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Research Article

Genetic differentiation of *Wolffia globosa* in China

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Abstract *Wolffia* Horkel ex Schleid. (Lemnaceae) includes the world's smallest angiosperms. Morphologically, their bodies are extremely simplified, making classification difficult and long-disputed. No reports about the genetic structure of these clonal-dominant aquatic plants have been published until now. In this study, of 247 samples from 42 populations spanning representative locations in China, two chloroplast haplotypes (*glo-cp* and *un-cp*) and 66 amplified fragment length polymorphism genotypes were identified. Based on wide sampling, cpDNA haplotypes, and amplified fragment length polymorphism profiles, we found that there are two genetic lineages (*glo-* and *un-* lineages) of *Wolffia* species in China. Genotypic and genetic diversity of *Wolffia* species are high compared with other clonal plants (Simpson's index, $D = 0.97$; Nei's diversity, $H = 0.1835$). Different spatial structure patterns were detected between *glo-* and *un-* lineages. Positive autocorrelation at short distances (<400 km) and slightly negative autocorrelations at larger distances (>1500 km) were detected within the *glo*-lineage, but no significant spatial genetic structure was detected beyond 100 km within the *un*-lineage. Overall, spatial genetic analysis of *W. globosa* revealed significant autocorrelation within short distances, indicating that restricted gene flow might be one of the most important factors in shaping genetic structure.

Key words AFLP, chloroplast DNA, genetic differentiation, spatial genetic structure, *Wolffia*.

Species of *Wolffia* Horkel ex Schleid. are the world's smallest angiosperms and important freshwater plants with aquaculture applications (Skillicorn et al., 1993). The plants are extremely reduced, and the whole bodies consist merely of "fronds"—simplified leaves with a size less than 1 mm (Landolt, 1986; Bernard et al., 1990). Only a few characters are available for comparison in these plants, such as size, shape, and pigmentation of the fronds, making taxonomic identification difficult. Based on limited morphological and anatomical characters, Landolt (1986, 1994) classified the genus into 11 species. Although there is the deficiency of distinguishing morphological characteristics in *Wolffia*, strong genetic divergence was detected in this genus based on allozyme and chloroplast DNA analyses (Crawford & Landolt, 1995; Les et al., 2002). For the *Wolffia* flora of China, species identification was often controversial due to different authors focusing on different characters. Li (1979) recognized one species, *W. arrhiza* (L.) Horkel ex Wimm., in *Flora Reipublicae Popularis Sinicae*. Based on morphological characters and chloroplast *matK* sequences, Huang (2007) found two *matK* haplotypes of the Chinese *Wolffia* species, one identical to the published sequence of *W. globosa* by Les et al. (2002); the other represented a newly found haplotype. Therefore, the use of more molecular markers, particularly more informative chloroplast DNA sequences, to settle the questionable taxonomic identification in *Wolffia* plants has become necessary.

The genetic structure of *Wolffia* species is also an attractive issue, because of the unique life features and breeding system of *Wolffia*. As an extremely small angiosperm, it propagates clonally as rapidly as doubling within 1 or 2 days (Landolt, 1986; Jordan et al., 1996). However, whether the plants hold sexual reproduction is mysterious, as flowering of the plants occurs very locally and rarely (Crawford et al., 1996). Additionally, although some authors speculated that the aquatic plants, free-floating on the surface of freshwater, might disperse by way of birds, fish stocks, or hurricanes (Landolt, 1986; Barrett et al., 1993; Crawford & Landolt, 1993), they have not identified the dispersal model, the genetic diversity, or the spatial genetic structure.

Molecular markers have shown potential in revealing genetic structure, involving genetic diversity, mating, and breeding systems. In the present study, the genetic structure of Chinese *Wolffia* plants was investigated on large-scale sampling using molecular markers. The molecular markers applied are amplified fragment

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length polymorphism (AFLP), a powerful DNA marker with the advantages of high resolution and reproducibility, and suitable for assessing the genetic structure of aquatic clonal plants, particularly when the genomic sequences are unknown (Lamote et al., 2002; Lamote et al., 2005; Lambertini et al., 2008; Bog et al., 2010).

