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Genetic delimitation and population structure of three *Trapa* taxa from the Yangtze River, China

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ABSTRACT

Water chestnuts (genus *Trapa*) are an annual floating-leaved aquatic plants widely distributed in the Old World. The taxonomy of the genus is extremely confusing worldwide. The plants of *Trapa* are abundant in lakes of mid-to-lower reaches of the Yangtze River, China. The genetic relationship and diversity among three common *Trapa* species in the area were evaluated using amplified fragment length polymorphism (AFLP) markers. A total of 249 unambiguous bands, of which 192 (77.1%) were polymorphic, was produced with four pairs of primers. The genetic relationship estimated by different approaches (NJ tree, STRCTURE, PCA and UPGMA) consistently indicated that all the three *Trapa* taxa formed genetically distinct groups, which confirmed the taxonomic status of the three separate species (*T. quadrispinosa*, *T. japonica* and *T. bispinosa*). The three *Trapa* taxa appeared to possess low level of gene diversity (H_E = 0.073–0.107) compared with the perennial aquatic macrophytes in the same habitat and range distribution. The main factor responsible for that was the habitat deterioration of recent years combined with the annual life history of the plants. Evident genetic structure was found among populations for each *Trapa* taxon, contributing more than 50% of the total gene diversity. The high genetic differentiation could be due to the restricted gene flow among populations (Nm = 0.165–0.243) and the high degree of inbreeding (F_{IS} = 0.482–0.503) in their reproductive system. These results were important in relation to conservation management of *Trapa* taxa.

1. Introduction

Trapa L. (Trapaceae), known as water chestnut, is a genus of annual floating-leaved aquatic plants widely distributed in subtropical and temperate regions of Africa, Asia, and Europe and was introduced into Australia and North America (Chen et al., 2007). Its seeds are important food for humans and animals in East and Southeast Asia due to the high content of starch (Suriyagoda et al., 2007; Hoque et al., 2009). However, the taxonomy of the genus is extremely confusing world-wide because of the wide variability in morphological traits of the genus and the limited diagnostic characters (Yu, 1998; Kim et al., 2010). Cook summarized that the genus contained only one polymorphic or up to ca. 20 species in the world (Cook, 1990). The classification of Chinese *Trapa* is also problematic. Flora Republicae Popularis Sinicae documented 15 species and 11 varieties in the genus (Wan, 2000), while only two species were reported in its revised edition, the Flora of China (Chen et al., 2007). The confused situation has greatly hindered further genetic researches of the genus. As valuable food and natural resources, the genetic diversity of *Trapa* populations should be investigated in order to effectively conserve or manage them. However, previous genetic researches only focused on the classification of the genus (Bao et al., 2004; Jiang and Ding, 2004; Takano and Kadono, 2005; Kim et al., 2010), and few efforts have been made on the genetic diversity and population structure of *Trapa* populations.

Trapa plants are common in northeastern and southern part of China, occurring in freshwater wetlands, sluggish reaches of rivers, lakes, ponds and estuaries (Wan, 2000; Chen et al., 2007). The plants have floating leaves and submersed ones. The floating leaves have spongy and swollen float in petioles, which allows the foliage to form a rosette on the surface of the water. The plants are capable of extensive clonal propagation (Groth et al., 1996). Additionally,

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Table 1
Locations of the three *Trapa* taxa.

Site (code)	Latitude (N)	Longitude (E)	Species (population code)	Vouchers and collector
Changhu Lake (CH)	30.374	112.338	*T. quadrispinosa* (CHsj)	Chenyuanyuan0013, Chen et al. (2012)
			T. japonica (CHqj)	Chenyuanyuan0011, Chen et al. (2012)
			T. bispinosa (CHl)	Chenyuanyuan009, Chen et al. (2012)
Yuhu Lake (YH)	29.950	112.205	*T. quadrispinosa* (YHsj)	Chenyuanyuan0016, Chen et al. (2012)
Honghu Lake (HH)	29.857	113.405	*T. quadrispinosa* (HHsj)	Chenyuanyuan0022, Chen et al. (2012)
			T. japonica (HHqj)	Chenyuanyuan0024, Chen et al. (2012)
			T. bispinosa (HHl)	Chenyuanyuan0020, Chen et al. (2012)
Xiliang Lake (XL)	29.979	114.127	*T. quadrispinosa* (XLsj)	Chenyuanyuan0026, Chen et al. (2012)
			T. japonica (XLqj)	Chenyuanyuan0025, Chen et al. (2012)
			T. bispinosa (XLl)	Chenyuanyuan0027, Chen et al. (2012)
Yezhu Lake (YZ)	30.826	114.086	*T. quadrispinosa* (YZsj)	Chenyuanyuan0029, Chen et al. (2012)
			T. bispinosa (YZl)	Chenyuanyuan0030, Chen et al. (2012)
Donghu Lake (DH)	30.575	114.387	*T. quadrispinosa* (DHsj)	Chenyuanyuan0034, Chen et al. (2012)
			T. japonica (DHqj)	Chenyuanyuan0032, Chen et al. (2012)
			T. bispinosa (DHl)	Chenyuanyuan0033, Chen et al. (2012)
Liangzi Lake (LZ)	30.425	114.541	*T. quadrispinosa* (LZsj)	Chenyuanyuan0037, Chen et al. (2012)
			T. japonica (LZqj)	Chenyuanyuan0036, Chen et al. (2012)
			T. bispinosa (LZl)	Chenyuanyuan0035, Chen et al. (2012)
Ouhuasai Lake (OH)	30.685	117.136	*T. quadrispinosa* (OHsj)	Chenyuanyuan0049, Chen et al. (2012)
Longgan Lake (LG)	30.023	116.261	*T. japonica* (LGqj)	Chenyuanyuan0044, Chen et al. (2012)
			T. bispinosa (LGl)	Chenyuanyuan0041, Chen et al. (2012)
Shengjin Lake (SJ)	30.381	117.059	*T. quadrispinosa* (SJsj)	Chenyuanyuan0055, Chen et al. (2012)
			T. japonica (SJqj)	Chenyuanyuan0051, Chen et al. (2012)
Poyang Lake (PY)	29.515	116.172	*T. quadrispinosa* (PYsj)	Chenyuanyuan0059, Chen et al. (2012)
			T. japonica (PYqj)	Chenyuanyuan0058, Chen et al. (2012)

Trapa plants are considered to be of high degree of autogamy because of the selfing-pollinating behavior before flowering (Arima et al., 1999). Because of the similar vegetative characters in *Trapa* plants, the morphology of fruits (nuts) offers the best diagnostic criteria for the classification of *Trapa*. Variations of fruit morphology, including fruit size, the number of spines, the shape of lower protuberances and tubercles on the surface of the fruits and so on, have been used for recognition of various taxa (Staszkiewicz and Wojcicki, 1979, 1981; Chung et al., 1987a, 1987b; Kadono, 1987).

