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Comparison of the extent of genetic variation of *Vallisneria natans* and its sympatric congener *V. spinulosa* in lakes of the middle–lower reaches of the Yangtze River

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ABSTRACT

Vallisneria natans and *Vallisneria spinulosa* are two morphologically very similar and sympatrically dominant submerged macrophytes in lakes of the middle–lower reaches of the Yangtze River. Genetic variation was compared based on a total of 196 individuals from six *V. natans* populations and 201 individuals from seven *V. spinulosa* populations. Using eight ISSR primers, a total of 139 and 129 DNA fragments were generated with 121 being polymorphic in *V. natans* and 99 in *V. spinulosa*. The two species maintained higher genetic variation both at the species and population levels in comparison with other aquatic macrophytes. A higher level of genetic diversity among populations was found in *V. natans* than in *V. spinulosa*: the percentage of polymorphic loci (*PPL*) in *V. natans* was 52–62% vs. 38–47% in *V. spinulosa*; gene diversity (*H*) was 0.21 in *V. natans* vs. 0.17 in *V. spinulosa*.

Both an analysis of molecular variance (AMOVA) and *F*-estimation (F_{ST}) showed that most of the total genetic variation resided within populations of both species (AMOVA: 85% and 80%; F_{ST}: 0.132 and 0.202), indicating low genetic differentiation between populations. Principal coordinates analysis (PCA) indicated evident gene flow between populations of both species. The outcrossing reproductive mode and pervasive gene flow might have played important roles in maintaining high genetic diversity and in shaping low population differentiation of the two *Vallisneria* species, while the extent of clonal growth might account for the different levels of population divergence between them.

1. Introduction

Shallow lakes with dense areas of submerged plants often have clear water and low concentrations of nutrients and phytoplankton (e.g. Havens et al., 2004; Caraco et al., 2006), suggesting that submerged plants are important for the ecological recovery of eutrophic water bodies. The middle–lower reaches of the Yangtze River form the largest floodplain in China, with thousands of shallow lakes that are generally less than 3 m deep. Most of these lakes can sustain a rich variety of submerged plants. However, due to recent eutrophication and excessive human disturbance, the population size and distribution of submerged plants such as *Vallisneria* spp. have decreased (Wang et al., 2005). The continued decline of submerged vegetation will further worsen aquatic conditions for fish and other wildlife in these areas. It appears urgent to take action and re-introduce submerged plants to restore environmental conditions, allowing wildlife to subsequently re-colonize naturally.

Since *Vallisneria* spp. are thought to provide food for waterfowl, as a nursery habitat for fish, a substrate for invertebrates, and have a strong influence on water quality, they are used as pioneer plants in freshwater ecosystem restoration and have attracted attention in recent years (e.g. by Korschgen et al., 1997; Li et al., 2005; Ke and Li, 2006; Xie et al., 2006a, 2007; Xiao et al., 2007; Wang et al., 2008; Wu et al., 2009). Three species of the genus *Vallisneria* are recorded in China: *Vallisneria spinulosa* Yan, *Vallisneria denseserrulata* (Makino) Makino, and *Vallisneria natans* (Lour.) Hara. *V. spinulosa* is thought to be endemic to China, only occurring in the middle–lower reaches of the Yangtze River; *V. denseserrulata* is restricted to China and Japan; and *V. natans* is a cosmopolitan species, especially

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in tropical and subtropical zones (Xie et al., 2006a,b, 2007). In China, these can be found in different freshwater locations including lakes, ponds, rivers and paddy fields.

V. natans, *V. spinulosa* and *V. denseserrulata* are very similar morphologically, making it difficult to distinguish them clearly in the field without flowers or fruit (Les et al., 2008). Especially *V. natans* and *V. spinulosa* usually occur sympatrically and form mixed populations in the middle–lower reaches of the Yangtze River. These two species have somewhat different life histories, however. For example, Chen et al. (2008b) found that *V. natans* flowered earlier than *V. spinulosa* and produced fewer vegetable tubers, suggesting that stronger clonal growth occurs in *V. spinulosa*. This would be expected to result in population genetic variation differences, since clonal growth can have a strong influence on population diversity and genetic structure (Ellstrand and Roose, 1987).

