species, using *S. guayanensis* as the outgroup, was similar to the tree reconstructed under the NJ approach with regard to species-level phylogenetic relationships (Figs S1, S2). MP analysis retained 100 trees, 235 steps in length with a consistency index (CI) = 0.92 and retention index (RI) = 0.95. Each species except *S. lichuanensis* formed a clade with bootstrap values of 98–100% in the NJ tree and 96–100% in the MP tree. *Sagittaria lichuanensis* was paraphyletic. The unrooted TCS network for all *Sagittaria* plastid DNA haplotypes (Fig. 2) was consistent with those recovered using MP and NJ.

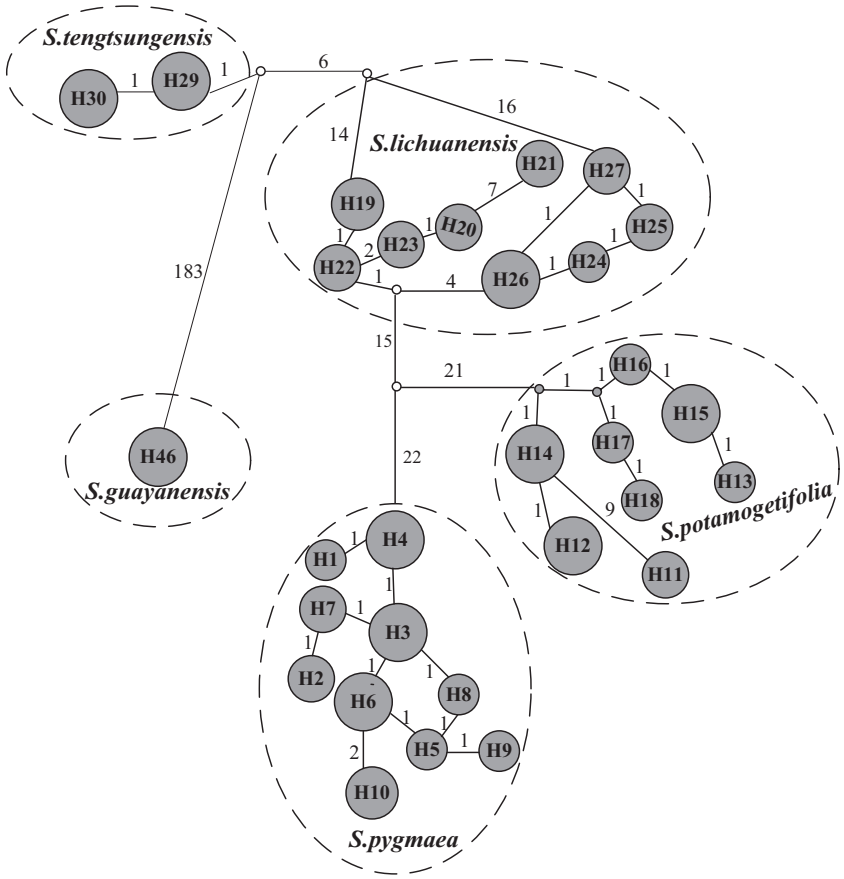

Figure 2. Statistical parsimony network of 29 plastid DNA haplotypes of four endemic *Sagittaria* species and one haplotype (H46) of the outgroup (*S. guayanensis*) sampled in China. Each numbered circle represents a unique haplotype (H1–H27, H29, H30), with circle size reflecting the haplotype frequencies. The number beside the line connecting the haplotype circles indicates the mutational steps between haplotypes. Dashed lines indicate haplotypes attributed to the same species.

The MP and NJ trees based on the 11 haplotypes from the ITS region produced identical topologies with regard to phylogenetic relationships among species (Figs S3, S4). Each species except *S. lichuanensis* formed a clade. The *S. potamogetifolia* clade was supported by a high bootstrap value for MP (96%) and NJ (97%). Again, *S. lichuanensis* was paraphyletic. The topologies based on these 11 haplotypes using both MP and NJ methods were different from the topologies revealed using the 29 plastid DNA haplotypes; for example, with plastid haplotypes, *S. pygmaea* was sister to the other three species, whereas with nrDNA haplotypes, *S. potamogetifolia* was sister to the rest. *Sagittaria lichuanensis* and *S. tengtsungensis* showed a close relationship in all the molecular phylogenetic trees based on both datasets (Figs S1–S4).

Divergence time estimates

The results of divergence time estimates between species from the plastid DNA dataset suggested that the split between *S. tengtsungensis* and *S. lichuanensis* occurred 7.1 Mya [95% high posterior density (HPD) interval of 1.1–13.9 Mya] and *S. potamogetifolia* diverged from the lineage including *S. tengtsungensis* and *S. lichuanensis* 8.3 Mya (95% HPD: 0.4–17.5 Mya). The split between *S. pygmaea* and the lineage including *S. tengtsungensis*, *S. lichuanensis* and *S. potamogetifolia* took place at *ca*. 13.7 Mya (95% HPD: 2.2–21. 9 Mya) (Fig. 3A). Within *S. tengtsung*ensis, branching events took place in the mid-Pleistocene. In contrast, *S. pygmaea*, *S. potamogetifolia* and *S. lichuanensis* underwent a relatively earlier radiation in the late Miocene, with most divergences in the Plio-Pleistocene (data not shown).