The mid-to-lower reaches of the Yangtze River include the largest floodplain in China. This area contains thousands of shallow lakes and sustains a rich variety of aquatic macrophytes (Wang and Dou, 1998), which makes the area one of the six biodiversity hotspots in the large river ecoregions of the world (Olson and Dinerstein, 1998). As one of the main distribution ranges of *Trapa* in China, several *Trapa* taxa grow in mixed populations in lakes of this area during our field investigation (2011). Among them, the most common *Trapa* taxa were *T. japonica* Flerow, *T. bispinosa* Roxb. and *T. quadrispinosa* Roxb (taxonomy follows Flora Republicae Popularis Sinicae; Wan, 2000). According to morphological features described in Flora Republicae Popularis Sinicae (Wan, 2000), their fruits show obvious differences among the three species. For *T. bispinosa* and *T. quadrispinosa*, their fruits are nuts with two and four barbed spines, respectively, and the tubercles on the nut hulls are not obvious. *T. japonica* has the two-horned nuts with the pseudo horns replaced by blunt protuberances. Additionally, the snout of *T. japonica* nuts is more obvious than that of *T. bispinosa* and *T. quadrispinosa*. All three taxa were included in the species *T. natans* L in Flora of China (Chen et al., 2007). The three *Trapa* taxa grow in mixed populations in most lakes. What is the extent of hybridization and gene flow among congeners in these mixed populations?

Molecular markers, due to the high polymorphism and resolution, have been considered a preferred method for identifying species and evaluating the genetic structure of plant populations in the past years (Nybom, 1994, 2004). In the present study, amplified fragment length polymorphism (AFLP) was employed for the analysis of genetic relationships and genetic diversity among populations of the three commonly-found *Trapa* taxa in the mid-to-lower reaches of the Yangtze River. The specific questions addressed are: (I) Can AFLP markers discriminate among the three *Trapa* taxa? (II) What is the level of genetic diversity in the *Trapa* populations? (III) Is there any genetic structure within *Trapa* taxa and, if so, are the populations in different lakes completely isolated, or is there any gene flow among populations? The baseline genetic information will be beneficial for the conservation and sustainable utilization of the natural resources in the area.

2. Materials and methods

2.1. Sample collection

Eleven lakes from the mid-to-lower reaches of the Yangtze River were extensively investigated in the autumn of 2011. The foliar rosettes of *Trapa* with matured seeds were randomly collected at intervals of at least 4 m. The morphological characters of fruits and leaves were measured and recorded. The samples were identified according to the description of Flora Republicae Popularis Sinicae (Wan, 2000). All the voucher specimens have been logged in the Plant Herbarium, Wuhan Botanical Garden, Chinese Academy of Sciences (HIB). For each taxon, samples from the same lake were handled as a population. For most populations, 16–22 individuals were sampled in every population. In all, a total of 482 individuals were collected from the three taxa, including ten (213 individuals), eight (140 individuals) and seven (128 individuals) populations of *T. quadrispinosa*, *T. japonica* and *T. bispinosa*, respectively (Table 1; Fig. 1). Young leaves from the foliar rosettes were immediately dried in silica gel and brought back to our laboratory for DNA extraction.

2.2. DNA extraction and AFLP analysis

Total genomic DNA was extracted from 0.3 to 0.5 g of dried leaves using the modified CTAB (Cetyltrimethyl Ammonium Bromide) protocol outlined in Doyle and Doyle (1987). The AFLP analysis was performed essentially as described by Vos et al. (1995). Total genomic DNA (200 ng) was digested using 3 units of *Eco*RI and *Mse*I endonuclease mixture (New England Biolabs, Ipswich, MA, USA) in a total volume of 20 μL for 3 h at 37 °C and digests were confirmed by electrophoresis on 1.5% agarose gels. Then, 10 μL of ligation solution containing 4 pM *Eco*RI adaptor, 40 pM

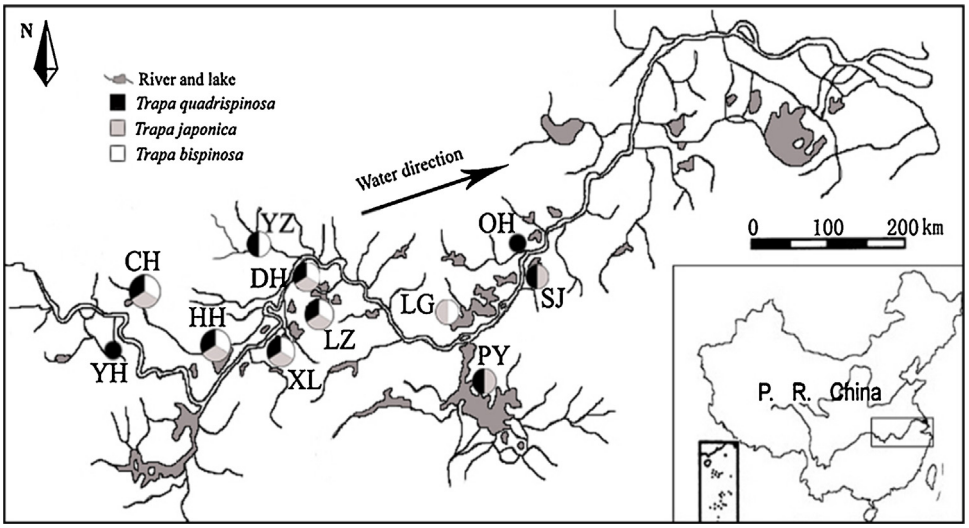

Fig. 1. Geographical distribution of the *Trapa* populations. See Table 1 for lake abbreviations.