The present study was designed to investigate the genetic diversity levels of the two *Vallisneria* species in lakes of the middle–lower reaches of the Yangtze River by DNA molecular markers with careful field sampling during the flowering period. Following an unsuccessful attempt to estimate genetic variations using the SSR markers developed by Chen et al. (2006), this time we used inter-simple sequence repeat (ISSR) assays. The method applies the principle of SSR-anchored polymerase chain reaction (PCR) amplification by designed primers that can randomly amplify DNA fragments of the inter-repeat regions. Since prior DNA sequence information is not required, it is a particularly useful method for studying species whose sequence information is not known (Zietkiewicz et al., 1994; Jones et al., 2009). Although the dominance of the ISSR marker limits the utilization of the method for estimating heterozygosity and mating systems, it is commonly used in studies of population genetics, taxonomy and phylogeny of many plant species (e.g., Meimberg et al., 2006; Angelone et al., 2007).

V. spinulosa has considerable genetic variation and hardly any population genetic differentiation, probably due to extensive hydrologic connectivity among populations by the Yangtze River (Chen et al., 2007), suggesting that in practice any population can be used as stock for re-introduction. In a comparison with the sympatric and phenotypically similar congener *V. spinulosa*, we addressed the following questions: (1) does *V. natans* also hold rather genetic variation and present a similar pattern of population divergence as *V. spinulosa*? (2) can we find evidence of interpopulation gene flow in *V. natans*?

2. Materials and methods

2.1. Sample collection

Of the 26 characters used to classify species in the genus of *Vallisneria* by Les et al. (2008, their Table 2), only two, i.e. the number of locules/anthers and the form of the fruit cross-section, can be used as reliable identifiers of *V. natans* and *V. spinulosa* under field conditions. Thus, the useful time to collect and separate the two species in the field should be during the flowering period. In September to October, 2007, when both species flowered, we sampled plant material from 12 lakes in the middle–lower reaches of the Yangtze River using the above characters for identification.

At each population, if the plants were distributed continuously, one 500 m × 2 m transect was established and one individual was randomly collected at 5 m intervals (Chen et al., 2007). When plants occurred in patches, one individual was collected every 5 m in each patch. A total of 196 samples from 6 populations of *V. natans* and 201 samples from 7 populations of *V. spinulosa* were collected (Table 1). Plant material was stored dry in silica gel and brought back to the laboratory for DNA extraction.

2.2. Total DNA extraction

Total genomic DNA was isolated from 0.5 g of silica-dried leaf tissue using a modification of the hexadecyl trimethyl-ammonium bromide (CTAB) extraction procedure of Doyle and Doyle (1987). Leaf material was powdered with liquid nitrogen, mixed with 2 mL extraction buffer (1.4 M NaCl, 100 mM Tris–HCl (pH 8.0), 20 mM EDTA, 2% (V/V) CTAB and 2% 2-mercaptoethanol) at 65 °C, and incubated at 65 °C for 30 min with gentle shaking every 5 min. Proteins were extracted twice with 2 mL of chloroform:isoamylalcohol (24:1), then centrifuged at 10,000 × *g* for 2 min. RNase (10 μg mL^{-1}) was added to the supernatant and incubated for 2 h at 37 °C. The mixture was centrifuged at 10,000 × *g* for 2 min. The sediment was washed twice in 70% ethanol, air-dried, resuspended in 100 μL 0.1 × TE, and then stored at −20 °C.

Table 1
Sample size and parameters of genetic diversity of *Vallisneria* populations. *G* = number of genotypes detected; *PPL* = percentage of polymorphic loci; *H* = Nei's gene diversity.