The divergence time estimates between species based on the ITS dataset were similar to those estimated from the plastid dataset. The split between *S. tengtsungensis* and *S. lichuanensis* was estimated at 11.1 Mya. The split between *S. pygmaea* and *S. potamogetifolia* took place at 11.8 Mya. The lineage

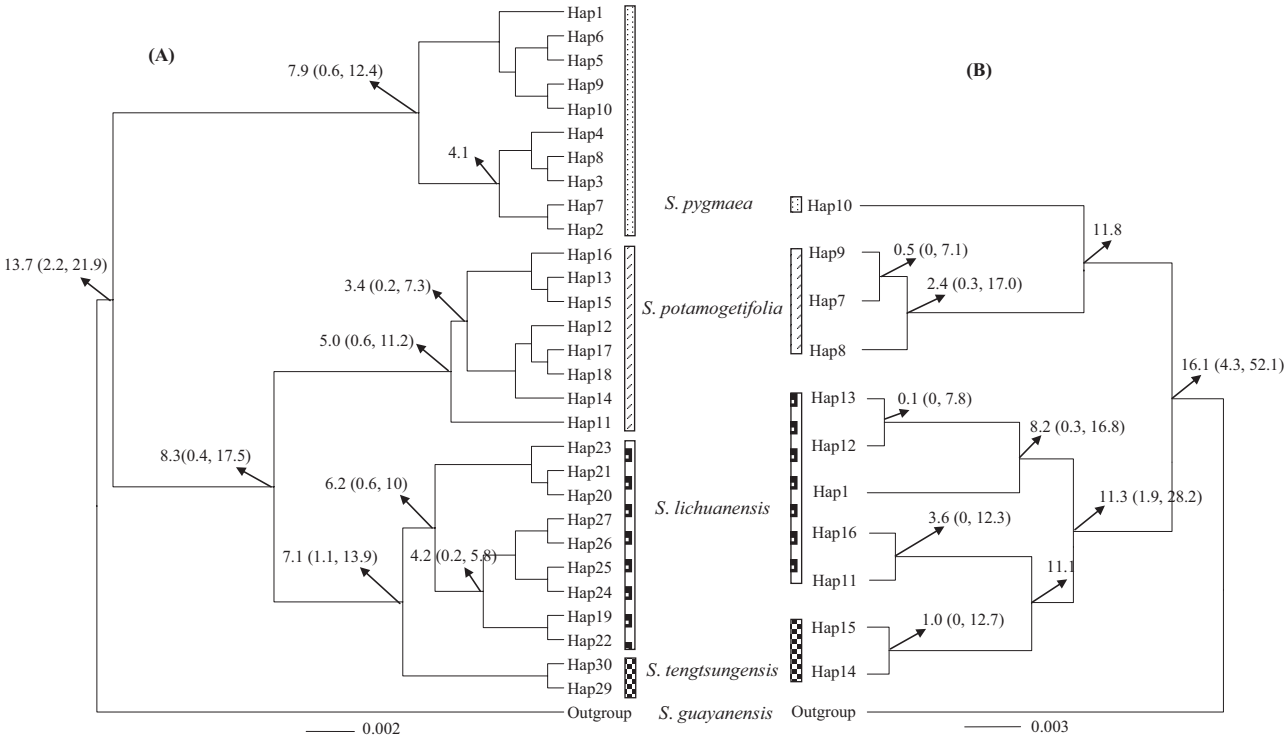

Figure 3. Bayesian analyses of the divergent time (Mya, indicated above branches) between the main lineages of the four studied *Sagittaria* species (A, plastid DNA; B, ITS). 95% HPD values are shown when the bootstrap value of the branch is > 50% based on 1000 replicates.

including *S. pygmaea* and *S. potamogetifolia* diverged from the lineage including *S. tengtsungensis* and *S. lichuanensis* at 16.1 Mya (95% HPD: 4.3–52. 1 Mya) (Fig. 3B).

Biogeographical history

The reconstruction of ancestral geographical distribution areas of genetic lineages, based on the BBM analyses, is shown in Figure 4. Our analyses revealed nine dispersal events and four vicariance events, which could be used to explain the observed biogeographical patterns of endemic *Sagittaria* species. The reconstruction suggested that a common ancestor of the four endemics was probably distributed in the east China region (B). From the BBM analyses, *S. pygmaea* and *S. potamogetifolia* were also suggested to have originated in the east China region (B); although the current distribution range of *S. lichuanensis* is mainly around the Wuyishan mountains in this region, the BBM analyses suggested its origin to be the central China region (C), whereas *S. tengtsungensis* was suggested to have originated in the Hengduan Mountains region (G) (Fig. 4).

