Effects of hydrological connection and human disturbance on genetic variation of submerged *Vallisneria natans* populations in four lakes in China*

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Abstract   With the increase in the need for flood prevention and lake resource used by humans, the construction of floodgates and sluices has changed the hydrological connection between rivers and lakes, and between adjacent lakes. In river-disconnected lakes, exploitation and use of lake resources have resulted in water quality decline and mechanical disturbance intensification to a different degree. Of the large number of river-disconnected lakes in the middle-lower reaches of the Changjiang (Yangtze) River, the Futou Lake, and the Xiliang Lake lie close together and are, historically, directly connected, and so do Liangzi Lake and Baoan Lake. The extent of human disturbance is severe in the Futou Lake and the Baoan Lake, but relatively mild in the Xiliang Lake and Liangzi Lake. The freshwater rosette-forming submerged plant *Vallisneria natans* is one of the dominant species in the four lakes. Using microsatellite markers, we studied the genetic variation of *V. natans* subpopulations in lakes with different intensities of human disturbance and historical direct hydrological connections. Our results showed that human disturbance decreased plant density and clonal growth in *V. natans*, but might increase genetic and clonal diversity at a subpopulation level and enhance gene flow among subpopulations by sexual propagule movement. Under similar climatic conditions, different intensities of disturbance seem to have such a high selective potential to differentiate genetically adjacent lake populations that they outperform the forces of gene flow through historical direct hydrological interconnection, which tends to produce genetic homogeneity. Our findings imply that human disturbance has a profound effect on the evolutionary process of natural populations of submerged plants. Moreover, increased subpopulation genetic diversity can enhance resistance and resilience to environmental disturbances. To a certain degree, we could expect that disturbed populations have the possibility of restoring spontaneously if humans cease to perturb natural ecosystems in the future.

Keyword: river-disconnected lakes; annual plant; microsatellite marker; clonal diversity; gene flow

1 INTRODUCTION

With the increase in the need for flood prevention and lake resource used by humans, the construction of floodgates and sluices has changed the hydrological connection between rivers and lakes, and between adjacent lakes (Wang and Dou, 1998; Lacoul and Freedman, 2006; Chen et al., 2009). Many lakes, historically connected with rivers, have become disconnected and some, isolated in recent decades (Wang and Dou, 1998). The hydrologic variations of the lakes, caused by sluices, are markedly different from the original natural ones (Lacoul and Freedman, 2006). In fact, floodgates and sluices reduce peak flow and the magnitude of water level fluctuation (Nilsson et al., 2010), and prolong water retention time, thus accumulating more pollutants in the lake water. Additionally, artificial control of water level via sluices facilitates intense fishing and favors lake enclosures for cultivation of certain crops, resulting in anthropogenic mechanical disturbance and water

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Table 1 The environmental information on the four sampling lakes of *Vallisneria natans*

	Futou Lake	Xiliang Lake	Liangzi Lake	Baoan Lake
Area[a] (km²)	114.7	72.1	304.3	48.0
Average water depth[a] (m)	2.90	1.94	4.16	3.40
Average temperature[a] (°C)	16.2	16.8	16.8	16.5
Average precipitation[a] (mm)	1 531.4	1 370.0	1 263.4	1 283.0
High water period[b]	Apr.–Sep.	Apr.–Sep.	Apr.–Sep.	Apr.–Sep.
Hydrological history				
Ancient lake[b]	Futou and Xiliang Lakes were components of one ancient lake		Liangzi and Baoan Lakes were components of another one ancient lake	
Water system[b]	Jinshui River system	Jinshui River system	Liangzi Lake water system	Liangzi Lake water system
The year and the sluice gate disconnected with Changjiang River[b]	1935, Jinshui Gate	1935, Jinshui Gate	1926, Minxin and Minsheng Gates	1926, Minxin and Minsheng Gates
The year when direct waterway disrupted between adjacent lakes and lake becoming isolated[b]	1950	1950	1964	1964
Human disturbance				
Intensity of human disturbance[c]	Severe	Relatively mild	Relatively mild	Severe
Water transparency[d] (m)	0.6	1.4	1.5	0.7
Total N/P in water[d] (mg/L)	1.118/0.177	0.394/0.092	0.366/0.057	1.116/0.161

[a]: referred to Wang and Dou (1998); [b]: referred to Committee for Lake Records of Hubei Province (2014); [c]: the human disturbance includes aquatic plant harvest intensity, harrowing clam intensity, enclosing intensity, herbivorous fish stocking, unherbivorous fish stocking (Jin et al., 1999; Peng et al, 2004), and vegetation destruction (Yang et al., 2017); [d]: the data were collected when plants sampled.

quality decline to different extents (Qin, 2002). Therefore, human activities greatly change the aquatic habitat conditions of submerged plants (Chambers et al., 2007; Rejmánková, 2011). Environmental characteristics of aquatic habitats influence the occurrence of submerged species and their life-history traits (recruitment, growth, and reproduction) (Bornette and Puijalon, 2010). Moreover, sluices affect the hydrochory of submerged plants by acting as physical barriers to dispersal (Nilsson et al., 2010). Hence, anthropogenic changes in aquatic environmental conditions may affect the genetic variation of submerged species in lakes.

With the reputation of being "the province with thousands of lakes", the Hubei Province, in central China, does indeed have a large number of shallow lakes lying in a subtropical monsoon climate (Committee for Lake Records of Hubei Province, 2014). Of the lakes in the middle reach of the Changjiang (Yangtze) River (the longest river in China), the Futou Lake and the Xiliang Lake lie close together, and both belong to the water system of the Jinshui River (Fig.1, Table 1). The two lakes were components of one large ancient lake before the 16th century and, historically, directly connected to each other by a narrow waterway until the 1950s (Committee for Lake Records of Hubei Province,

2014). Their water mingles with the Jinshui River before reaching the Changjiang River. Similarly, Liangzi Lake and Baoan Lake are also closely related, historically, to a large ancient lake and remain directly interconnected, belonging to the same water system (Liangzi Lake Water System). Their water mingles with the Changgang River, which drains into the Changjiang River.

Historically, the above four lakes naturally connected with the mainstream of the Changjiang River and lake water level fluctuated seasonally with the rise and fall of the Changjiang River. To reduce flooding around the lakes, sluice gates were built in the estuaries of the Changgang and Jinshui Rivers in 1926 and 1935, respectively (Committee for Lake Records of Hubei Province, 2014) (Table 1). Since then, the four lakes have been disconnected from the Changjiang River, resulting in the termination of natural seasonal lake water fluctuations. In the decades that followed, to better regulate the water level in each lake, the direct waterway connection between the neighboring lakes was cut off and more sluice gates were built on the rivers connected to each of them, resulting in each lake becoming isolated and the amplitude of water level fluctuation largely reduced.