*Mse*I adaptor, and 70 unit of T_4 DNA ligase (New England Biolabs) were added to the digests in a total volume of 20 μL. The ligation mixture was incubated overnight at 20 °C in a thermocycler. The resulting DNA (template DNA) was then diluted 1:5 in TE buffer. PCR pre-amplification was performed in a 20 μL solution containing 2 μL 10 × reaction buffer (Takara, Kyoto, Japan), 3 mM $MgCl_2$, 0.20 mM dNTPs (Takara), 40 ng of primer *Eco*RI + 1, 40 ng of primer *Mse*I + 1, 0.5 units of *Taq* polymerase (Takara) and 5 μL of template DNA for 25 cycles of the thermal profile: 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min. For selective PCR amplification, six *Eco*RI + 3/*Mse*I + 3 primer combinations (Table 2) were chosen among 78 sets that were screened for variability, and the 25-fold diluted pre-amplification product was used as template. An aliquot of 2.5 μL diluted pre-amplification DNA was added to 7.5 μL of selective amplification cocktail (20 ng *Eco*RI + 3 primer, 20 ng *Mse*I primer, 0.20 mM dNTPs, 1 μL 10 × reaction buffer, 0.5 units of *Taq* polymerase, 1.5 mM $MgCl_2$), and amplified with the thermal cycle profile: one cycle with 94 °C for 4 min, 65 °C for 1 min and 72 °C for 1 min, then 12 cycles of 94 °C for 30s, 65 °C (decreasing by 0.7 °C) for 1 min and 72 °C for 1 min, followed by 23 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min. The amplification products added with 7.5 μL loading buffer (98% formamide, 10 mM EDTA (pH 8.0), 0.25% bromophenol blue and 0.25% xylene cyanol) were denatured at 94 °C for 5 min and electrophoresed on a 6% denaturing polyacrylamide gel on a 40 × 45 cm EC 160 DNA Sequencing System (Thermo). Silver staining was conducted according to protocol of the Silver SequenceTM (Promega) with slight modification. To minimize genotyping errors, 10 samples amplified poorly were discarded. We also compared genotypes obtained for individual from two separate PCRs and from duplicate DNA isolations from a subset of 24. To reflect the reliability of genotypes used for analysis, the error rate, estimated at 0.015, was calculated as the number of samples with mismatched genotypes/total number of samples genotyped after removal of eight markers that tended to appear distinct differences in phenotypic comparisons.

2.3. Data analysis

Only intense and unambiguous bands (60–500 bp) were manually scored as presence (1) or absence (0), and the data matrix of the AFLP phenotypes was constructed for further analysis. By using GenoType/GenoDive (Meirmans and van Tienderen, 2004), samples were assigned to the same multilocus genotypes and considered to be clonemates if their AFLP profiles were identical (threshold 0). With the same software, the clonal diversity within populations was calculated, including (1) the number of genotypes, *G*; (2) Simpson's diversity index, *D* which expresses the probability that two randomly sampled individuals are genotypically different; (3) Genotypic evenness, *E*, which expresses the distribution of multilocus genotypes. All the following genetic analyses were based on the multilocus genotype data set.

Firstly, data were analyzed using hierarchical Bayesian method of Foll and Gaggiotti (2008) to detect outlier loci with BAYESCAN (http://cmpg.unibe.ch/software/byescan/). The parameters were set to 10 pilot runs of 5000 iterations and additional burn-in of 50 000 iterations. We identified outliers based on the 95% posterior probabilities and removed the outliers loci before genetic analyses. To show the relationships among *Trapa* taxa, we conducted the following four calculations. Firstly, the dissimilarity matrix of multilocus genotypes was calculated by the program DARwin 6.0.9

Table 2
DNA sequences of amplified fragment length polymorphism (AFLP) primers, adaptors and number of (polymorphic) bands generated from each primer combination of three *Trapa* taxa.

Adaptor	Sequence	Total bands	Polymorphic bands	Polymorphism (%)
*Eco*RI adaptor	5′-CTCGTAGACTGCGTACC-3′			
	3′-CATCTGACGCATGGTTAA-5′			
*Mse*I adaptor	5′-GACGATGAGTCCTGAG-3′			
	3′-TACTCAGGACTCAT-5′			
*Eco*RI + 1/*Mse*I + 1 primer	5′-GTAGACTGCGTACCAATTCA-3′			
	5′-GACGATGAGTCCTGAGTAAC-3′			
*Eco*RI + 3/*Mse*I + 3 primer	M-CTG/E-AGG	59	45	76.3
	M-CTC/E-AGT	60	44	73.3
	M-CGA/E-AGG	64	50	78.1
	M- CGA/E-ACT	66	53	80.3

(Perrier and Jacquemoud-Collet, 2006) with 1000 re-samples for bootstrap support. The neighbor-joining (NJ) clustering of multilocus genotypes, based on the dissimilarity matrix, was computed by Mega 2 (Kumar et al., 2001). Secondly, a model-based Bayesian clustering method was used to assign individuals to populations by the program STRUCTURE 2.3 (Pritchard et al., 2000). This method identifies K (unknown) populations within a dataset and assigns each population/individual to one or more population/cluster if the individual is admixed. Markov's chain Monte Carlo simulation (MCMC) parameters were set for a burn-in period of 30 000 and a run length of 10^5 iterations under an admixture model with correlated allele frequencies within populations. The batch run function was carried out a total of 286 runs (11 runs each for 1–26 clusters, i.e. $K = 1–26$) in order to quantify the amount of variation of the likelihood of each K. We inferred K using the ad hoc statistic ΔK defined by Evanno et al. (2005). Thirdly, Principal Coordinate Analysis (PCA) was performed for multilocus genotypes and implemented in the program GenAlEx 6.2 (Peakall and Smouse, 2006). Finally, the UPGMA dendrogram of the populations was constructed based on the genetic distance (Nei, 1972) using TFPGA (Miller, 1997). By using GenAlEx 6.2, analysis of molecular variance (AMOVA) was performed to assess the partitioning of genotypic variation among and within taxa.

The markers that were scored as "0" for all members of one taxon were deleted to estimate genetic diversity within each taxon. Percentage of polymorphic loci (P_p), Nei's gene diversity (H_E)(Nei, 1973), Shannon's index (I)(Shannon and Weaver, 1949) and gene differentiation (G_{ST})(Nei, 1987) were estimated using the program POPGENE version 1.31 (Yeh et al., 1997), assuming complete selfing within population ($F_{IS} = 1$) because water chestnuts are considered to have high rate of self-pollinating (Kadono and Schneider, 1986; Arima et al., 1999). We also used the Bayesian approach (Holsinger et al., 2002) to directly estimate F_{ST} without prior knowledge of the F_{IS}. The f (analogous to F_{IS}) and θ_B (analogous to F_{ST}) were calculated under different models implemented in the HICKORY version 1.0 program (Holsinger and Lewis, 2003). Several runs were conducted with default sampling parameters (burn-in = 50 000, sample = 250 000, thin = 50) to ensure consistency of results (Tero et al., 2003). The average level of gene flow (Nm) among populations was indirectly estimated by a traditional method based on F_{ST} value [$Nm = (1 - F_{ST})/4F_{ST}$] (Slatkin and Barton, 1989). The geographical distances among populations were calculated using the program Geographic Distance Matrix Generator_v1.2.3 (Biodiversity Informatics Facility, http://biodiversityinformatics.amnh.org/open_source/gdmg/). A Mantel test (Mantel, 1967) was used to assess if there was an association between genetic distance and geographical distances for the populations, and the significance of the test was also determined, using the program TFPGA with 1000 permutations (Miller, 1997).