DISCUSSION

Divergence among and within species

Arising from the phylogenetic analyses (MP, NJ and haplotype network), both the plastid DNA and the nrITS haplotypes (except for *S. lichuanensis* in the MP analysis) were grouped by species, not by geographical areas, and no haplotype was shared among species (Figs S1–S4, Fig. 2). AMOVAs of plastid DNA sequence variations also revealed significant differentiation among species, showing that a great amount of variation resided among species (85.78%) and only 5.08% represented differences among regions (Table 4). The Mantel test also demonstrated that there were no associations ($r^2 = 0.962$, $P > 0.01$) between genetic differentiation and geographical distances between populations across the distribution range of all the species. The lack of significant geographical structure to the genetic variation might be the result of the distribution of *S. pygmaea* being sympatric with most of the populations of the other three Chinese endemic species. This geographical distribution pattern might have resulted in frequent dispersal of these species (see below). *Sagittaria lichuanensis* appeared to be paraphyletic in the NJ analysis of plastid DNA data (Fig. S2) and the MP analysis of nrDNA data (Fig. S3).

Based on differences in the distribution range and a set of morphological characters, Chen, Sun & Wang (1984) and Chen (1989) named *Sagittaria* plants from central China (Lichuan county) and Wuyi mountain region of south-eastern China as two endemic species, *S. lichuanensis* and *S. wuyiensis* J.K.Shen. However, given the similarity in many morphological characters, e.g. leaves, flowers and bulbs in the sheath, Wang *et al*. (2010) regarded them as one species, *S. lichuanensis*. In this study, based on both plastid DNA and nrDNA sequence variations, we found great differentiation between populations of *S. lichuanensis*. It is possible that two distinct species exist in the extant populations of these plants, but further detailed checks are needed using data from multiple subjects.

Species-level divergence time estimates

Our estimates of divergence times drawn from plastid and ITS datasets showed a similar range of diversification time among the four endemic *Sagittaria* species in the SJFR (Fig. 3); for example, the divergence times between species occurred in the late Tertiary (plastid DNA: 7.1–13.7 Mya; ITS: 11.1–16.1 Mya). These results support the hypothesis that modern species in the Northern Hemisphere originated mostly in the Tertiary or earlier (Hewitt, 2000; Pennington *et al*., 2004; Willis & Niklas, 2004; Rull, 2006, 2008) and therefore do not support the hypothesis that the Chinese endemic *Sagittaria* species might have originated during the Quaternary (Chen, 1989). Several previous phylogenetic or phylogeographical studies in the SJFR of China also implied that the studied genus or species originated in the Tertiary (Song *et al*., 2009; Su *et al*., 2011). For example, Song *et al*. (2009) conducted a phylogeographical study on populations of *Alcippe morrisonia* in southern China and the common ancestor of these populations was dated to 11.6 Mya, which falls in the late Tertiary. Su *et al*. (2011) studied phylogenetics and evolutionary divergence times in *Apterosperma* Hung T.Chang and *Euryodendron* Hung T.Chang (Theaceae) that are endemic to southern China. They provided evidence for a Tertiary origin of *Euryodendron* (the divergence was dated at 20.51 Mya, which falls in the Miocene). Li *et al*. (2014) studied the palaeobiogeography of the aquatic *Nelumbo* Adans. (Nelumbonaceae) based on fossil evidence and they revealed that the origin of tubers and the differentiation of ecotypes in this genus in the SJFR might have occurred in the Eocene. The cooling climate and increasing seasonality of East Asia in the Eocene may have favoured these speciation processes in *Nelumbo*. In the course of the Tertiary, a profound global climate shift took place from a Cretaceous/early Palaeogene 'greenhouse' world to the current 'icehouse' world, with major cooling events at the Eocene–Oligocene boundary (~34 Mya) and the mid-Miocene (~15 Mya) (Miller, 1992; Zachos *et al*., 2001; Eldrett *et al*., 2009; Liu *et al*., 2009b; Zhang *et al*., 2011). In addition, Neogene mountain uplift events could also have contributed to the plant diversification (Zachos *et al*., 2001; Sanmartín, Enghoff & Ronquist, 2011; Su *et al*., 2011). These historical mountain-building events and/or global climate shifts may have promoted strong population structure and consequently led to speciation in *Sagittaria* in China.