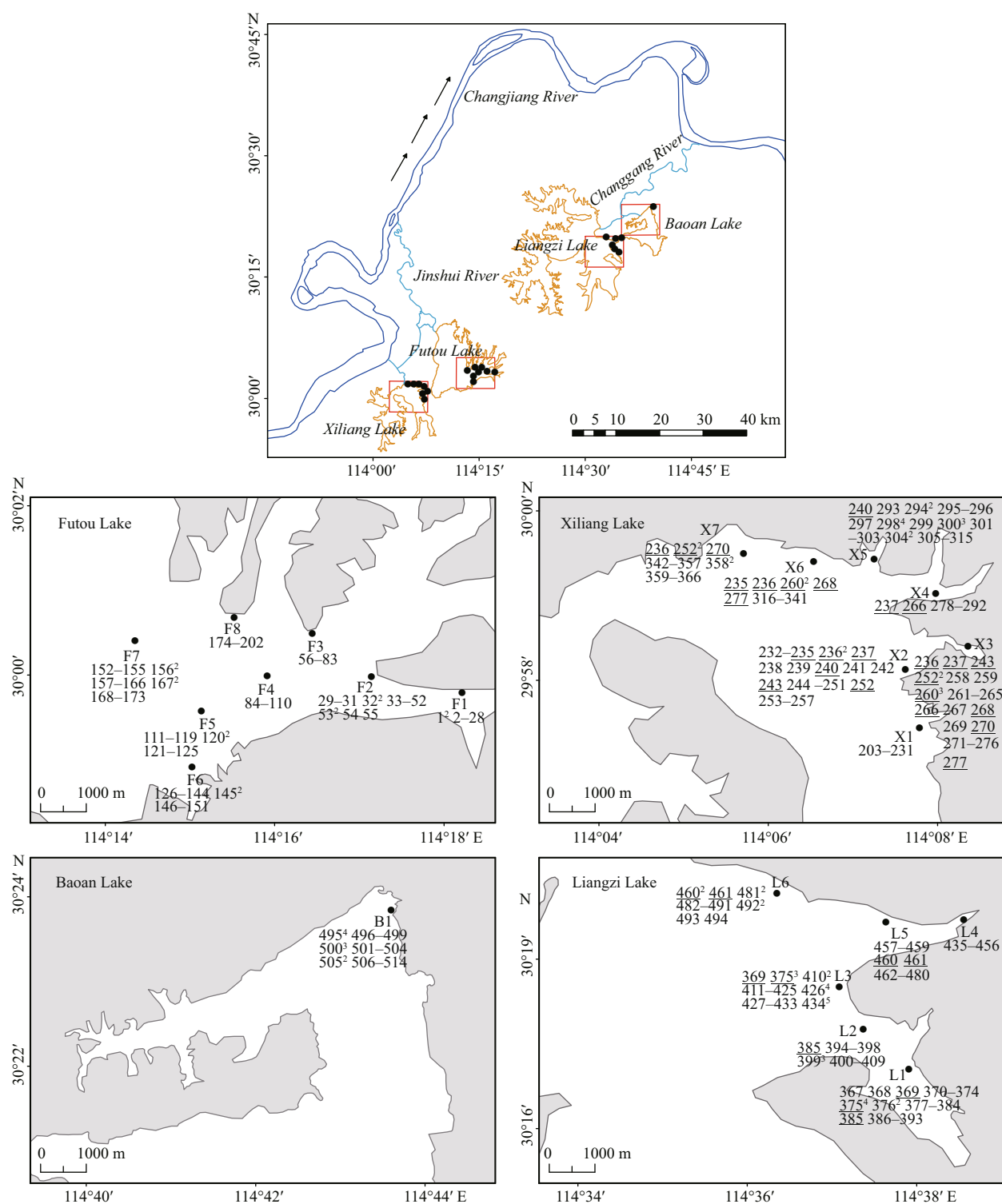Since the sluice gates were built and water level

Fig.1 Location of the sampling lakes of *Vallisneria natans* (the arrows indicate the direction of water flow) and distribution of multilocus genotypes (MLGs) in each subpopulation

Samples belonging to the same MLGs are represented by the same normal numbers among subpopulations. Numbers in superscript are the ramet number of each MLG in each subpopulation. Normal numbers underlined denote MLGs shared between two or more subpopulations.

artificially regulated, exploitation and use of the lake resources by humans have increased greatly, resulting in an increase in the deterioration of water quality and in the intensity of mechanical disturbance in the lakes.

The extent of human disturbance is severe in the Futou and Baoan Lakes, but relatively mild in the Xiliang and Liangzi lakes (Jin et al., 1999; Peng et al, 2004; Yang et al., 2017) (Table 1). In Baoan Lake,

some submerged plants had disappeared at the local scale, so some recovery measurements, such as planting materials originating from the own lake, were conducted at the end of the 1990s (Jin et al., 1999). Relatively, the other lakes experienced recovery cultivation at much smaller scales. Perhaps, the plant genetic variation in the Baoan Lake may not be as natural as that in the other three lakes. Therefore, this study mainly focused on the plant samples from the three lakes but the Baoan Lake.

Vallisneria natans (Lour.) H. Hara (Hydrocharitaceae), native to China, is a cosmopolitan submerged species, found especially in the tropical and subtropical zones (Wang et al., 2010; Zhou et al., 2016). This plant is one of the dominant species in natural submerged communities (Chen et al., 2008; Wang et al., 2010) and one of the pioneer species in the restoration of degraded ecosystems (Xie et al., 2007) in the lakes of the Changjiang River Basin. *V. natans* has a rosette growth form with multiple belt-like leaves and thrives in clear waters (Bornette and Puijalon, 2010). Decreasing light can lower the survival rates of *V. natans* seedlings (Zhou et al., 2016), resulting from a possible sensitive response to changes in water depth and quality. *V. natans* is a dioecious ephydrophilous species (pollination occurs on the surface of the water) (Zhou et al., 2016) and its sexual reproduction is greatly dependent on aquatic environmental conditions. Different aquatic environmental characteristics may favor different alleles in different populations (Slatkin, 1987), hence increasing genetic differentiation. Conversely, similar aquatic environmental conditions and shared selection pressure might facilitate the production of genetically similar populations (Turner et al., 2018). *V. natans* overwinters mainly by seeds and reproduces mainly by sexual seedling in the middle-lower reaches of the Changjiang River (Chen et al., 2008; Wang et al., 2010; Zhou et al., 2016). In the vegetative growth season, new ramets are produced through the extension of stolons. When the stolons break, vegetative propagules are produced. Therefore, dispersal via both seeds and vegetative propagules can contribute to gene flow at the subpopulation and population levels. Sluices may considerably reduce gene flow by hydrochory between lake populations, thus increasing genetic differentiation among isolated lakes. Therefore, changes in lake water environments and in hydrological connections due to human activities may simultaneously affect the genetic variation of *V. natans*.