Species	Locality	Sample size	*G*	*PPL* (%)	*H* ± sd
Vallisneria natans					
NSVn	Niushan Lake, Hubei Province (E114°31.1′/N31°20.7′)	29	29	55	0.24 ± 0.23
BAVn	Baoan Lake, Hubei Province (E114°43.9′/N30°12.1′)	30	29	52	0.22 ± 0.22
PYVn	Poyang Lake, Jiangxi Province (E115°53.5′/N29°14.2′)	32	32	62	0.28 ± 0.23
LGVn	Longgan Lake, Hubei Province (E116°01.4′/N29°57.1′)	35	35	53	0.24 ± 0.23
DPVn	Dongpu Lake, Anhui Province (E117°12.1′/N31°52.7′)	44	44	58	0.23 ± 0.22
DSVn	Dianshan Lake, Shanghai city (E120°55.1′/N31°06.9′)	26	24	56	0.23 ± 0.24
Mean				56	0.24 ± 0.20
Total		196	193	87	0.32 ± 0.21
V. spinulosa					
HHVs	Honghu Lake, Hubei Province (E113°23.2′/N290°50.7′)	26	24	47	0.21 ± 0.23
DCVs	Diaocha Lake, Hubei Province (E113°43.2′/N30°43.2′)	32	32	44	0.19 ± 0.22
XLVs	Xiliang Lake, Hubei Province (E114°26.5′/N30°19.3′)	28	27	42	0.18 ± 0.22
BAVs	Sympatric with BSVn	36	34	45	0.21 ± 0.23
LGVs	Sympatric with LGVn	26	23	38	0.17 ± 0.22
DPVs	Sympatric with DPVn	25	25	38	0.18 ± 0.23
DSVs	Sympatric with DSVn	28	25	39	0.16 ± 0.21
Mean				42	0.18 ± 0.21
Total		201	190	77	0.28 ± 0.22

Table 2
ISSR primers and the total bands/polymorphic bands in *Vallisneria* species.

Primer	Sequence (5′–3′)	Anneal temperature (°C)	No. of total bands/polymorphic bands		No. of species-specific bands	
			V. natans	*V. spinulosa*	*V. natans*	*V. spinulosa*
807	$(AG)_8T$	53	17/14	17/14	3	3
810	$(GA)_8T$	52	15/13	13/12	3	1
811	$(GA)_8C$	54	17/14	18/11	3	4
836	$(AG)_8YA$	54	11/10	13/10	1	2
840	$(GA)_8YT$	53	16/14	16/11	2	2
841	$(GA)_8YC$	55	18/15	16/12	6	3
842	$(GA)_8YG$	55	21/18	23/16	4	5
880	$GG(AG)_2(GA)_2G(GA)_2$	53	13/11	13/9	3	3
Total			138/121	129/99	27	23

2.3. ISSR PCR amplification

A total of 100 ISSR primers, acquired from the University of British Columbia SSR Primer (RAPD) Synthesis Project Oligonucleotide Set 100/9, were screened with the PCR amplification of three DNA samples from each population. Eight primers that amplified clear and repeatable bands were used in the study (Table 2). The ISSR PCR reactions were carried out in a volume of 20 μL containing 50 ng of genomic DNA, 20 mM $MgCl_2$, 0.25 mM each dNTP, 0.2 mM ISSR primer (Sangon Inc.), 2% dimethyl sulfoxide, and 0.5 unit of Taq DNA polymerase (Tiangen Inc.).

PCR cycling parameters included an initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 54 °C, extension for 1 min at 72 °C, and a final extension for 10 min at 72 °C. All PCR reactions were performed in an Eppendorf Mastercycler gradient thermocycler (Eppendorf Inc.). The PCR products were separated on 6% polyacrylamide denaturing gels. After electrophoresis, the bands were visualized using the procedure described by Song et al. (2006).