Biogeographical history

Resulting from the generic phylogenetic and historical biogeographical studies of Alismataceae, based on multiple DNA sequences, Chen *et al*. (2012) suggested that *Sagittaria* originated in the Afrotropical area. An African origin of *Sagittaria* agrees with the view that the genus originated in tropical swamp and lake areas of Gondwanaland (Chen, 1989). The genus was also suggested to have originated during the late Eocene and Oligocene and the ancestors of *Sagittaria* in south-eastern Eurasia may be the result of eastward dispersal from the Afrotropical area (Chen *et al*., 2012). The ancestors of *Sagittaria* may have arrived in south-eastern China before the complete disintegration of Gondwana (Chen, 1989). In this study, our reconstruction of the ancestral geographical distribution areas of four endemic *Sagittaria* species based on BBM analyses showed that the common ancestor was probably distributed in the east China region (Fig. 4), supporting the hypothesis that the ancestors of *Sagittaria* in China might have originated in the southeast region (Chen, 1989).

From the BBM analyses, the endemic species *S. pygmaea*, *S. potamogetifolia* and *S. lichuanensis* were inferred to have originated in the east China region, central China region and the Hengduan Mountains region, respectively (Fig. 4). Moreover, *S. tengtsungensis* most likely originated in the Hengduan Mountains region. With the retreat of the Tethys Sea, the Hengduan Mountains and the central and southeastern hills in China were formed during the late Cretaceous (Tao, 1992; Ying, 2001; Sun, 2002). These regions are characterized by great diversity in topography, climate and ecological conditions and escaped the direct effect of repeated Pleistocene continental glaciations (Wang & Ge, 2006). These regions, characterized by high diversity and endemism, were probably refugia for plant species in general (Wang & Ge, 2006). In addition, the great diversity in topography and ecological conditions may have provided opportunities for plant speciation (Ying, 2001). Many taxa (genera or species) are thought to have originated in these regions as a result of the alternation of topography and climate between the Miocene and Quater-

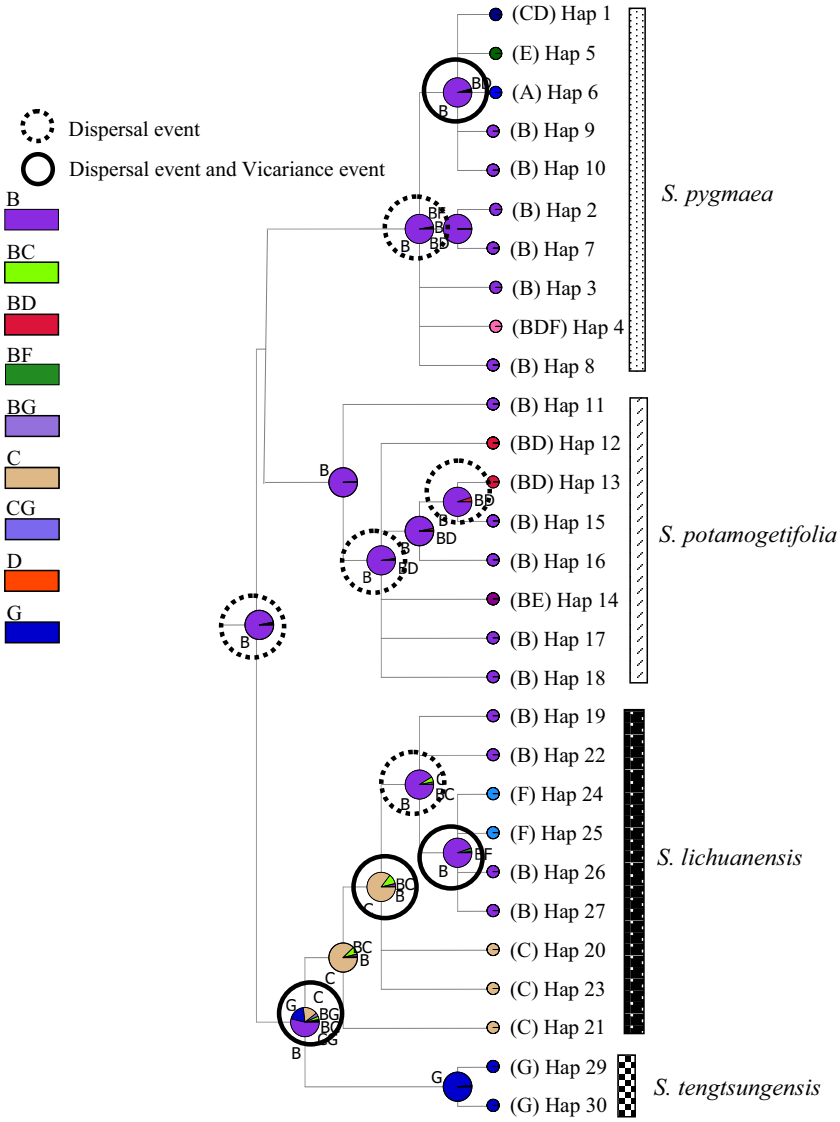

Figure 4. Results of the reconstruction of the ancestral geographical distribution areas of four endemic *Sagittaria* species. Letters above nodes (A–G) indicate the inferred ancestral areas of corresponding clades. A–G, geographical regions in China are as indicated in Figure 1, based on Wu (1979).

nary (Chen *et al*., 2005; Wang, Yang & Liu, 2005; Liu *et al*., 2006; Wang, Liu & Miehe, 2007; Zhang, Volis & Sun, 2010; Sun *et al*., 2012). The four endemic *Sagittaria* species in the SJFR may also represent species derived from the ancestors of *Sagittaria* in these regions.