The aim of our study was (1) to evaluate the genetic diversity of *V. natans* at the subpopulation level in each lake, (2) to identify the pair of lake populations, which has a closer genetic relationship between each other, and (3) to determine what factors (hydrological connection, human disturbance intensity or other factors) may have a relatively greater effect on genetic variation in *V. natans*. We expect that the results will enhance our understanding of the impact of human activities on the genetic variation of submerged plants.

2 MATERIAL AND METHOD

2.1 Plant sample

In June 2017, we sampled 6–8 subpopulations (152–209 individuals) of *V. natans* from each of the Liangzi, the Xiliang, and the Futou lakes (Fig.1). Considering that the Baoan Lake experienced cultivation at a relatively large scale, only one subpopulation (30 individuals) was sampled from that lake. However, since the cultivated plants originated from the lake itself, the samples collected could provide some references for genetic analyses of *V. natans*. The distance between adjacent sampling sites (subpopulations) in each lake was approximately 2 km. Distances between samples within subpopulations were approximately 1.5–2 m. The sample sizes of the subpopulations were 16–37 (Table 2), depending on the areas being continuously covered by this species in the sample sites. In total, we collected 584 samples. One leaf, representing one sample, was taken from each plant. Leaf samples were desiccated with allochroic silica gel and stored until DNA extraction. We recorded the latitude and longitude of each sampling site using GPS. Water transparency and the concentrations of total N and total P in the lake water (Table 1) were measured. They showed the extent of human disturbance in terms of water quality when plant samples were collected.

2.2 DNA extraction and PCR amplification

Total genomic DNA was extracted from dry leaf samples following a modified cetyltrimethylammonium bromide (CTAB) protocol (Saghai-Maroof et al., 1984). Using microsatellite (simple sequence repeats, SSR) primer selection, we found that our samples could amplify bands using all the primers developed by Wang et al. (2011). However, the polymorphism of

Table 2 The parameters of genetic and clonal diversity of *Vallisneria natans* from four lake populations

Subpopulation	N	A	A_e	I	H_o	H_e	P (%)	G	G_e	R	P_g(%)	G_r	N_r	G_s	S_s
Futou Lake															
F1	29	3.667	2.871	1.127	0.391	0.647	100	28	27.1	0.964	96.4	1	2.0	0	0
F2	29	3.667	2.446	1.002	0.310	0.567	100	27	25.5	0.929	92.6	2	2.0	0	0
F3	28	4.000	3.072	1.186	0.417	0.675	100	28	28.0	1.000	100.0	0	0	0	0
F4	27	4.500	2.773	1.169	0.401	0.629	100	27	27.0	1.000	100.0	0	0	0	0
F5	16	3.667	2.864	1.094	0.354	0.631	100	15	14.2	0.933	93.3	1	2.0	0	0
F6	27	4.167	2.523	1.054	0.377	0.582	100	26	25.1	0.962	96.2	1	2.0	0	0
F7	24	3.667	2.499	1.024	0.396	0.594	100	22	20.6	0.913	90.9	2	2.0	0	0
F8	29	3.833	2.457	0.996	0.305	0.573	100	29	29.0	1.000	100.0	0	0	0	0
Mean (SD)	26 (4.4)	3.896 (0.308)	2.688 (0.237)	1.081 (0.074)	0.369 (0.042)	0.612 (0.039)	100 (0)	25 (4.7)	24.6 (4.9)	0.963 (0.035)	96.2 (3.6)	0.9 (0.8)	1.3 (1.0)	0 (0)	0 (0)
Total	209	4.500	3.090	1.244	0.368	0.662	100	202	195.9	0.966	96.5	7	–	0	–
Xiliang Lake															
X1	29	4.333	2.525	1.049	0.397	0.579	100	29	29.0	1.000	100.0	0	0	0	0
X2	27	4.167	1.921	0.870	0.327	0.459	100	26	25.1	0.962	96.2	1	2.0	6	2.7
X3	27	3.667	1.578	0.631	0.290	0.333	83	24	20.8	0.885	90.0	2	2.5	9	2.4
X4	17	3.500	1.789	0.682	0.314	0.362	83	17	17.0	1.000	100.0	0	0	2	2.5
X5	31	4.000	2.013	0.787	0.425	0.420	100	24	18.1	0.767	82.6	4	2.8	1	2.0
X6	32	4.000	2.056	0.877	0.333	0.478	100	31	30.1	0.968	96.2	1	2.0	5	2.4
X7	30	3.667	1.914	0.750	0.272	0.417	100	28	26.5	0.931	92.0	2	2.0	2	3.5
Mean (SD)	28 (5.0)	3.905 (0.302)	1.971 (0.291)	0.806 (0.140)	0.337 (0.055)	0.435 (00.081)	95 (8.3)	26 (4.6)	23.8 (5.2)	0.930 (0.083)	93.8 (6.2)	1.4 (1.4)	1.6 (1.1)	3.6 (3.2)	2.2 (1.1)
Total	193	4.833	2.241	0.978	0.339	0.484	100	164	125.4	0.849	93.9	10	–	12	–
Liangzi Lake															
L1	31	4.333	1.821	0.819	0.258	0.437	100	27	21.4	0.867	92.6	2	3.0	3	2.0
L2	19	3.333	1.854	0.791	0.228	0.451	100	17	14.4	0.889	93.8	1	3.0	2	2.0
L3	37	4.000	1.688	0.705	0.248	0.366	100	27	17.8	0.722	84.0	4	3.5	2	2.0
L4	22	4.000	2.253	0.912	0.364	0.494	100	22	22.0	1.000	100.0	0	0	0	0
L5	24	3.500	1.972	0.762	0.410	0.416	83	24	24.0	1.000	100.0	0	0	2	2.0
L6	19	3.833	1.622	0.644	0.298	0.329	100	16	14.4	0.833	78.6	3	2.0	2	2.0
Mean (SD)	25 (7.2)	3.833 (0.365)	1.868 (0.226)	0.772 (0.093)	0.301 (0.072)	0.416 (0.060)	97 (6.9)	22 (4.8)	19.0 (4.1)	0.885 (0.106)	91.5 (8.6)	1.7 (1.6)	1.9 (1.6)	1.7 (1.0)	1.7 (0.8)
Total	152	4.667	2.192	0.958	0.296	0.490	100	128	91.7	0.841	93.8	8	–	6	–
Baoan Lake															
B1	30	2.833	1.650	0.578	0.133	0.344	100	20	15.0	0.655	70.0	6	2.7	–	–
Mean of 22 subpopulations (SD)	27 (5.3)	3.833 (0.374)	2.189 (0.457)	0.887 (0.185)	0.329 (0.073)	0.490 (0.112)	97.7 (6.0)	24.3 (4.7)	22.4 (5.3)	0.917 (0.097)	93.0 (7.9)	1.5 (1.6)	1.6 (1.2)	1.7 (2.4)	1.2 (1.2)
Total of 22 subpopulations	584	6.667	3.626	1.416	0.328	0.678	100	514	409.9	0.880	94.0	–	–	–	–

N: sample size; A: number of alleles; A_e: number of effective alleles; I: Shannon's information index; H_o: observed heterozygosity; H_e: unbiased expected heterozygosity; P: percentage of polymorphic loci; G: number of MLGs; G_e: effective number of MLGs; R: genotypic richness, $R=(G–1)/(N–1)$; P_g: proportion of single-genet; G_r: number of the MLGs sampled repeatedly; N_r: average number of the ramets per MLG sampled repeatedly in each subpopulation; G_s: number of the MLGs shared among subpopulations; S_s: average number of the subpopulations sharing the same MLG. –: the statistical variable is meaningless.