ISSR fragments were scored as either presence (1) or absence (0) of the putative homologous bands. Several precautions were taken, as suggested by Bonin et al. (2004), to ensure the good quality of the ISSR dataset. First, the reproducibility of all the ISSR markers was investigated by carrying out 3–5 repeated analyses of all the steps from DNA isolation to data scoring, for three individuals per population, totaling 27 individuals. This allowed us to identify any markers that could not be scored reliably. Markers that were scored differently in more than 3 of the 27 individuals were removed from the data set. Second, markers with more than 10% missing data in the complete dataset were removed. Third, to exclude low-quality DNA samples, the proportion of missing data per individual was calculated and individuals with more than 15% missing data were removed from the dataset. Fourth, the primers that produced bands that appeared in the negative control (H_2O) in two or more replicates were removed, even though the bands in the negative controls were typically much weaker than the bands in real samples. In the next step, the genotyping error rate per individual was calculated as the ratio of the observed number of differences in 3–5 replicates to the total number of comparisons (2.18%; Pompanon et al., 2005).

2.4. Data analysis

Samples with identical multilocus genotypes were each considered to be a single clone and were counted once per population to constitute the genet data set. All genetic analyses were based on the genet data set. Standard measures of genetic diversity, including *PPL* and *H* (Nei, 1978) were calculated for each population and for overall populations using the POPGENE program ver. 1.31 (Yeh et al., 1999). The average *PPL* and *H* among populations were compared using a *t*-test to determine whether there was any significant difference in population genetic variations between the two species.

To estimate the degree of genetic differentiation between populations, F_{ST} was employed using the method of Weir and Cockerham (1984) with the POPGENE program ver. 1.31 (Yeh et al., 1999). AMOVA was also used to estimate the distribution of genetic variation among and within populations. The variance components were tested statistically by nonparametric randomization tests using 999 permutations. A Mantel test was then performed with 999 permutations on two distance matrices to examine whether the genetic distance between a population pair increased as a linear function of isolation by distance measured. We measured geographical distance between populations in kilometer that was metric then used a 10 log transformation. Finally, PCA was performed to visually examine the genetic covariance across all samples. AMOVA, Mantel test and PCA were calculated using GENALEX 6.0 (Peakall and Smouse, 2006).

The relationships between population genetics parameters and the log of population sample size were explored with least-square linear regressions using the REG procedure of STATISTICA 6.0 (StatSoft Inc., 2001). The average of pairwise population F_{ST} was compared using a *t*-test to determine whether there was any significant difference in population genetic divergence between the two species.

3. Results

3.1. Genetic variation

A total of 138 and 129 bands were generated with the eight ISSR primers. Of these, 121 and 99 were polymorphic and 27 and 23 were species-specific in *V. natans* and *V. spinulosa*, respectively (Table 2). Of the multilocus genotypes, 193 were scored from the 196 *V. natans* samples and 190 were scored from the 201 *V. spinulosa* samples (Table 1). No widespread multilocus genotypes were found among populations in either species.

Genetic diversity varied between populations: *PPL* ranged from 52% to 62% in *V. natans* populations, and from 37% to 47% in *V. spinulosa* populations (Table 1). The *H* index indicated that the PYVn population had the greatest variation (0.28), while the BAVn population showed the least variation (0.22) for *V. natans*. In *V. spinulosa*, the *H* values between populations ranged from 0.21 in the HHVs population to 0.16 in the DSVs population. The *PPL* and *H* values both indicated that *V. natans* had relatively higher genetic variation than *V. spinulosa* (average $PPL = 56\%$ vs. 42%, *t*-test, $p < 0.001$; average $H = 0.21$ vs. 0.17, *t*-test, $p < 0.001$). Regression analyses showed no positive relationships between genetic variation and population sample size ($r = 0.302$, $p = 0.561$ for *H*, and $r = 0.227$, $p = 0.666$ for *PPL* in *V. natans*; $r = 0.267$, $p = 0.563$ for *H*, and $r = 0.472$, $p = 0.285$ for *PPL* in *V. spinulosa*).

Table 3
Analysis of molecular variance for individuals of *Vallisneria natans* and *V. spinulosa* based on ISSR markers (significance tests after 999 permutations).