Within-species demographic history

The within-species divergence times of most of the previously studied species in the SJFR in China fall in the Quaternary and the climatic oscillations during this period may have promoted their range fragmentation, vicariance and population isolation (Qiu *et al*., 2011). However, the within-species lineage divergences of four *Sagittaria* species from China are estimated to have taken place around the late Miocene and mostly fall in the Plio-Pleistocene. For example, within *S. tengtsung*ensis, the branching events took place in the mid-Pleistocene, whereas within *S. pygmaea*, *S. potamogetifolia* and *S. lichuanensis*, most divergences took place in the Plio-Pleistocene. Thus, the geological events and changing climatic conditions before the Quaternary (Miller, 1992; Zachos *et al*., 2001; Eldrett *et al*., 2009; Liu *et al*., 2009b; Sanmartín *et al*., 2011; Su *et al*., 2011; Zhang *et al*., 2011) may also have been major factors that led to the within-species lineage divergences. However, the current distribution

of Chinese *Sagittaria* species may have been profoundly shaped by the climatic oscillations of the Quaternary. Mismatch distribution analyses indicated that each species has experienced a recent range expansion (Table 3). Tajima's D and Fu's F_S tests for each species deviated non-significantly from expectations of neutrality; they also indicated that there may have been a recent expansion (Table 3). The phylogeographical pattern of plastid DNA haplotypes of each species, except for *S. tengtsungensis* (with only two haplotypes), demonstrates 'star-like' clusters that were the result of haplotypes being linked to a central haplotype (Fig. 2). These results concurred with previous within-species phylogeographical studies on *Sagittaria* species in China (e.g. *S. trifolia* L., Chen *et al*., 2008; *S. potamogetifolia*, Tan *et al*., 2008; *S. lichuanensis*, Liu *et al*., 2010). This relatively simple pattern could be explained by populations that had experienced expansion after glaciations, but had insufficient time to form a more complicated structure (Dynesius & Jansson, 2000).

In most parts of China, there was no massive ice sheet during Quaternary glacial periods (Ying, Zhang & Boufford, 1993; Axelrod *et al*., 1996; Ying, 2001; Qiu *et al*., 2011). In addition, due to bird-mediated passive transport, dispersal of sexual and asexual propagules of aquatic plants is generally assumed to be common (Santamaria, 2002). All *Sagittaria* species studied in this study are clonal aquatic herbs with potentially long-distance dispersal abilities. Considering this, it is likely that dispersal should have been frequent enough to ensure rapid range expansions following glacial retreat. The range expansions could have begun from populations in different refugia. However, no specific refugial areas could be deduced because there was no significant geographical structure in the genetic variation. Although several haplotypes were unique to single populations, in the haplotype network of each species the central haplotypes were widely distributed in the sampled populations (Table 1; Fig. 2). The range expansions might have replaced the pre-existing genetic structure and swamped evidence of refugia.

CONCLUSION AND PERSPECTIVES

Based on the phylogeographical analyses of four endemic *Sagittaria* species in the SJFR, we revealed that the species-level divergence times fall in the mid- to late Tertiary, supporting the view of modern species in the Northern Hemisphere having originated mostly in the Tertiary (Hewitt, 2000; Pennington *et al*., 2004; Willis & Niklas, 2004; Rull, 2006, 2008). Our findings do not support the hypothesis of a Quaternary origin of Chinese endemic *Sagittaria* species (Chen, 1989). The regions with great diversity in topography, climate and ecological conditions (Wang & Ge, 2006), such as the Hengduan Mountains, central China and east China regions, were inferred to be the areas where the endemics originated. Due to the range expansions in each species, the current distribution range of Chinese *Sagittaria* species may thus have been profoundly modified by the climatic oscillations in the Quaternary. Although great efforts have been made to infer the biogeographical history of the endemic *Sagittaria* species in the SJFR in this study, the historical biogeography for the whole genus may suffer from undersampling. For example, species from the other distribution centres of endemic species (e.g. North America and South America) were not included. Whether the species from the other regions also display a similar biogeographical history as revealed for the SJFR of mainland China will require re-evaluation in future studies.

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REFERENCES

Axelrod DI, Al-Shehba I, Raven PH. 1996. History of the modern flora of China. In: Zhang AL, Wu SG, eds. *Floristic characteristics and diversity of East Asian plants*. New York: Springer, 43–55.

Chen JK. 1989. *Systematic and evolutionary studies on* Sagittaria *from China*. Wuhan: Wuhan University Press.

Chen JK, Sun XZ, Wang HQ. 1984. New taxa of *Sagittaria* L. from Hubei. *Bulletin of Botanical Research* **4:** 129–132.