V. natans primers was lower than that of some primers developed from the congeneric species *V. spinulosa* Yan (Chen et al., 2006) and *V. americana* Michaux (Burnett et al., 2009). Therefore, we finally selected three pairs of primers from *V. spinulosa* (VS01, VS03, and VS29) and three pairs from *V. americana* (Vaam_ATG002, Vaam_AAG_X013, and Vaam_AAG_X051) (Supplementary Table S1), which could amplify highly polymorphic bands.

Amplification of SSR was carried out in 20 μL containing a mix of *Taq* polymerase (0.1 U/μL), dATP, dCTP, dGTP, dTTP (0.4 mmol/L each), and a buffer (ComWin Biotech, Beijing, China). PCRs were performed in a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). A denaturation period of 5 min at 95 °C was followed by 34 cycles of 50 s at 94 °C, 50 s at optimum annealing temperature, 60 s at 72 °C, and for final extension, 10 min at 72 °C. PCR products were analyzed by Tianyi Huiyuan Biotechnology Co. Ltd. (Wuhan, China), and the length of DNA fragments was determined using GeneMarker software (version 1.3; SoftGenetics, State College, PA, USA).

2.3 Data analyses

Vallisneria natans is a diploid species. The DNA fragment lengths of the amplification products were used. Analyses of genetic diversity (A, number of alleles; A_e, effective number of alleles; I, Shannon's information index; H_o, observed heterozygosity; H_e, unbiased expected heterozygosity; P, percentage of polymorphic loci, allele frequency), unbiased genetic identity, principal coordinate analyses (PCoA), and analyses of molecular variance (AMOVA) were performed using GenAlEx 6.5 (Peakall and Smouse, 2012). The highest allele frequency per locus per subpopulation was more than 25%, so the allele frequencies ≥25% were considered as dominant allele frequencies. Pairwise genetic differentiation (F_{ST}) were calculated using the values of the variance component estimates (CV) in AMOVA. Nm, the theoretical number of migrants per generation, was calculated using the equation $Nm=[(1/F_{ST})-1]/4$. Although estimation of Nm via F_{ST} is now generally considered problematic, F_{ST} measures may provide reasonable estimates of Nm in cases where the spatial scale is small (Whitlock and McCauley, 1999). Therefore, among subpopulations within each lake, Nm was used to estimate gene flow.

The genetic identity matrix of subpopulations was used for a cluster analysis according to the upweighted pair-group method with arithmetic averages (UPGMA) using NTSYSpc 2.0 (Rohlf, 1998). Scatter diagrams from the principal coordinate analysis (PCoAs) of individual samples were drawn in SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA).

Individuals with the same multilocus genotype (MLG) were treated as a clone (genet). Clone assignment and clonal diversity analysis (G, number of MLGs; G_e, effective number of MLGs) were performed using GenoType and GenoDive (Meirmans and Van Tienderen, 2004). We calculated the other parameters of clonal diversity: R, genotypic richness, where $R=(G-1)/(N-1)$ and N is the number of samples (Dorken and Eckert, 2001); P_g, proportion of single-genet; G_r, number of the MLGs sampled repeatedly; N_r, average number of ramets per MLG sampled repeatedly in each subpopulation; G_s, number of MLGs shared among subpopulations; S_s, average number of subpopulations sharing the same MLG.

Bayesian cluster analyses of population structure were performed using STRUCTURE 2.3 (Pritchard et al., 2000) to determine the number of genetic clusters, using the admixture model with independent allele frequencies. We tested K in 10 independent runs from 1 to 10 without using sampling location as a prior to assessing convergence of ln$P(D)$ (10 000 burn-in and 100 000 Markov Chain Monte Carlo replicates in each run). We uploaded the files of the project data to the website of Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester/, June 9, 2020) to obtain the distribution of ΔK as a function of K (Earl and vonHoldt, 2012). The best number of clusters was determined based on the ΔK method (Evanno et al., 2005).

3 RESULT

3.1 Genetic diversity and clonal diversity

Generally, the average values of expected heterozygosity (H_e) and genotypic richness (R) in all subpopulations studied were 0.490 and 0.917, respectively (Table 2). Among the lakes studied, the genetic diversity, at the subpopulation level, was the highest in the Futou Lake (mean A_e=2.688, I=1.081, H_e=0.612) and lower in both the Xiliang Lake (mean A_e=1.971, I=0.806, H_e=0.435) and the Liangzi Lake (mean A_e=1.868, I=0.772, H_e=0.416). Only one subpopulation of Baoan Lake had a very low level of genetic diversity (A_e=1.650, I=0.578, H_e=0.344). The total genetic diversity of all subpopulations studied in each lake showed the same trend as the average

Table 3 Analysis of molecular variance (AMOVA) and *F*-statistics of *Vallisneria natans* in three lake populations

Source of variation	d.f.	SSD	CV	% Total	P value	F_{IS}	F_{IT}	F_{ST}	Nm
Futou Lake						0.402	0.450	0.080	2.876
Among subpopulations	7	76.69	0.16	8	<0.001				
Among individuals	201	520.78	0.74	37	<0.001				
Within individuals	209	231.00	1.11	55	<0.001				
Total	417	828.47	2.01						
Xiliang Lake						0.234	0.309	0.098	2.313
Among subpopulations	6	57.16	0.14	10	<0.001				
Among individuals	186	305.09	0.31	21	<0.001				
Within individuals	193	196.50	1.02	69	<0.001				
Total	385	558.74	1.47						
Liangzi Lake						0.287	0.415	0.180	1.142
Among subpopulations	5	76.18	0.27	18	<0.001				
Among individuals	146	234.11	0.36	24	<0.001				
Within individuals	152	135.00	0.89	58	<0.001				
Total	303	445.30	1.52						

d.f.: degree of freedom; SSD: sum of squared deviations; CV: variance component estimates; % total: percentage of total variation. Pairwise genetic differentiation between subpopulations (F_{ST}), inbreeding coefficient within individuals relative to the total (F_{IT}), and inbreeding coefficient within individuals relative to the subpopulation (F_{IS}) were calculated using the values of CV in AMOVA. $Nm=[(1/F_{ST})-1]/4$, the theoretical number of migrants per generation.