3. Results

3.1. AFLP polymorphism and genetic relationship among taxa

Four primer pairs resolved a total of 249 unambiguous bands, of which 192 (77.1%) were polymorphic. The number of markers generated by each primer pair was similar (59–66), and the percentage of polymorphic fragments varied from 73.3 to 80.3%. By using the markers, a total of 450 unique multilocus genotypes were identified from the 482 individuals. No multilocus genotype was shared by two populations. Using BAYESCAN, among the 249 loci, a total of 15, 2 and 0 were detected as outlier loci for *T. quadrispinosa*, *T. japonica* and *T. bispinosa*, respectively.

The cluster analyses (NJ, STRUCTURE and PCA) consistently showed the 450 multilocus genotypes clearly clustered into three

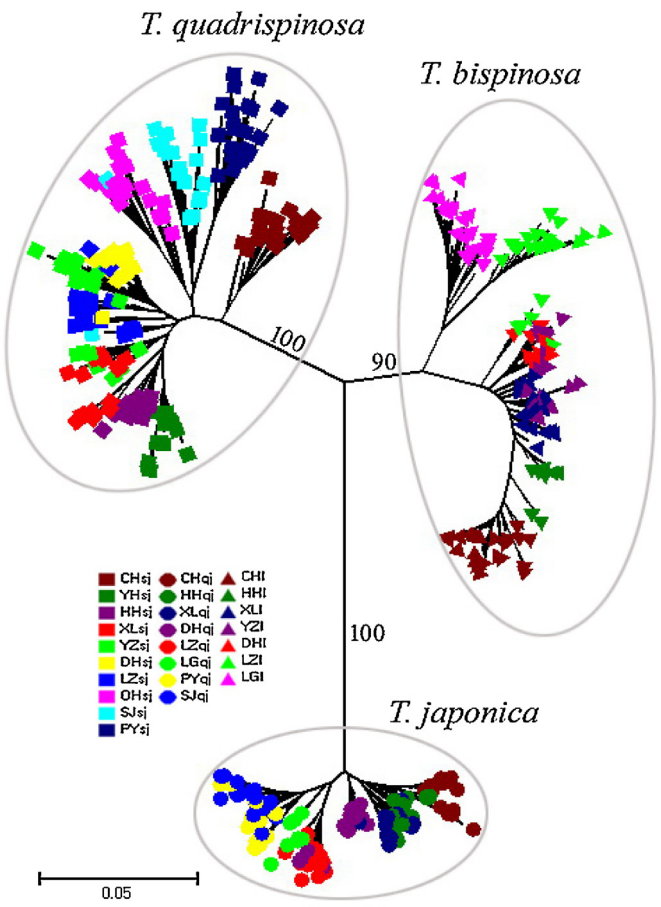

Fig. 2. Neighbor-joining tree based on the dissimilarity matrix (Perrier and Jacquemoud-Collet, 2006) for 450 multilocus genotypes of the *Trapa* taxa. Numbers beside branches indicate bootstrap values (1000 replicates).

major clusters which was well consistent with the three defined taxa. Additionally, the analyses also showed a close relationship between *T. quadrispinosa* and *T. bispinosa*. High bootstrap values (90–100%) were found in the three clusters in the NJ analysis (Fig. 2). In the Bayesian clustering analysis, $\triangle K$ indicated that three clusters best explained the genetic relationship of the multilocus genotypes (Fig. 3a, b). The second-highest modal value of ΔK was at $K = 2$, suggesting a close relationship between *T. quadrispinosa* and *T. bispinosa* (Fig. 3a, b). In the scatter plot of a PCA, the first and second principal coordinates accounted for 56.71% and 26.17% of the total variance, respectively, and distinguished three species groups (Fig. S1).

The UPGMA dendrogram for the 25 populations yielded a very similar result to the above analyses. In the UPGMA dendrogram (Fig. 4), each taxon has its own distinct branch. *T. japonica* was strongly supported by a bootstrap value of 85%, and the cluster consisting of *T. quadrispinosa* and *T. bispinosa* was well supported by a bootstrap value of 92%. Within the second cluster, *T. quadrispinosa* and *T. bispinosa* were supported by a bootstrap value of 99% and 95%, respectively. Within each cluster, the adjacent populations tended to clustered together.

3.2. Genetic diversity and clonal diversity within each taxon

After excluding the outlier loci and the loci scored as "0" for all samples of one taxon, the four primer pairs resolved a total of 183, 192 and 199 bands for *T. quadrispinosa*, *T. japonica* and *T. bispinosa*, respectively, for further calculation about genetic diversity within each taxon.