Chen JM, Liu F, Wang QF, Motley TJ. 2008. Phylogeography of a marsh herb *Sagittaria trifolia* (Alismataceae) in China inferred from cpDNA *atp*B-*rbc*L intergenic spacers. *Molecular Phylogenetics and Evolution* **48:** 168–175.

Chen LY, Chen JM, Gituru RW, Temame TD, Wang QF. 2012. Generic phylogeny and historical biogeography of Alismataceae, inferred from multiple DNA sequences. *Molecular Phylogenetics and Evolution* **63:** 407–416.

Chen ST, Guan KY, Zhou ZK, Olmstead R, Cronk Q. 2005. Molecular phylogeny of *Incarvillea* (Bignoniaceae) based on ITS and *trnL-F* sequences. *American Journal of Botany* **92:** 625–633.

Chou YW, Thomas PI, Ge XJ, LePage BA, Wang CN. 2011. Refugia and phylogeography of *Taiwania* in East Asia. *Journal of Biogeography* **38:** 1992–2005.

Clement M, Posada D, Crandall K. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **29:** 1657–1660.

Demesure B, Sodzi N, Petit RJ. 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Molecular Ecology* **4:** 129–131.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochemistry Bulletin, Botanical Society of America* **19:** 11–15.

Drummond AJ, Suchard MA, Xie D, Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29:** 1969–1973.

Dynesius M, Jansson R. 2000. Evolutionary consequences of changes in species' geographical distributions driven by Milankovitch climate oscillations. *Proceedings of the National Academy of Sciences USA* **97:** 9115–9120.

Ehlers J, Gibbard PL. 2007. The extent and chronology of Cenozoic global glaciations. *Quaternary International* **164–165:** 6–20.

Eldrett JS, Greenwood DR, Harding IC, Huber M. 2009. Increased seasonality through the Eocene to Oligocene transition in northern high latitudes. *Nature* **459:** 969–973.

Excoffier L, Laval G, Schneider S. 2006. *ARLEQUIN version 3.1: a software for population genetic data analysis*. Computational and Molecular Population Genetics Laboratory, Institute of Zoology, University of Berne, Switzerland.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783–791.

Fu YX. 1997. Statistical tests of neutrality against population growth, hitchhiking and background selection. *Genetics* **147:** 915–925.

Haggard KK, Tiffney BH. 1997. The flora of the early Miocene Brandon Lignite, Vermont, USA. VIII. *Caldesia* (Alismataceae). *American Journal of Botany* **84:** 239–252.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41:** 95–98.

Harpending HC. 1994. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology* **66:** 591–600.

Hewitt G. 2000. The genetic legacy of the Quaternary ice ages. *Nature* **405:** 907–913.

Keener BR. 2005. *Molecular systematics and revision of the aquatic monocot genus* Sagittaria *(Alismataceae)*. PhD Dissertation, the University of Alabama, Tuscaloosa.

Les DH, Schneider EL. 1995. The Nymphaeales, Alismatidae, and the theory of an aquatic monocotyledon origin. In: Rudall PJ, Cribb PJ, Cutler DF, Humphries CJ, eds. *Monocotyledons: systematics and evolution*. Kew: Royal Botanic Gardens, 23–42.

Li Y, Smith T, Svetlana P, Yang J, Jin JH, Li CS. 2014. Paleobiogeography of the lotus plant (Nelumbonaceae: *Nelumbo*) and its bearing on the paleoclimatic changes. *Palaeogeography, Palaeoclimatology, Palaeoecology* **399:** 284–293.

Liu F, Zhao SY, Li W, Chen JM, Wang QF. 2010. Population genetic structure and phylogeographic patterns in the Chinese endemic species *Sagittaria lichuanensis*, inferred from cpDNA *atpB-rbcL* intergenic spacers. *Botany* **88:** 886–892.

Liu JQ, Wang YJ, Wang AL, Hideaki O, Abbott RJ. 2006. Radiation and diversification within the *Ligularia–Cremanthodium–Parasenecio* complex (Asteraceae) triggered by uplift of the Qinghai–Tibetan Plateau. *Molecular Phylogenetics and Evolution* **38:** 31–49.

Liu KB. 1988. Quaternary history of the temperate forests of China. *Quaternary Science Reviews* **7:** 1–20.

Liu YF, Wang Y, Huang HW. 2009a. Species-level phylogeographical history of *Myricaria* plants in the mountain ranges of western China and the origin of *M. laxiflora* in the Three Gorges mountain region. *Molecular Ecology* **18:** 2700–2712.

Liu ZH, Pagani M, Zinniker D, Deconto R, Huber M, Brinkhuis H, Shah SR. 2009b. Global cooling during the Eocene–Oligocene climate transition. *Science* **323:** 1187–1190.

Miller KG. 1992. Middle Eocene to Oligocene stable isotopes, climate, and deep-water history: the terminal Eocene event? In: Prothero DR, Berggren WA, eds. *Eocene–Oligocene climatic and biotic evolution*. Princeton: Princeton University Press, 160–177.