Table 4 Pairwise genetic differentiation (F_{ST}) among lake populations of *Vallisneria natans*

	Futou Lake	Xiliang Lake	Liangzi Lake	Baoan Lake
Futou Lake	0			
Xiliang Lake	0.254	0		
Liangzi Lake	0.274	0.205	0	
Baoan Lake	0.259	0.372	0.403	0

All pairwise F_{ST} values were significant at $P=0.001$.

subpopulation genetic diversity (Futou Lake, total H_e=0.662; Xiliang Lake, total H_e=0.484; Liangzi Lake, total H_e=0.490).

The average clonal diversity, at the subpopulation level, was considerably higher in the Futou Lake (mean G_e=24.6, R=0.963) and the Xiliang Lake (mean G_e=23.8, R=0.930), compared to that in the Liangzi Lake (mean G_e=19.0, R=0.885) (Table 2). Differently, the total clonal diversity of all subpopulations in the Xiliang Lake (total R=0.849) was similar to that in the Liangzi Lake (total R=0.841), which was considerably lower than that in the Futou Lake (total R=0.966). Only one subpopulation of Baoan Lake had relatively very low clonal diversity (R=0.655).

The number of the MLGs sampled repeatedly and the average number of ramets per MLG sampled repeatedly was the highest in the subpopulations of the Liangzi Lake (mean G_r=1.7, N_r=1.9), medium in the Xiliang Lake (mean G_r=1.4, N_r=1.6), and the lowest in the Futou Lake (mean G_r=0.9, N_r=1.3) (Fig.1, Table 2). The proportion of single-genet (P_g) was in the converse order to the values of G_r and N_r. The number of MLGs shared among subpopulations and the average number of subpopulations sharing the same MLG was the highest in the Xiliang Lake (mean G_s=3.6, S_s=2.2), but no MLGs were shared among the Futou Lake subpopulations (Fig.1).

3.2 Genetic variation among subpopulations within each lake

The results of AMOVA showed that the genetic variation was much less among subpopulations (8%–18%) than within subpopulations in each lake, especially low among the subpopulations of the Futou and Xiliang Lakes (Table 3). Therefore, the values of Nm, calculated on the basis of subpopulation differentiation, were higher in the Futou Lake (2.876) and the Xiliang Lake (2.313), compared to the Liangzi Lake (1.142).

3.3 Genetic relationships among lake populations

The values of pairwise genetic differentiation among lake populations (F_{ST}) ranged from 0.205 to 0.403 (Table 4). Based on genetic identity, the subpopulations originating from each lake were clustered together (Fig.2). Among the four lake populations, the subpopulations from the Xiliang
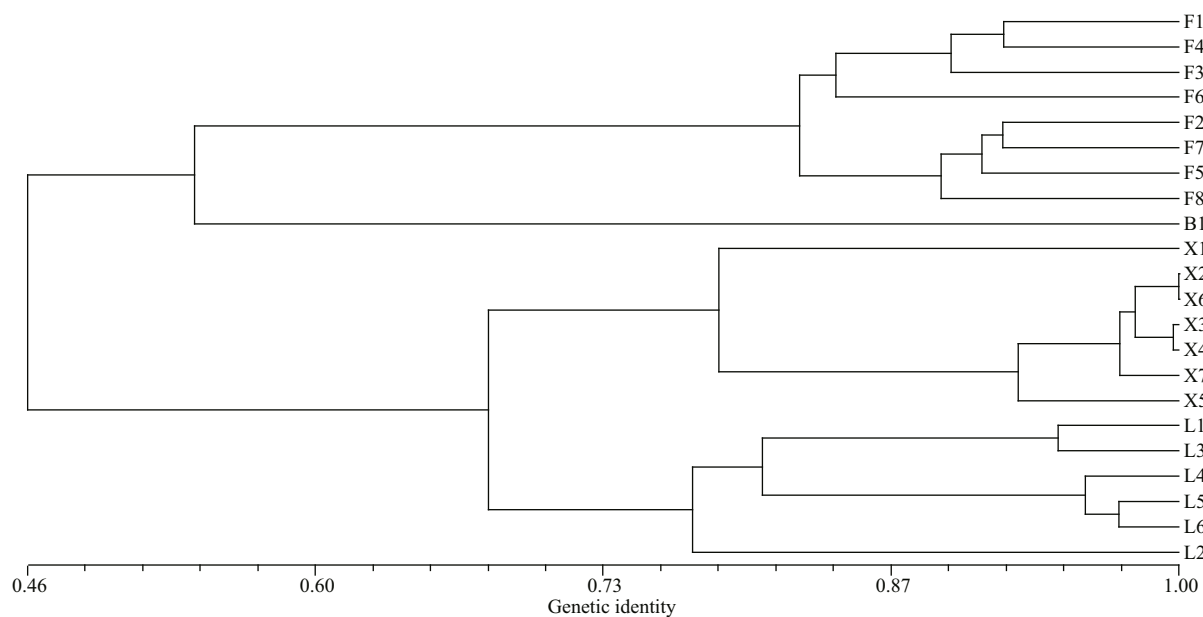
Fig.2 Dendrograms of subpopulations of *Vallisneria natans* from four lakes using Nei's unbiased genetic identity coefficients
F1–F8, Futou Lake; X1–X7, Xiliang Lake; L1–L6, Liangzi Lake; B1, Baoan Lake.
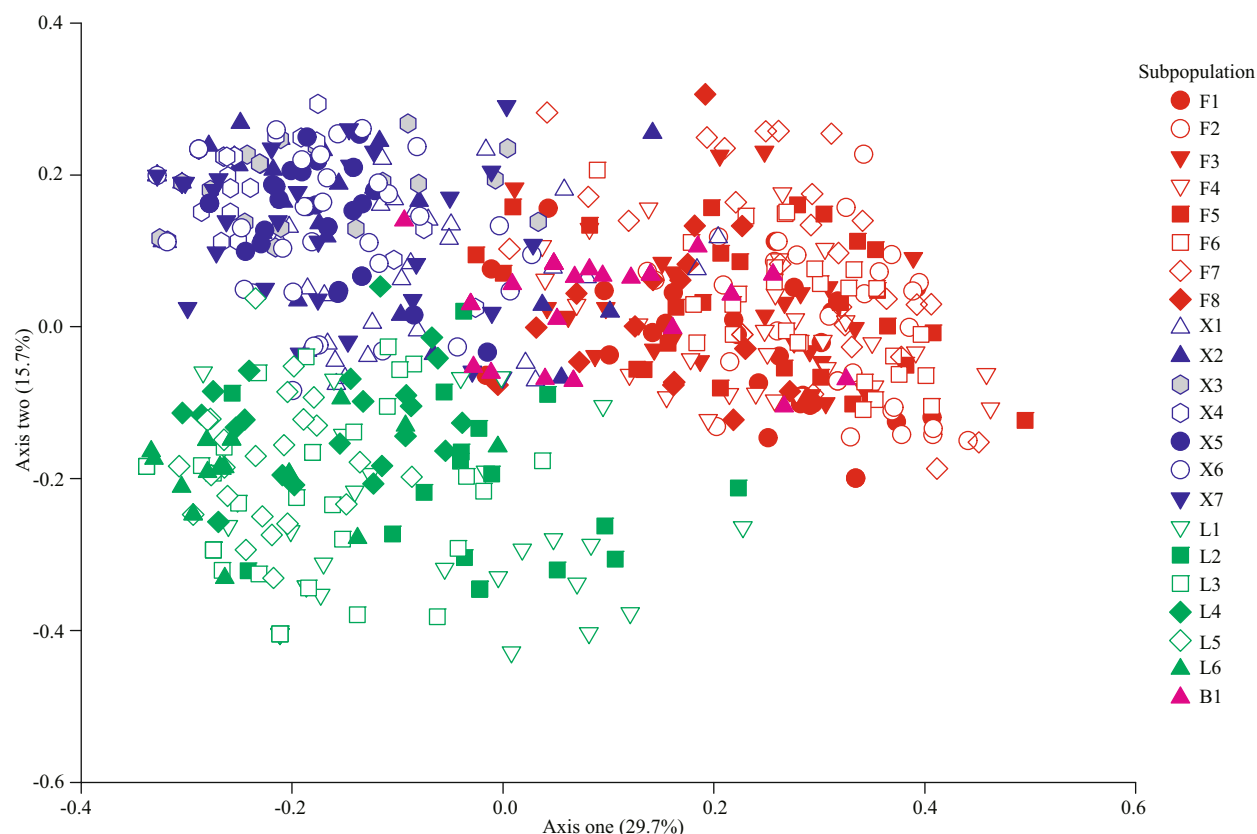