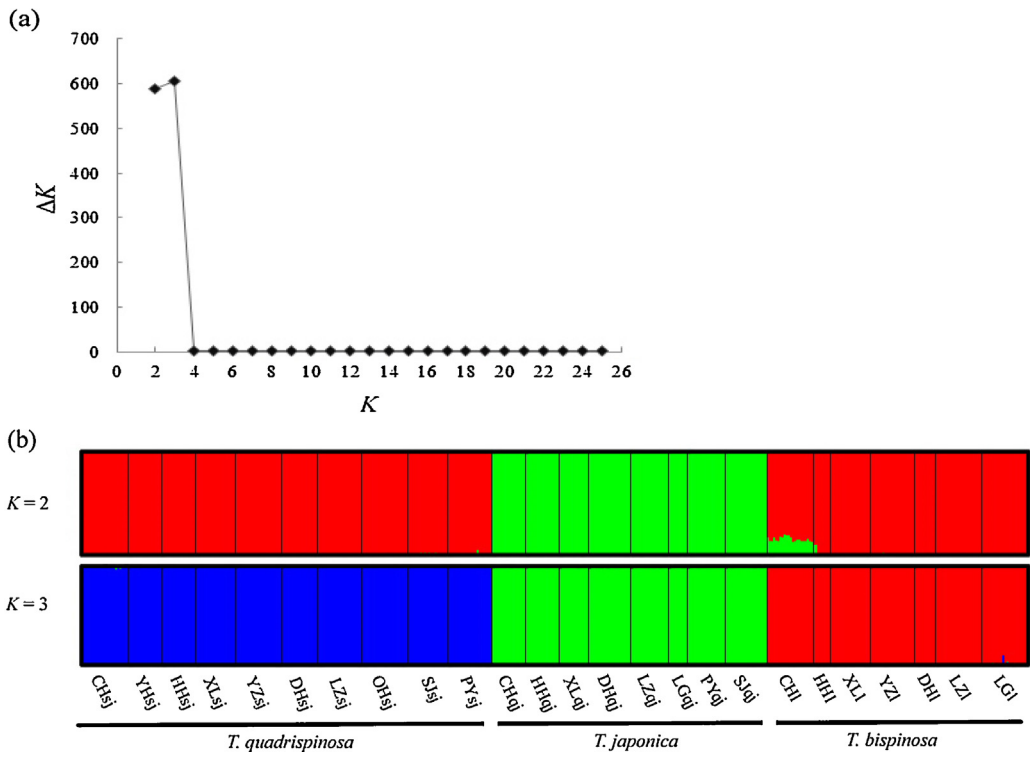

Fig. 3. Structure analysis of AFLP data for *Trapa* taxa inferred by a Markov's chain Monte Carlo Bayesian clustering method: (a) Values of ΔK (Evanno et al., 2005) are plotted against $K = 2$–25; (b) Each individual is represented by a vertical line, which is partitioned into $K = 2$ and 3 differently colored segments that represent the individual's estimated membership fractions in clusters. Vertical black lines separate individuals of the 25 different populations. Populations are labeled below the figure according to the abbreviations used in Table 1.

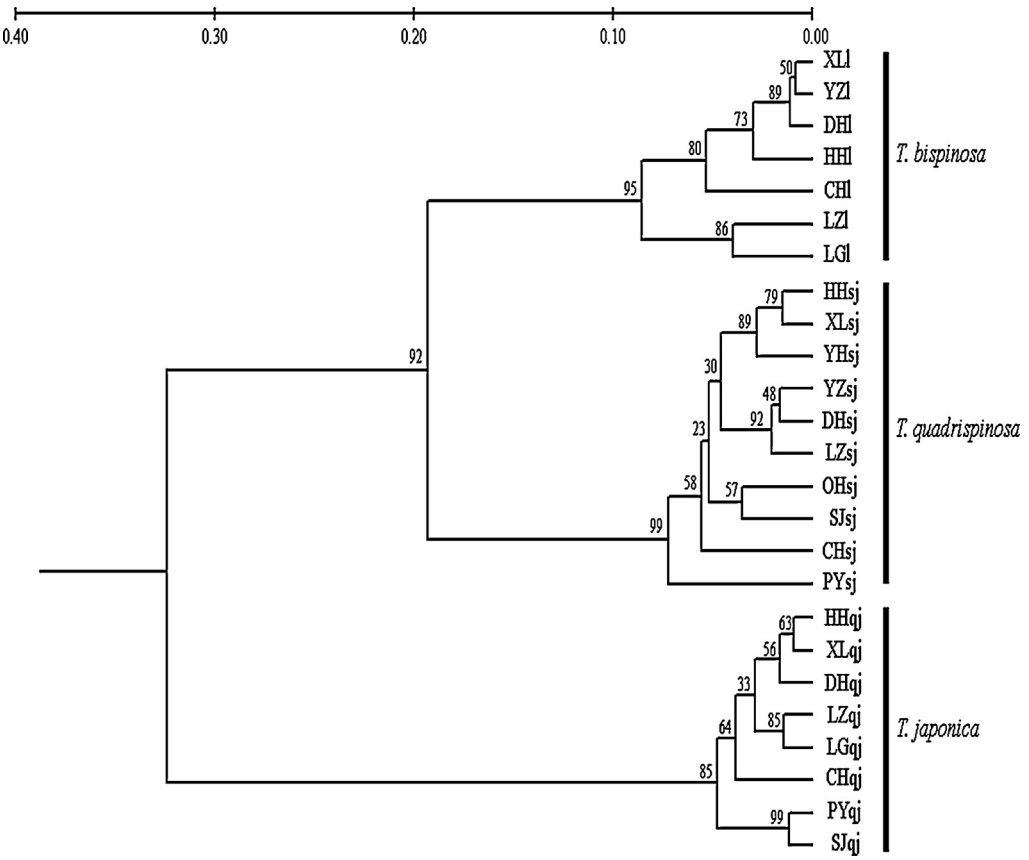

Fig. 4. UPGMA dendrogram of the *Trapa* populations based on Nei's (1972) genetic distance. Numbers beside branches indicate bootstrap values (1000 replicates).

Table 3
Results of clonal diversity and genetic diversity for wild populations of *Trapa* taxa (numbers in parentheses are standard deviations; see Table 1 for description of population abbreviation).

Population	*N*	*G*	*D*	*E*	P_p (%)	H_E	*I*
T. quadrispinosa							
CHsj	22	22	1.000	1.000	15.85	0.048 (0.130)	0.074 (0.189)
YHsj	19	16	0.982	0.903	8.74	0.025 (0.088)	0.039 (0.134)
HHsj	22	16	0.965	0.796	12.02	0.034 (0.101)	0.054 (0.154)
XLsj	22	19	0.987	0.910	16.94	0.046 (0.123)	0.073 (0.180)
YZsj	22	22	1.000	1.000	18.58	0.059 (0.138)	0.090 (0.203)
DHsj	22	17	0.974	0.837	11.48	0.032 (0.102)	0.051 (0.153)
LZsj	22	21	0.996	0.960	12.02	0.037 (0.114)	0.057 (0.167)
OHsj	22	22	1.000	1.000	18.58	0.065 (0.150)	0.096 (0.217)
SJsj	19	19	1.000	1.000	22.40	0.071 (0.146)	0.108 (0.216)
PYsj	21	21	1.000	1.000	23.50	0.065 (0.139)	0.101 (0.205)
Mean	21.3	19.5	0.990	0.941	16.01	0.048 (0.016)	0.074 (0.024)
Total	213	195	0.999	0.912	52.46	0.102 (0.148)	0.168 (0.221)
T. japonica							
CHqj	17	16	0.993	0.951	8.33	0.025 (0.090)	0.039 (0.136)
HHqj	16	16	1.000	1.000	8.33	0.032 (0.110)	0.048 (0.161)
XLqj	14	14	1.000	1.000	6.77	0.025 (0.097)	0.037 (0.141)
DHqj	21	20	0.995	0.959	8.33	0.025 (0.093)	0.039 (0.139)
LZqj	18	18	1.000	1.000	6.77	0.023 (0.090)	0.035 (0.134)
LGqj	10	9	0.978	0.926	9.38	0.036 (0.114)	0.053 (0.167)
PYqj	22	18	0.983	0.896	7.29	0.030 (0.113)	0.044 (0.161)
SJqj	22	20	0.991	0.931	9.38	0.029 (0.102)	0.044 (0.150)
Mean	17.5	16.4	0.992	0.958	8.07	0.028 (0.004)	0.042 (0.006)
Total	140	131	0.999	0.947	29.17	0.073 (0.144)	0.115 (0.215)
T. bispinosa							
CHl	22	22	1.000	1.000	8.54	0.028 (0.101)	0.042 (0.148)
HHl	9	8	0.972	0.920	6.03	0.024 (0.096)	0.035 (0.140)
XLl	22	19	0.983	0.849	7.04	0.020 (0.085)	0.031 (0.125)
YZl	21	21	1.000	1.000	12.06	0.033 (0.104)	0.051 (0.154)
DHl	10	10	1.000	1.000	8.04	0.029 (0.103)	0.043 (0.151)
LZl	22	22	1.000	1.000	24.12	0.068 (0.138)	0.106 (0.207)
LGl	22	22	1.000	1.000	17.59	0.053 (0.150)	0.081 (0.192)
Mean	18.3	17.7	0.994	0.967	11.92	0.036 (0.016)	0.056 (0.026)
Total	128	123	0.999	0.951	37.69	0.107 (0.171)	0.164 (0.250)