Nei M, Tajima F. 1983. Maximum likelihood estimation of the number of nucleotide substitutions from restriction sites data. *Genetics* **105:** 207–217.

Nylander JAA. 2004. *MrModeltest v2*. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Pennington RT, Lavin M, Prado DE, Pendry CA, Pell SK, Butterworth CA. 2004. Historical climate change and speciation: neotropical seasonally dry forest plants show patterns of both Tertiary and Quaternary diversification. *Philosophical Transactions of the Royal Society of London, Series B: Biological Science* **359:** 515–537.

Qian H. 2002. A comparison of the taxonomic richness of temperate plants in East Asia and North America. *American Journal of Botany* **89:** 1818–1825.

Qian H, Ricklefs RE. 2000. Large-scale processes and the Asian bias in species diversity of temperate plants. *Nature* **407:** 180–182.

Qian H, Ricklefs RE. 2004. Geographic distributions and ecological conservatism of disjunct genera of vascular plants in eastern Asia and eastern North America. *Journal of Ecology* **92:** 253–265.

Qiu YX, Fu CX, Comes HP. 2011. Plant molecular phylogeography in China and adjacent regions: tracing the genetic imprints of Quaternary climate and environmental change in the world's most diverse temperate flora. *Molecular Phylogenetics and Evolution* **59:** 225–244.

Qiu YX, Sun Y, Zong M, Zhang XP, Lee J, Lee JK, Fu CX, Comes HP. 2009. Molecular phylogeography of East Asian *Kirengeshoma* in relation to Quaternary climate change and land-bridge configurations. *New Phytologist* **183:** 480–495.

Rambaut A, Drummond AJ. 2007. *Tracer v1.5 [online].* Available at: http://beast.bio.ed.ac.uk/Tracer

Rambaut A, Drummond AJ. 2009. *TreeAnnotator v1.5.3: MCMC output analysis [online]*. Available at: http://beast.bio.ed.ac.uk/TreeAnnotator

Ronquist F, Hulsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Rosenberg MS, Anderson CD. 2011. PASSAGE: pattern analysis, spatial statistics, and geographic exegesis. Version 2. *Methods in Ecology and Evolution* **2:** 229–232.

Rull V. 2006. Quaternary speciation in the Neotropics. *Molecular Ecology* **15:** 4257–4259.

Rull V. 2008. Speciation timing and Neotropical biodiversity: the Tertiary–Quaternary debate in the light of molecular phylogenetic evidence. *Molecular Ecology* **17:** 2722–2729.

Sang T, Crawford DJ, Stuessy TF. 1997. Chloroplast phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *American Journal of Botany* **84:** 1120–1136.

Sanmartín I, Enghoff H, Ronquist F. 2011. Patterns of animal dispersal, vicariance and diversification in the Holarctic. *Biological Journal of the Linnean Society* **73:** 345–390.

Santamaria L. 2002. Why are most aquatic plants widely distributed? Dispersal, clonal growth and small-scale heterogeneity in a stressful environment. *Acta Oecologia* **23:** 137–154.

Schneider S, Excoffier L. 1999. Estimation of demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. *Genetics* **152:** 1079–1089.

Shen L. 2002. Glacial refugia and postglacial recolonization patterns of organisms. *Acta Ecological Sinica* **22:** 1983–1990.

Small RL, Ryburn JA, Cronn RC, Seelanan T, Wendel JF. 1998. The tortoise and the hare: choosing between noncoding plastome and nuclear *Adh* sequences for phylogeny reconstruction in a recently diverged plant group. *American Journal of Botany* **85:** 1301–1315.

Song G, Qu YH, Yin ZH, Li SH, Liu NF, Lei FM. 2009. Phylogeography of the *Alcippe morrisonia* (Aves: Timaliidae): long population history beyond late Pleistocene glaciations. *BMC Evolutionary Biology* **9:** 143.

Su YJ, Liao WB, Wang T, Sun YF, Wei Q, Chang HT. 2011. Phylogeny and evolutionary divergence times in *Apterosperma* and *Euryodendron*: evidence of a Tertiary origin in south China. *Biochemical Systematics and Ecology* **39:** 769–777.

Sun H. 2002. Tethys retreat and Himalayas–Hengduanshan Mountains uplift and their significance on the origin and development of the Sino-Himalayan elements and alpine flora. *Acta Botanica Yunnanica* **3:** 273–288.

Sun YS, Wang AL, Wan DS, Wang Q, Liu JQ. 2012. Rapid radiation of *Rheum* (Polygonaceae) and parallel evolution of morphological traits. *Molecular Phylogenetics and Evolution* **63:** 150–158.

Swofford DL. 1998. *PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4*. Sunderland, MA: Sinauer Associates.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123:** 585–595.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30:** 2725–2729.