Fig.3 Scatter plots of principle coordinate analyses (PCoA) for individuals of *Vallisneria natans* in four lakes
Subpopulation F1–F8, Futou Lake; X1–X7, Xiliang Lake; L1–L6, Liangzi Lake; B1, Baoan Lake.

Lake and from the Liangzi Lake had relatively close genetic relationships, while only one subpopulation of the Baoan Lake was clustered together with the Futou Lake subpopulations.

According to the PCoA results, the individuals originating from each lake tended to have close genetic relationships (Fig.3). Along axis one, which explained 29.7% of the total genetic variation, the
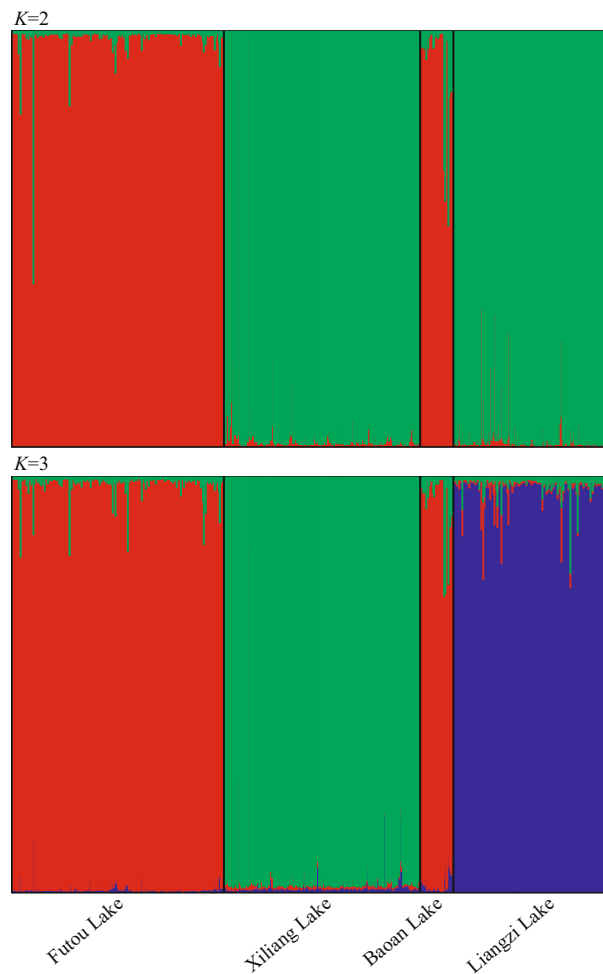
K=2

K=3

Fig.4 Estimated genetic structure of *Vallisneria natans* populations in four lakes, inferred by a Markov chain Monte Carlo clustering (STRUCTURE) at the individual level

Black lines indicate different population origins.

individuals from the Futou Lake (severely disturbed) were different from those of the Liangzi and Xiliang Lakes (mildly disturbed). All individuals from the Baoan subpopulation were more or less within the range of Futou Lake individuals.

The Bayesian cluster analyses suggested *K*=2 as the optimal number of clusters based on the value of Δ*K* (Supplementary Fig.S1). The individuals of the Xiliang and Liangzi Lakes mainly belonged to a genetic cluster different from those of the Futou and Baoan Lakes (Fig.4). The result of *K*=3 was also presented due to the high value of Δ*K*. When *K*=3, the individuals of the Futou and Baoan Lakes still belonged to the same cluster, but those of the Xiliang Lake were genetically differentiated from those of the Liangzi Lake.

The analyses of the allele frequency showed that the dominant allele frequencies (≥25%) were generally similar among the subpopulations within each lake (Table 5). Among the four lakes, the dominant allele frequencies of Xiliang subpopulations exhibited a similarity to those from the Liangzi Lake at some loci, e.g. VS01, VS03 and Vaam_AAG_ X013. Additionally, the Liangzi population had the most private alleles.

4 DISCUSSION

4.1 Genetic diversity, clonal diversity, and genetic variation within populations

The values of genetic diversity for all subpopulations of *V. natans* studied in the present study (mean H_e=0.490, total H_e=0.678, 584 individuals) were higher than those detected in 196 individuals from six lake populations of this species (mean H_e=0.24, total H_e=0.32) in the middle-lower reaches of the Changjiang River using inter-simple sequence repeat (ISSR) primers (Wang et al., 2010). The difference in values may be related to the higher variability of SSR markers used in the present work than when using ISSR markers, in addition to our larger sample size. Compared with the congeneric *V. americana* in Chesapeake Bay (mean H_e=0.53, mean *R*=0.57) (Lloyd et al., 2011), *V. natans* in the four lakes studied in this work had slightly lower genetic diversity but higher clonal diversity for all subpopulations (mean *R*=0.917). Lloyd et al. (2011) used the same type of molecular markers (SSR) and a comparable sample size (mean *N*=26) as our study (mean *N*=27) but larger distances between individual samples (5–10 m) within each site. The high clonal diversity in this study indicates smaller clone size and less vigorous clone growth in *V. natans* than in *V. americana*. The limited clone growth of *V. natans* agrees with the previous finding that in natural populations only a low proportion of *V. natans* plants can produce a few ramets (Chen et al., 2008).