	Source of variation	d.f.	SS[a]	Variance components	Percentage of variation	*p*-Value
V. natans	Among populations	5	956	5.3	25	<0.001
	Within populations	190	3021	16.2	75	<0.001
V. spinulosa	Among populations	6	1066	5.7	30	<0.001
	Within populations	194	2542	13.1	70	<0.001

[a] SS, the total sum of squared deviations.

3.2. Genetic structure

The coefficients of genetic differentiation between populations (F_{ST}) were 0.132 in *V. natans* and 0.202 in *V. spinulosa*. This indicates that a greater proportion of the genetic variance resided between individuals within populations, while a smaller proportion resided between populations of the two species. Genetic differentiation between populations was greater in *V. spinulosa* than in *V. natans* (*t*-test for average F_{ST}, $p < 0.001$). AMOVA analysis revealed a similar pattern of population genetic divergence in the two species (Table 3). The Mantel test detected weak correlations between genetic distance and geographical distance for the populations of both species ($r^2 = 0.183$, $p = 0.090$ for *V. natans*; $r^2 = 0.271$, $p = 0.070$ for *V. spinulosa*).

An examination of the proportions of genetic diversity indicated that 37% occurred within species, whereas about twice as much (63%) occurred between species. PCA for the two species gave similar results: the first two axes of the PCA explained 71% and 11% of the total genetic variation. Consequently, the two-dimensional PCA diagram showed the individuals arranged into distinct groups with respect to species (Fig. 1a). In the separate PCA, for *V. natans* only the first two axes explained 30% and 22% of the total genetic variation. For *V. spinulosa*, 36% and 18% of the total genetic variation were explained by the first two axes of the PCA. The PCA diagram showed that in both species, some individuals were scattered among populations, although most individuals of a population clustered together (Fig. 1b and c). Co-occurring populations of the two species did not show the same spatial pattern, for example, the populations from Dongpu Lake (DPVn vs. DPVs) and Dianshan Lake (DSVn vs. DSVs) were scattered in different regions of the plot (Fig. 1d).