N, sample size; *G*, the number of genotypes; *D*, Simpson's diversity index; *E*, genotypic evenness; P_p, percentage of polymorphic bands; H_E, Nei's gene diversity; *I*, Shannon and Weaver's index.

At the species level, the highest genetic variability was found in *T. quadrispinosa* (P_p = 52.46%, H_E = 0.102, *I* = 0.168), and the lowest was in *T. japonica* (P_p = 29.17%, H_E = 0.073, *I* = 0.115). At the population level, low genetic variation was found in all populations from the three taxa, with P_p ranging from 6.03% for HHl of *T. bispinosa* to 24.12% for LZl of *T. bispinosa*, H_E from 0.020 for XLl of *T. bispinosa* to 0.071 for SJsj of *T. quadrispinosa*, and *I* from 0.031 for XLl of *T. bispinosa* to 0.108 for SJsj of *T. quadrispinosa* (Table 3).

A total of 195, 131 and 123 unique multiloc us genotypes was identified for *T. quadrispinosa*, *T. japonica* and *T. bispinosa*, respectively. Within each taxon, no multilocus genotype was shared by two populations. The Simpson's clonal diversity index (*D*) was 0.999 for the three taxa, and the genotypic evenness (*E*) ranging from 0.912 for *T. quadrispinosa* to 0.951 for *T. japonica*.

3.3. Genetic structure within each taxon

The θ_B values generated by the Bayesian approach from the three different models are presented in Table 4. For all the three taxa, the smallest mean DIC, with 1622.052 for *T. quadrispinosa*, 693.252 for *T. japonica* and 911.042 for *T. bispinosa*, was obtained under the *f*-free model, suggesting that the *f*-free model is more suitable than other models for the three taxa. Therefore, θ_B = 0.507, 0.574 and 0.604 for *T. quadrispinosa*, *T. japonica* and *T. bispinosa*, respectively, were determined to be an unbiased estimate of population genetic differentiation under the *f*-free model, with an inbreeding coefficient about 0.5 for the three taxa. Under the assumption of total selfing, the coefficient of gene differentiation (G_{ST} = 0.523, 0.612 and 0.640 for *T. quadrispinosa*, *T. japonica* and *T. bispinosa*, respectively) was obtained, which is close to the θ_B values derived from the *f*-free model. Thus, the results suggested that natural *Trapa* populations had a high degree of inbreeding, and there was a strong genetic differentiation within the three taxa. Estimation of gene flow suggested a very low gene exchange rate among the populations, with *Nm* = 0.243, 0.185 and 0.165 for *T. quadrispinosa*, *T. japonica* and *T. bispinosa*, respectively, based on θ_B (analogous to F_{ST}).

The data from AMOVA further revealed a significant genetic differentiation across the sampled distribution of *Trapa* taxa. When the total variance was partitioned into three hierarchical levels, the largest variance 69.67% of the total molecular variation was attributed to inter-taxon differentiation and 17.66 and 12.68% variance were found within taxa and within populations, respectively. For each taxon, more than 50% of the total molecular variation was attributed to inter-population differentiation (Table 5). Mantel test revealed that a significant correlation between genetic distances and geographical distances existed in *T. quadrispinosa* (r = 0.552 P = 0.002, Fig. 5a), *T. japonica* (r = 0.703, P = 0.002, Fig. 5b) and *T. bispinosa* (r = 0.852, P = 0.002, Fig. 5c).

4. Discussion

4.1. Species relationships

Based on 232 AFLP bands, analyses of genetic relationships using different approaches (NJ tree, STRUCTURE, PCA and UPGMA) consistently indicated that all the three *Trapa* taxa formed genetically distinct groups. This means that, although the *Trapa* taxa

Table 4
Wright's *F* statistics calculated for populations of *Trapa* taxa under different models based on a Bayesian approach (θ_B is analogous to Wright's F_{ST}, *f* is analogous to F_{IS}, and DIC is deviance information criterion).

Models	θ_B				*f*				DIC
	Mean	SD	2.5%	97.5%	Mean	SD	2.5%	97.5%	
T. quadrispinosa									
Full model	0.462	0.021	0.422	0.503	0.029	0.033	0.001	0.117	1648.674
$f=0$ model	0.457	0.020	0.419	0.496	0.000	–	–	–	1649.028
$\theta_B=0$ model	0.000	–	–	–	0.340	0.146	0.112	0.653	7111.453
f-free model	0.507	0.023	0.461	0.548	0.503	0.287	0.029	0.976	1622.052
T. japonica									
Full model	0.540	0.030	0.481	0.598	0.082	0.123	0.001	0.462	700.307
$f=0$ model	0.530	0.028	0.475	0.584	0.000	–	–	–	704.118
$\theta_B=0$ model	0.000	–	–	–	0.566	0.210	0.176	0.953	3308.652
f-free model	0.574	0.029	0.515	0.628	0.502	0.289	0.027	0.978	693.252
T. bispinosa									
Full model	0.574	0.026	0.526	0.626	0.086	0.117	0.001	0.452	919.782
$f=0$ model	0.564	0.022	0.519	0.608	0.000	–	–	–	924.928
$\theta_B=0$ model	0.000	–	–	–	0.558	0.157	0.276	0.879	5272.231
f-free model	0.604	0.025	0.551	0.650	0.482	0.287	0.023	0.970	911.042

Table 5
Analysis of molecular variance (AMOVA) for the three *Trapa* taxa using AFLP (statistical significance is based on 999 permutations).