In the Futou Lake, which was severely disturbed by humans, *V. natans* exhibited the highest genetic diversity at the subpopulation (mean H_e=0.612) and the total lake population (total H_e=0.662) levels among the four lakes. However, the genetic diversity of *V. natans* was low but similar in the Xiliang (mean H_e=0.435, total H_e=0.484) and the Liangzi (mean H_e=0.416, total H_e=0.490) Lakes, and both were affected by mild disturbance. This result is consistent with the influence of environmental disturbances on the seagrass *Zostera marina* as reported by Kim et al. (2019), where the highest genetic diversity was

Table 5 The frequency of dominant alleles (frequency ≥25%) in each subpopulation and of private alleles in each lake population at each SSR locus of *Vallisneria natans* (the values ≥50% in bold)

Locus Allele	The frequency of dominant alleles (frequency ≥25%) in each subpopulation																						The frequency of private alleles in each lake population			
	F1	F2	F3	F4	F5	F6	F7	F8	B1	X1	X2	X3	X4	X5	X6	X7	L1	L2	L3	L4	L5	L6	Futou	Baoan	Xiliang	Liangzi
Vaam_ATG002																										
A	**0.60**	**0.53**	0.29	0.31	0.34		0.31	0.36	**0.98**								**0.69**	**0.76**	0.38							
B		0.27			0.35					0.36					0.33	0.25				**0.50**	0.42	0.47				
C	0.28			0.35	0.47		0.42	0.34		**0.53**	**0.76**	**1.00**	**1.00**	**0.81**	**0.64**	**0.73**	0.29		0.47	0.45	0.31	0.32				
E																										0.04
Vaam_AAG_X013																										
B		0.25								0.47																
C	0.29	0.34	0.39	0.33	0.41		0.43													0.25						
D	**0.52**	**0.53**	0.36	**0.59**	0.38	**0.65**	**0.65**	**0.55**	**0.53**	**0.66**	**0.76**	**0.76**	**0.71**	**0.74**	**0.75**	**0.85**	**0.85**	**0.84**	**0.93**	**0.84**	**0.65**	**0.89**				
Vaam_AAG_X051																										
A	**0.53**		0.48	0.43		0.33																				
B																	**0.65**	**0.58**	**0.73**	**0.61**	**0.73**	**0.87**				**0.69**
C		0.45				0.46	0.48	0.48	**0.87**	0.36	**0.80**	**0.85**	**0.79**	**0.79**	**0.75**	**0.78**										
D							0.42																		0.13	
E										0.48																
H																										0.05
J					0.38																					
K																										0.04
VS01																										
A			0.48		0.25			0.52	**0.87**																	
B										0.33	**0.67**	**0.81**	**0.82**	**0.55**	**0.72**	**0.87**	**0.82**	**0.74**	**0.78**	**0.50**	**0.90**	**0.74**				
C	**0.52**	**0.50**	0.30	**0.70**	0.38	**0.74**	0.44																			
D																										
E		0.34			0.28		0.27																			
F														0.27											0.05	
G									**0.52**																	
VS03																										
A		**0.84**	0.30	**0.56**	**0.81**	**0.65**	**0.73**	**0.81**																	**0.61**	
B										0.38																
C	0.33		0.32			0.26			0.42																	
D									0.45		**0.54**	**0.65**	0.47		0.45	0.37		**0.68**		0.43	**0.54**	**0.79**				
E			0.34											0.34												
F														0.34			**0.71**		**0.73**							
G																				0.25						
H																		0.28			0.25					
VS29																										
A	0.33		0.36	0.44	0.25				**0.53**	**0.73**	**0.79**	**0.74**	**0.70**	**0.82**	**0.97**	**0.77**	**0.67**	**0.53**	0.26	**0.91**	**0.93**	**1.00**				
B					0.37	0.44																				
C	0.45	**0.62**	**0.55**	0.43	**0.53**	**0.54**	0.48	0.43									0.30	0.40	0.66							

The alleles whose frequency is less than 25% are not shown at the subpopulation level.

detected in the population with the greatest disturbances. In fact, with disturbances, especially anthropogenic ones, the general plant response is an increase in sexual reproductive effort, thus increasing genetic diversity to confer higher resilience to plants under unfavorable conditions (Reusch, 2006; Cabaco and Santos, 2012; Kim et al., 2019). The contribution of sexual reproduction of *V. natans* in the severely disturbed Futou Lake is supported by very few MLGs sampled repeatedly (mean G_r=0.9), a high proportion of single-genet (mean P_g=96.2%), and high genotypic richness (mean R=0.963) in the subpopulations. When collecting samples, we observed that *V. natans* had a lower plant density and produced fewer ramets in the Futou Lake compared to the Xiliang and Liangzi Lakes. Relatively stable aquatic environments in mildly disturbed lakes facilitate the production of more ramets, such as seagrass *Z. Marina* (Kim et al., 2019). In contrast, unstable environments in the severely disturbed lake repress the formation of dense ramets but provide opportunities for seedling recruitment (Reusch, 2006), if the aquatic environment can meet the growth and reproduction needs of *V. natans*. Additionally, the diversity level differences between the populations might be affected by recovery cultivation, but the effect may be limited to a very small extent. In the Futou Lake, strong gene flow (Nm=2.876) among subpopulations may also be a contributor to the high genetic diversity. Moreover, gene exchanges mainly depend on the movement of sexual propagules and even pollens in terms of no MLGs shared (G_s=0) among subpopulations in that lake. As an annual plant in the region studied (Chen et al., 2008), *V. natans* overwinters by seeds and can produce a great quantity of seeds (321 seeds per fruit, 5 fruits per female flowering ramet, Zhou et al., 2016). Fruits of *Vallisneria* species can float in water after detachment from the mother plants (Liu et al., 2005) and hence, offering a wider dispersal range. Being a species exhibiting epihydrophily, the large number of pollen produced by *V. natans* also contribute to the species' dispersal through water by migrating within the populations (Wang et al., 2010), although the distances travelled by the pollen are limited to several meters (Lloyd et al., 2018). However, *Vallisneria* fruits contain seeds sired by many different fathers (seven fathers on average) (Lloyd et al., 2018) and contribute to increasing genetic diversity at the subpopulation level.

In the mildly disturbed Liangzi Lake, *V. natans* had considerably low clonal diversity at the subpopulation (mean R=0.885) and total lake population (total R=0.841) levels, compared to the Futou Lake. *V. natans* plants from the Liangzi Lake had larger clone sizes (G_r=1.7, N_r=1.9) and a lower proportion of single-genet (P_g=91.5%) than those from the Futou Lake. In less disturbed environments, submerged *V. natans* plants produce larger clones and more ramets, like the seagrass *Enhalus acoroides*. In a study carried out by Yu et al. (2019), *E. acoroides* had the largest clone with only three ramets in a seriously disturbed plot, while in a less disturbed plot, 10.9% of the genets contained more than three ramets and the largest clone covered about 15 m². Large clones may decrease the opportunity for seedling recruitment, thus decreasing genetic diversity within populations, reducing gene flow (Nm=1.142 in the Liangzi Lake), and enlarging differentiation among subpopulations to a certain degree.