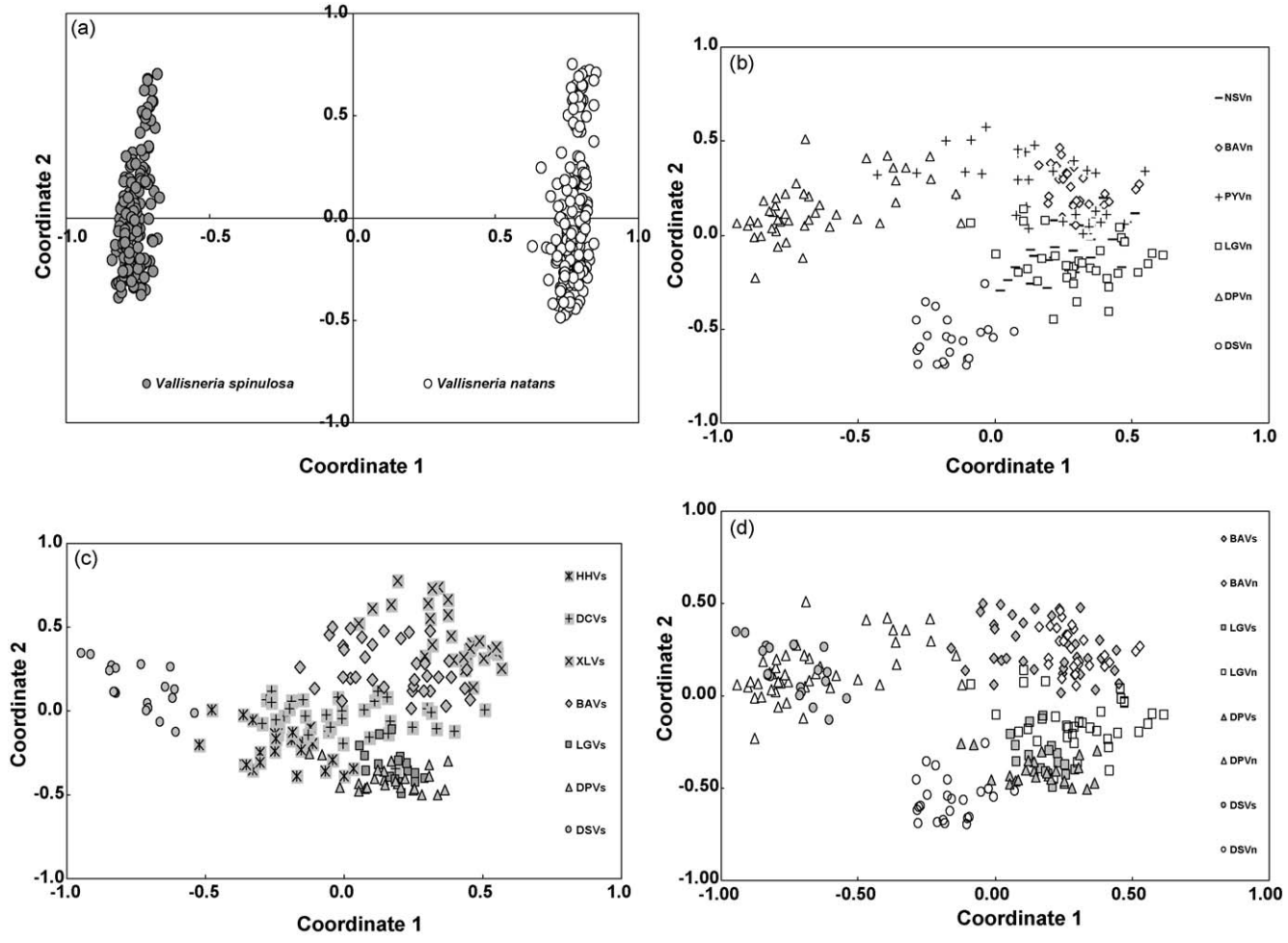

Fig. 1. PCA results showing *Vallisneria* individuals based on ISSR multilocus genotypes in respect to individual component score to the first two coordinates: (a) all 397 *Vallisneria* samples, the coordinate 1 and 2 explained 71% and 11% of the total genetic variation; (b) 196 individuals of *V. natans* from six populations, the coordinate 1 and 2 explained 30% and 22% of the total genetic variation. (c) 201 individuals of *V. spinulosa* from seven populations, the coordinate 1 and 2 explained 36% and 18% of the total genetic variation. (d) Individuals from co-occurred populations of the two species. See Table 1 for reference to the population codes, locations, and sample sizes.

4. Discussion

4.1. Genetic diversity in Vallisneria

The mean values of genetic diversity at the population level in *V. natans* ($H = 0.24$) and *V. spinulosa* ($H = 0.18$) found in the present work, and also the value reported by Chen et al. (2007) for *V. spinulosa* ($H = 0.21$) from the same region, are comparable to those for long-lived perennial species ($H = 0.25$) and widespread angiosperm species ($H = 0.22$) reported by Nybom (2004). Unlike the above, several other aquatic macrophytes distributed in the same area possess lower genetic diversity, e.g. *Potamogeton maackianus* ($H = 0.15$; Li et al., 2004), *Ceratopteris pteridoides* ($H = 0.14$; Dong et al., 2007) and *Ottelia alismoides* ($H = 0.04$; Chen et al., 2008a).