Summarily, we investigated the genetic differentiation of Chinese *Wolffia* species by using cpDNA sequences and AFLP markers in the present study, focusing on: (i) genetic differentiation of *Wolffia* in China; (ii) genotypic and genetic diversity levels of *Wolffia* in China; and (iii) the spatial genetic structure pattern of *Wolffia* species in China.

1 Material and methods

1.1 Plant material

Wolffia species are distributed over almost the whole of China, except the northwestern regions. In this study, 42 ponds were sampled, covering most distribution regions in China from the southern island, Hainan Island, to the northeastern province, Jilin (18.25–43.88°N, 100.48–125.35°E). The collections were divided into seven groups according to different geographic districts in China. They are the northeast group (NE), north group (NC), east group (EC), central group (CC), southwest group (SW), south group (SC), and Hainan group (HN). The latter group (HN) is geographically separated from the SC group by the Qiongzhou Strait (Fig. 1, Table 1).

Sampling was randomly carried out around each target pond, and individuals from the same pond were considered as belonging to the same population. Apart from the Chinese *Wolffia* species, four plant materials, kindly provided by Elias LANDOLT (Geobotanical Institute ETH, Zurich, Switzerland) including *W. arrhiza* (L.) Horkel ex Wimm., *W. neglecta* Landolt, *W. australiana* (Benth.) Hartog & Plas, and *W. cylindracea* Landolt, were also involved in the study.

Aseptic *Wolffia* strains were established under laboratorial conditions. The fronds were treated with 1% sodium hypochlorite solution (NaClO) for 3 min, then single fronds were picked up separately and cultured as a monoclonal line in Hoagland's medium (Hoagland & Arnon, 1950) with a slight modification, in that the K^+ concentration was increased to 0.01 mol/L.

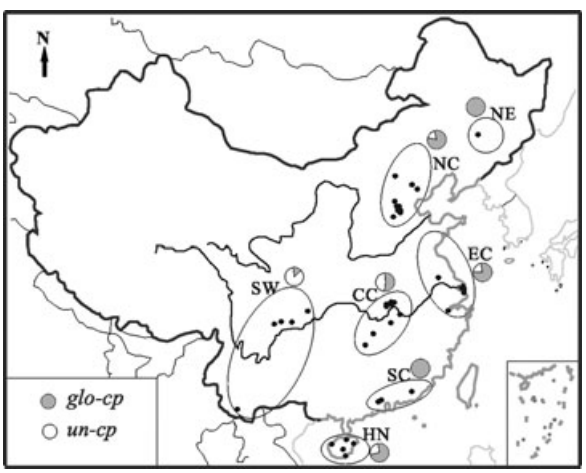

Fig. 1. Collection sites of populations of *Wolffia* species in China (indicated by black dots). The pie charts indicate the distribution of two cpDNA haplotypes, *glo-cp* (light grey) and *un-cp* (white). The source of the base map was www.onegreen.net/maps/m/china.htm. CC, Central China; EC, East China; HN, Hainan; NC, North China; NE, Northeast China; SC, South China; SW, Southwest China.

Table 1 Locations, population symbols, cultured clone numbers (N) and identified genotypes (G) of *Wolffia* collected in China