The clonal diversity of *V. natans* in the Xiliang Lake (mean R=0.930, total R=0.849) was almost as high as that in the Futou Lake at the subpopulation level, but as low as that in the Liangzi Lake (total R=0.841) at the total lake population level. The difference in genotypic richness between the two levels in the Xiliang Lake may be explained by relatively strong gene exchanges via vegetative propagules among subpopulations (mean G_s=3.6, S_s=2.2, Nm=2.313). Although *V. natans* does not produce overwintering turions (Chen et al., 2008; Zhou et al., 2016), ramets produced by stolon disruption in the vegetative growth season can migrate via hydrochory within populations.

Only one subpopulation of *V. natans* in the Baoan Lake had very low genetic (H_e=0.344) and clonal (R=0.655) diversity and a very low proportion of single-genet (P_g=70%), indicating that restoration experience may decrease genetic diversity at a subpopulation level.

Therefore, under severely disturbed conditions, *V. natans* populations are maintained mainly by sexual reproduction through the production of genetically new individuals and continuous seedling recruitment, while under relatively mildly disturbed conditions, the contribution of vegetative reproduction plays an ineligible role in population dynamics and expansion, consistent with the performance of some seagrass species (Cabaco and Santos, 2012; Kim et al., 2019). Consequently, human disturbance might increase the genetic diversity of *V. natans* at the subpopulation level to a certain extent.

4.2 Genetic relationships among lake populations

Based on the genetic identity at the subpopulation level and the PCoA and Bayesian cluster analyses at the individual level, the samples originating from each lake were clustered together, implying genetic differentiation occurring among lake populations of *V. natans*. This tendency was also supported by the analyses of dominant allele frequency. The result is consistent with other studies on genetic variation in natural populations of submerged plants (Li et al., 2004; Wang et al., 2010; Han et al., 2014; Cao et al., 2017). As Laushman (1993) considered, lake habitats of submerged plants are 'islands' in a terrestrial 'sea', so little gene exchange would be expected between the habitats, and founder effects and genetic drift would be more pronounced under such conditions. Therefore, a great isolation may be observed among freshwater lake populations relative to most terrestrial species and coastal species (Laushman, 1993; Reusch, 2002; Yu et al., 2019).

Among the lakes, the subpopulations of *V. natans* from the Xiliang and the Liangzi Lakes, disturbed to a similarly mild extent, had a relatively close genetic relationship. Only one subpopulation of *V. natans* from the Baoan Lake was clustered together with those of the Futou Lake, which was severely disturbed. Furthermore, at the individual level, Bayesian cluster analyses showed a similar trend (K=2). The results indicate that the intensity of human disturbance might differentiate lake populations of *V. natans* under similar climatic conditions. Different intensities of disturbance in lake environments might produce different selective pressures on submerged plants; that is to say, the populations in the relatively mildly disturbed lakes (Xiliang and Liangzi Lakes) differentiate genetically from those in severely disturbed lakes (Futou and Baoan Lakes). For instance, nutrient contents in water can impact the competitive capability among plants (Bornette and Puijalon, 2010), and high nutrient levels in severely disturbed lakes might favor competitive clones while low nutrient levels in mildly disturbed lakes might favor tolerators. Natural selection favoring adaptations to local environmental conditions leads to genetic differentiation of local populations (Slatkin, 1987). This selective force due to different extents of human disturbance seems to be so strong that it outperforms the force of historical and current gene flow tending to produce genetic homogeneity (Slatkin, 1987) between neighboring lake populations with direct interconnecting waterways (Committee for Lake Records of Hubei Province, 2014). In addition, gene flow by bird-mediated dispersal might contribute to genetic relationships among freshwater populations (Chen et al., 2009; Cao et al., 2017). The close plant genetic relationship between the Liangzi and Xiliang Lakes might benefit from more frequent bird inter-migration, for the two lakes; more diverse aquatic vegetation attracts and sustains more birds (Peng et al., 2004; Yang et al., 2017).

The F_{ST} values between the neighboring lake populations were more than 0.25 (0.254 between the Futou and Xiliang Lakes; 0.403 between the Liangzi and Baoan Lakes), indicating very large differentiation between the historically directly interconnected lakes (Wright, 1978). Our observed pairwise population differentiation (F_{ST} 0.205–0.403) was higher than that estimated by Wang et al. (2010) for *V. natans* (F_{ST}=0.132) and *V. spinulosa* (F_{ST}=0.202), and by Chen et al. (2007) for *V. spinulosa* (F_{ST}=0.06), all of which were sampled from lakes of the middle-lower reaches of the Changjiang River. The increase in F_{ST} values over time might be related to the growing reduction of hydrological connectivity (Chen et al., 2009) between the sampled lakes. Wang et al. (2010) also considered that more divergence among *Vallisneria* populations would occur with further reduction in connectivity. Similar to the findings of Wang et al. (2010), no widespread MLG shared among lakes was detected in the present study, further suggesting that water-dispersed propagules are rarely transferred between populations and the linkage by hydrological connection between populations is becoming weak.

5 CONCLUSION

Our results showed that for freshwater-submerged *V. natans*, human disturbance decreased plant density and clonal growth but might increase the genetic and clonal diversity at a subpopulation level and enhance gene flow among subpopulations by sexual propagule movement. Under similar climatic conditions, different intensities of disturbance seem to have such a high selective potential to differentiate genetically adjacent lake populations that they outperform the forces of gene flow through historical direct hydrological interconnection, which tends to produce genetic homogeneity. Our findings imply that human disturbance has a profound effect on the evolutionary process of natural populations of submerged plants. Increased subpopulation genetic diversity, especially by increasing sexual reproduction, can enhance

resistance and resilience to environmental disturbances because subpopulations that are repeatedly damaged due to disturbance can rapidly reestablish sexually via seed germination. To a certain degree, we could expect that disturbed populations have the possibility of restoring spontaneously if humans cease to perturb natural ecosystems in the future.

6 DATA AVAILABILITY STATEMENT

Data of microsatellite (SSR) primer pairs used for DNA amplification in this study and the value of ΔK in Bayesian cluster analyses (STRUCTURE) are included in the supplementary information files of this published article. Data on allele information of *Vallisneria natans*, generated and analyzed during the current study, are available from the corresponding author upon reasonable request.

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Electronic supplementary material

Supplementary material (Supplementary Table S1 & Fig.S1) is available in the online version of this article at https://doi.org/10.1007/s00343-020-0277-1.