Tan B, Liu K, Yue XL, Liu F, Chen JM, Wang QF. 2008. Chloroplast DNA variation and phylogeographic patterns in the Chinese endemic marsh herb *Sagittaria potamogetifolia*. *Aquatic Botany* **89:** 372–378.

Tao JR. 1992. The Tertiary vegetation and flora and floristic regions in China. *Acta Phytotaxonmica Sinica* **130:** 23–43.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673–4680.

Tiffney BH. 1985. Perspectives on the origin of the floristic similarity between eastern Asia and eastern North America. *Journal of the Arnold Arboretum* **66:** 73–94.

Wang AL, Yang MH, Liu JQ. 2005. Molecular phylogeny, recent radiation and evolution of gross morphology of the rhubarb genus *Rheum* (Polygonaceae) inferred from chloroplast DNA *trnL-F* sequences. *Annals of Botany* **96:** 489–498.

Wang HW, Ge S. 2006. Phylogeography of the endangered *Cathaya argyrophylla* (Pinaceae) inferred from sequence variation of mitochondrial and nuclear DNA. *Molecular Ecology* **15:** 4109–4122.

Wang QF, Haynes RR, Hellquist CB. 2010. Alismataceae and Butomaceae. In: Wu ZY, Peter HR, eds. *Flora of China*. Beijing and St. Louis: Science Press and Missouri Botanical Garden Press, 84–90.

Wang YJ, Liu JQ, Miehe G. 2007. Phylogenetic origins of the Himalayan endemic *Dolomiaea*, *Diplazoptilon* and *Xanthopappus* (Asteraceae: Cardueae) based on three DNA regions. *Annals of Botany* **99:** 311–322.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols: a guide to methods and applications*. New York: Academic Press, 315–322.

Willis KJ, Niklas KJ. 2004. The role of Quaternary environmental change in plant macroevolution, the exception or the rule? *Philosophical Transactions of the Royal Society of London, Series B: Biological Science* **359:** 159–172.

Wu ZY. 1979. The regionalization of Chinese flora. *Acta Botanica Yunnanica* **1:** 1–22.

Wu ZY, Wu SGA. 1996. Proposal for a new floristic kingdom (realm) – the E. Asiatic kingdom, its delimitation and characteristics. In: Zhang AL, Wu SG, eds. *Proceedings of the First International Symposium on Floristic Characteristics and Diversity of East Asian Plants*. Beijing/Berlin: China Higher Education Press/Springer-Verlag, 3–42.

Ying TS. 2001. Species diversity and distribution pattern of seed plants in China. *Biodiversity Sciences* **9:** 393–398.

Ying TS, Zhang YL, Boufford DE. 1993. *The endemic genera of seed plants of China*. Beijing: Science Press.

Yu Y, Harris AJ, He XJ. 2011. *RASP (reconstruct ancestral state in phylogenies) 2011 1·1.* Available at: http://mnh.scu.edu.cn/soft/blog/RASP

Zachos J, Pagani M, Sloan L, Thomas E, Billups K. 2001. Trends, rhythms, and aberrations in global climate 65 Ma to present. *Science* **292:** 686–693.

Zhang YH, Volis S, Sun H. 2010. Chloroplast phylogeny and phylogeography of *Stellera chamaejasme* on the Qinghai-Tibet Plateau and in adjacent regions. *Molecular Phylogenetics and Evolution* **57:** 1162–1172.

Zhang Z, Nisancioglu KH, Flatoy F, Bentsen M, Bethke I, Wang H. 2011. Tropical seaways played a more important role than high latitude seaways in Cenozoic cooling. *Climate of Past Discussions* **7:** 965–996.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Strict consensus of the most parsimonious trees obtained by analysis of 29 plastid DNA haplotypes of four *Sagittaria* species in China. *Sagittaria guayanensis* was used as the outgroup. Bootstrap values (> 50%) based on 1000 replicates are indicated below branches.
Figure S2. Neighbour-joining tree of 29 plastid DNA haplotypes of four *Sagittaria* species sampled in China; *S. guayanensis* was used as the outgroup. Bootstrap values (> 50%) based on 1000 replicates are indicated below branches.
Figure S3. Strict consensus of the most parsimonious trees obtained by analysis of 11 nrDNA haplotypes of four *Sagittaria* species in China; *S. guayanensis* was used as the outgroup. Bootstrap values (> 50%) based on 1000 replicates are indicated below branches.
Figure S4. Neighbour-joining tree of 11 nrDNA haplotypes of four *Sagittaria* species sampled in China; *S. guayanensis* was used as the outgroup. Bootstrap values (> 50%) based on 1000 replicates are indicated below branches.
Figure S5. The estimates of the mutation rate of plastid DNA sequences of *Sagittaria*.
Figure S6. Estimates of the mutation rate of nrDNA (ITS region) sequences of *Sagittaria*.
Table S1. Collection details of accessions of species of Alismataceae used in the estimates of the mutation rate of *Sagittaria*.