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*p*-value
Among taxa	2	6513.179	21.492	69.67	<0.001
Among populations within taxa	22	2230.731	5.448	17.66	<0.001
Within populations	425	1662.477	3.912	12.68	<0.001
Among populations of *T. quadrispinosa*	9	947.112	5.161	52.01	<0.001
Within populations of *T. quadrispinosa*	185	880.785	4.761	47.99	<0.001
Among populations of *T. japonica*	7	575.072	4.874	63.27	<0.001
Within populations of *T. japonica*	123	348.012	2.829	36.73	<0.001
Among populations of *T. bispinosa*	6	846.358	7.864	65.29	<0.001
Within populations of *T. bispinosa*	117	478.529	4.090	34.21	<0.001

grew interspersed within the same water bodies, we detected no instances of hybridization among them. The data from AMOVA further revealed that most of the total molecular variation (69.67%) was attributed to inter-taxon differentiation. Therefore, for the three *Trapa* taxa, AFLP markers can serve as reliable tools to clarify taxon delimitation. Given the lack of hybridization between the *Trapa* taxa and the obvious morphological differences in their fruits, the three taxa should be recognized as separate species, which supports the taxonomic delimitation proposed in Flora Republicae Popularis Sinicae (Wan, 2000). Likewise, using combination of nuclear AP2 and chloroplast *trnL-F* region, Kim et al. (2010) clearly identified three *Trapa* species, including *T. bispinosa* from China, *T. japonica* and *T. incisa* from Korea.

In contrast to the present study, previous report based on nrDNA ITS sequence failed to identify the 19 individuals of five wild *Trapa* taxa from the mid-to-lower reaches of the Yangtze River (Bao et al., 2004). The discrepancy might be explained by the fact that the two marker systems produced different genetic profiles. Although the nrDNA ITS region has been the widely used sequence data at the interspecific level in plant phylogenetic studies, its variation is not always sufficient to resolve closely related species (Baldwin et al., 1995; Sang, 2002). In contrast, AFLP fragments are spread over the entire nuclear genome, and consequently AFLP generates large data sets that reliably reflect discrimination between entire individual genomes (Kim et al., 2009; Na et al., 2010).

For the three taxa, *T. japonica* formed a distinct group in cluster analysis (NJ tree, STRUCTURE and UPGMA), which was well supported by the results of PCA. The first principal coordinate (56.71%) readily distinguished *T. japonica* from the other two taxa. *T. quadrispinosa* and *T. bispinosa* were clearly separated from each other by the second principal coordinate (26.17%). The current genetic relationship is in accordant with the dendrogram of the Chinese *Trapa* species based on RAPD markers (Jiang and Ding, 2004).

4.2. Clonal diversity and genetic variation within taxa

Trapa plants are annual aquatic macrophytes capable of extensive clonal propagation (Groth et al., 1996). Unexpectedly, very high level of clonal diversity was found for the three taxa (Simpson's clonal diversity index, $D=0.999$). In many populations, all sampled individuals were genetically distinct. The research about the effects of population density on module demography showed that at low density, *Trapa* plants produced ten times as many ramets as those at high density (Groth et al., 1996). In the present study, the sites where we sampled from were densely colonized by *Trapa* plants and there was little space for the individuals to expand outward. Therefore, the high clonal diversity in this study could be explained by the large sampling interval (4m) which may be longer than the clonal distance of most individuals.

The mean values of genetic diversity at the population level in the three *Trapa* taxa ($H_E=0.028$-0.048) are far less than the average for plants ($H_E=0.22$ or 0.23) derived by the dominantly inherited markers (RAPD, AFLP and ISSR) (Nybom, 2004). Likewise, low intra-population genetic diversity was also found in the other annual aquatic plants in the same habitat and range distribution, such as submerged plant *Ottelia alismoides* (L.) ($H_E=0.042$, based on ISSR markers; Chen et al., 2008) and aquatic fern *Ceratopteris pteridoides* (Hook.) Hieron ($H_E=0.050$, based on AFLP markers; Chen et al., 2010). On the other hand, relatively high genetic diversity was found in perennial aquatic macrophytes from the mid-to-lower reaches of the Yangtze River. For example, using ISSR markers,

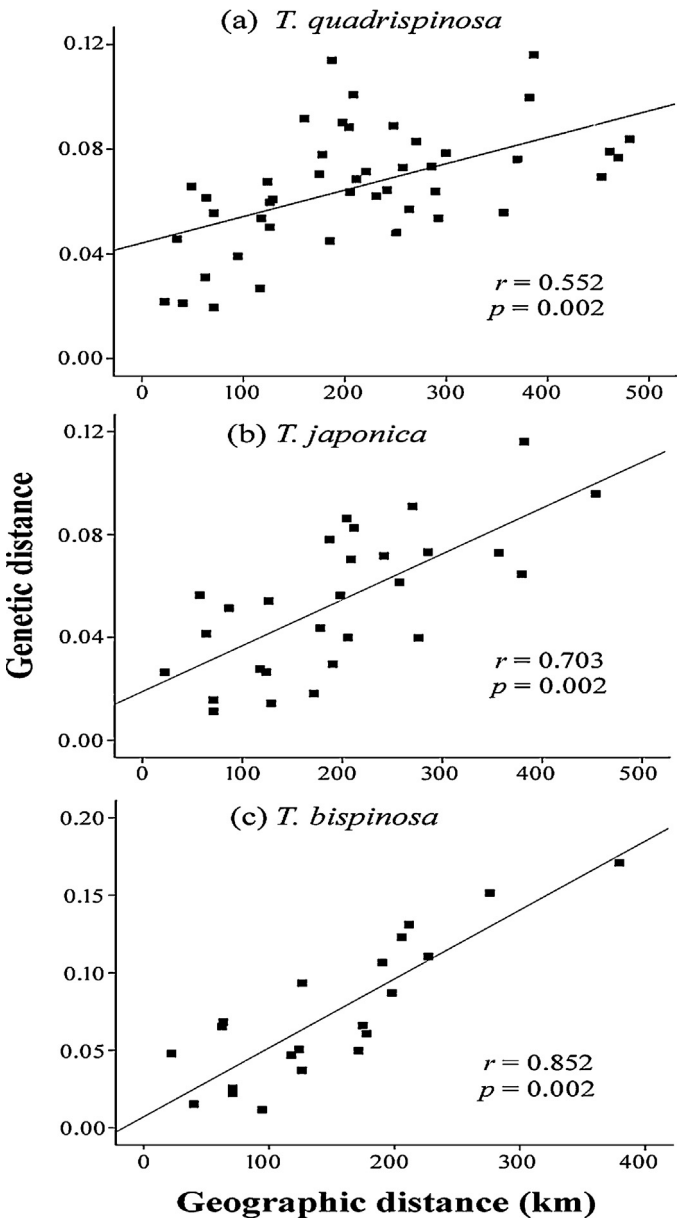

Fig. 5. Scatter plots of pairwise genetic distance (Nei, 1972) versus geographical distance (km) of all sampled populations of (a) *T. quadrispinosa*, (b) *T. japonica* and (c) *T. bispinosa*.