Considerable genetic variation has also been found in *Vallisneria americana* ($H = 0.34$; Lokker et al., 1994). This suggests that overall *Vallisneria* spp. may possess more genetic variation than other submerged plant species. Hamrick and Godt (1996) have suggested that obligate outcrossing reproduction is the main contributor. The outcrossing mating systems in *Vallisneria* breed complete sexual recombination seeds. A great number of *Vallisneria* seeds are produced every year because of their usually large population size, and seedling recruitment may be expected (Lokker et al., 1994; Li et al., 2005; Chen et al., 2007; Yuan and Zhang, 2007). In addition to large population size, prolonged population survival of subpopulations combined with high levels of connectivity among populations can all contribute to the high genetic variation in *Vallisneria* (Li et al., 2004; Nybom, 2004; Chen et al., 2007). Other hydrophilous angiosperms exhibit mixed mating with strong clonal growth, which is considered to lead to lower genetic diversity (Li et al., 2004; Dong et al., 2007; Chen et al., 2008a,b).

4.2. Population structure

The mean value of population differentiation for outcrossing species (F_{ST}) is 0.146 (Hamrick and Godt, 1989) and a value >0.25 of that can generally be regarded as indicating high population differentiation (Slatkin, 1993). By this criterion, *V. natans* showed low population differentiation ($F_{ST} = 0.132$) and *V. spinulosa* showed moderate population differentiation ($F_{ST} = 0.202$). AMOVA analysis also revealed that the greater proportion of genetic diversity was within populations in both species (Table 3). Again, this pattern is probably mainly shaped by the outcrossing mating system of *Vallisneria*.

Gene flow may also play an important role in shaping lower population differentiation in *Vallisneria* spp. This view is supported by the PCA results indicating evidence of interpopulation gene flow among some of the sampled populations in both *Vallisneria* species (Fig. 1b and c). In *Vallisneria*, gene flow can occur through pollen (staminal flower), seeds, tubers, turions and stolon fragments floating in the current (Lokker et al., 1994). Most of the sampled waterways in the present study are either directly or indirectly connected to the Yangtze River, at least historically. Such a high degree of hydrological connectivity would seem very likely to facilitate the long-distance dispersal of floating propagules and result in frequent interpopulation gene flow (Chen et al., 2007).

Our observed population differentiation ($F_{ST} = 0.132$ and 0.202) is higher than that estimated by Chen et al. (2007) for *V. spinulosa* ($F_{ST} = 0.06$) and for *V. americana* ($F_{ST} = 0.03$) (Lokker et al., 1994). This might be attributed to recent reduction in linkages between the sampled lakes. For example, there is now no waterway that connects Dongpu Lake and Dianshan Lake. As a consequence, the populations of both species from the two lakes presented relatively distinct genetic relationships each other (Fig. 1b and c). This point can also be partially supported by the Mantel test that shows marginal significant effects of geographical isolation on population divergence in both species ($p = 0.09$ and 0.07 respectively). If connectivity is further reduced, we would expect more among population divergence in *Vallisneria*, as occurred in other hydrophilous angiosperms (e.g., $F_{ST} > 0.481$; Les, 1991; Laushman, 1993). No widespread multilocus genotype was detected in the present study, suggesting that propagules are rarely transferred between populations and the linkage between populations is becoming weak.

4.3. Comparison of genetic variation between V. natans and V. spinulosa

The present study revealed that *V. natans* possesses higher genetic variation and lower population divergence than *V. spinulosa*. Such differences probably result from the different contributions of clonal growth to population colonization in the two species. Chen et al. (2008b) found that *V. spinulosa* was capable of extensive clonal propagation, while *V. natans* reproduced mainly by sexual seedling, and displays limited clonal growth. Our experiments (Wang et al., unpublished data) confirm that *V. natans* produces many more seeds but fewer ramets and tubers than *V. spinulosa*, and more seedling recruitment (51% vs. 27% of initial shoots) occurred in the experimental populations of *V. natans* than in *V. spinulosa*. The greater clonal growth in *V. spinulosa* populations might have reduced the effective population size, leading to the decreased local genetic diversity and relatively stronger population differentiation (Ellstrand and Roose, 1987). Other studies have suggested that repeated seedling recruitment, even at a low rate, is sufficient to maintain a high level of genotypic variation within plant populations (Pluess and Stocklin, 2004).

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