high genetic diversity was found in the two species of *Vallisneria* genus (for *V. natans* (Lour.) Hara, H_E = 0.24; for *V. spinulosa* Yan, H_E = 0.18; Wang et al., 2010). Estimates from the perennial species of *Potamogeton* genus in the areas also showed high level of genetic diversity (Li et al., 2004; Chen et al., 2006, 2009). Furthermore, high microsatellite variation was found in *Zizania latifolia* Turcz with H_E = 0.610 (Chen et al., 2012). During the past several decades, the areas of mid-to-lower reaches of the Yangtze River has suffered major destruction as the result of anthropogenic activities, which has led to habitat loss, degradation and fragmentation for aquatic macrophytes (Xie and Chen, 1999). Compared with annual or short-lived plants, perennial species are resilient to habitat destruction of recent years because there is not sufficient time for genetic bottleneck and inbreeding to erode the genetic diversity (Young et al., 1993; Lowe et al., 2005; Wei and Jiang, 2012). Therefore, the habitat deterioration of recent years combined with the annual life history may be responsible for the relatively low level of genetic diversity observed in the *Trapa* taxa.

4.3. Genetic structure within taxa

The coefficient of gene differentiation estimated by different approaches (G_{ST}, θ_B and AMOVA) consistently indicated the evident population structure among natural populations within the three *Trapa* taxa. The proportion of genetic differentiation among populations within each taxon accounted for more than 50% of total genetic diversity, which was typical of an annual and selfing plant species (mean G_{ST} = 0.553; see Hamrick and Godt, 1996). High genetic differentiation in the present study may explained by the lack of gene exchange among populations (Nm = 0.243, 0.185 and 0.165 for *T. quadrispinosa*, *T. japonica* and *T. bispinosa*, respectively). Because obviously pollen-mediated gene flow occurs at a local scale but diminishes logarithmically with increasing distance (Tero et al., 2003), the magnitude of genetic differentiation among populations is determined by the ability of a plant species to disperse its seeds or propagules. Long distance dispersal of seeds is not effective for *Trapa* because the seeds readily shatter after maturing and fall immediately to the bottom of water body. Only a small fraction of the seeds are carried on buoyant, detached floating ramets, in which manner seeds might be dispersed within a continuous water body (Groth et al., 1996). However, during the past 50 years, the historical continuous habitats in the mid-to-lower reaches of the Yangtze River have been broken up. The construction of dams and floodgates kept rivers and lakes separate. Additionally, for most lakes, the prevalent style of aquaculture is breeding fish or crab in enclosures, which divided the lakes into many small segments. Thus, it was difficult for the detached ramets of *Trapa* dispersed among different lakes, even in the same lake. When gene flow rate is greatly limited, population genetic differentiation will increase due to genetic drift (Slatkin, 1977; Hutchison and Templeton, 1999).

Species with predominantly self-fertilizing reproduction usually had a high genetic structure among populations (Hamrick and Godt, 1996). For *Trapa*, although a few insects have visited the blooming flowers, water chestnut is considered to a self-pollinating plant since its pollination possibly occurs before the flower opens (Arima et al., 1999). Controlled pollination experiments suggested that breeding system of *Trapa* plants mainly was selfing, supplemented by cross-pollination and apomixes (Kadono and Schneider, 1986; Ding et al., 1996). In the present study, high rate of inbreeding of the three taxa was directly obtained by Bayesian approach (F_{IS} = 0.503, 0.502 and 0.482 for *T. quadrispinosa*, *T. japonica* and *T. bispinosa*, respectively), which would partly responsible for the high genetic differentiation within the three *Trapa taxa*.

Under restricted gene flow, an isolation-by-distance (IBD) pattern of population structure probably developed (Hutchison and Templeton, 1999). The significant positive correlation between genetic and geographic distances detected in the three *Trapa* taxa indicated this IBD pattern influenced population differentiation in *Trapa* plants. The IBD pattern was well supported by the UPGMA dendrogram which showed that geographically adjacent populations tended to clustered together within each taxon. Two possible mechanisms could result in historical or current gene exchange among adjacent lakes: (i) native fishers could have dispersed *Trapa* seeds during their picking process. The fishers live in their boats and fish within neighboring lakes (Qian et al., 2009). During our field investigation, we observed that they frequently gatherd *Trapa* seeds as food, which might have resulted in seeds dispersal among the lakes. However, with the development of aquaculture and urbanization, only a handful of families retain the unstable life style (Qian et al., 2009); (ii) the detached ramets could have been dispersed by the seasonal floods which connected adjacent lakes. Whereas, due

to the construction of floodgates and dams, floods generally have been under control in recent decades.

4.4. Implications for management

Because of serious disturbances in the mid-to-lower Reaches of the Yangtze River, some once dominant or widespread aquatic macrophytes have gone locally extinct or become rare and endangered (Jian et al., 2001; Peng et al., 2003). Although *Trapa* plants are abundant in the areas, most of them are densely distributed in water regions nearby lake shores. In recent years, lake shores are often reclaimed massively into paddy fields and fishponds. Given the habitats of *Trapa* are critically vulnerable, conservation attention should be put in the wild populations of *Trapa*. Maintenance of genetic diversity is a major focus in conservation biology because genetic variation is important for a species to maintain its evolutionary potential to cope with ever-changing environments (Frankham et al., 2002). The information gained on the levels and distribution of genetic variation in the water chestnut populations can be used to suggest appropriate management strategies.

Our analyses showed that low level of genetic variation and evident population structure were found in the populations of the *Trapa* taxa. The results suggested that the numbers of populations in each *Trapa* taxon and individuals in each population should be almost equally considered for conservation managements because extinction of any population would reduce total genetic variability considerably. For *in situ* conservation, reclamation and fragmentation are major threats to the habitats of aquatic macrophytes growing in lakeside zones. Therefore, the activity of reclamation on wetlands should be properly controlled and monitored. For *ex situ* conservation, considering the evident genetic differentiation among the taxa and populations within each taxon, the plants and seeds should be separately planted or preserved according to their taxa and geographic origin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquabot.2016.09.009.

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