Group	Population	Latitude (°N)	Longitude (°E)	N	G
NE	JL1	43.88	125.35	7	2
NC	TJ1	39.40	117.05	3	3
	BJ1	39.92	116.46	10	1
	HB1	39.44	116.29	3	1
	HB2	39.20	116.32	2	1
	HB3	39.12	116.38	3	2
	HB4	39.12	116.38	4	2
	HB5	38.87	116.45	3	1
	HB6	38.72	116.08	2	1
	HB7	39.76	117.00	4	1
	HB8	39.76	117.00	3	1
	HB9	39.76	117.00	3	2
	HB10	39.90	117.90	3	1
EC	ZhJ1	30.26	120.19	3	1
	JS1	32.39	119.42	10	1
	SH1	31.22	121.48	3	1
	SH2	31.22	121.48	1	1
	SH3	31.22	121.48	3	2
	SH4	31.22	121.48	4	1
	SH5	31.22	121.48	12	2
CC	HuB1	30.52	114.31	5	2
	HuB2	30.52	114.31	4	2
	HuB3	30.52	114.31	11	2
	HuB4	30.52	114.31	12	3
	HuB5	30.52	114.31	5	1
	HuB6	30.38	114.87	3	1
	HuB7	29.87	114.28	4	1
	HuN1	28.21	113.00	10	1
	HuN2	29.37	113.09	5	2
SW	SCh1	29.36	103.44	2	2
	SCh2	29.36	103.44	4	1
	SCh3	29.23	104.46	6	2
	CHQ1	29.59	106.54	4	1
	YN1	22.01	100.48	12	1
SC	GD1	23.78	116.18	2	1
	GD2	23.05	113.11	10	2
	GD3	23.16	113.23	10	2
HN	HAN1	19.61	110.72	10	2
	HAN2	19.52	109.57	10	2
	HAN3	19.05	109.83	10	2
	HAN4	18.25	109.51	12	3
	HAN5	19.98	110.33	10	2

Sampling groups: CC, Central China; EC, East China; HN, Hainan; NC, North China; NE, Northeast China; SC, South China; SW, Southwest China.

1.2 Chloroplast DNA amplification and sequencing

Total genomic DNA from each monoclonal line was extracted using the CTAB method (Doyle & Doyle, 1987). The chloroplast DNA sequences chosen were *matK*, *rpl16* intron, *trnT-trnL*, *trnS-trnfM*, and *atpB-rbcL*. All the primers for polymerase chain reaction (PCR) amplification are listed in Appendix Table S1. The PCR program began with initial denaturation for 3 min at 95 °C, followed by 37 cycles each for 45 s at 94 °C, 45 s at 55 °C, and 1 min at 72 °C, and finished by an extension step at 72 °C for 10 min. The PCR amplified fragments were sequenced on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

1.3 Amplified fragment length polymorphism procedure

The AFLP procedure was carried out according to Vos et al. (1995) with little modification. Genomic DNA (approximately 0.2 μg) was digested by the restriction enzymes *Eco*RI and *Mse*I, and ligated with *Eco*RI and *Mse*I adapters at 37 °C for 6 h. Preselective amplifications were carried out using the prime combination *Eco*RI-A/*Mse*I-C, and seven selective amplification primer pairs were finally selected for analysis, *Eco*RI-AAG/*Mse*I-CAA, *Eco*RI-AAG/*Mse*I-CTC, *Eco*RI- AGA/*Mse*I-CTC, *Eco*RI-AGA/*Mse*I-CAA, *Eco*RI-AGA/ *Mse*I-CTT, *Eco*RI-ACG/*Mse*I-CTC, and *Eco*RI-AAC/*Mse*I-CTC. Primer sequences (without selective nucleotides) were *Eco*RI (5′-GACTGCGTACCAATTC-3′) and *Mse*I (5′-GATGA GTCCTGAGTAA-3′).

The AFLP fragments were separated by electrophoresis on 4% denaturing polyacrylamide gel and visualized by silver staining (Bassam et al., 1991). Only clear and reproducible AFLP fragments were scored as presence (1) or absence (0) to generate a binary matrix.

1.4 Analysis of chloroplast sequences

Chloroplast DNA sequences were aligned by CLUSTAL X version 1.81 (Thompson et al., 1997), with manual adjustment. The cpDNA sequences of other *Wolffia* species were available in GenBank (Accession Nos. AY034210–AY034220 and AY034287–AY034297). Two species from the most closed alien genus *Wolffiella* were used as outgroups (GenBank Accession Nos. AY034200, AY034202, AY131184, and AY131185).

The pairwise estimates of nucleotide divergence between sequences were calculated in MEGA4 (Tamura et al., 2007). Maximum parsimony, as implemented in PAUP* version 4.0b10 (Swofford, 2002) was used to infer phylogenies based on nucleotide substitutions in aligned sequences. Heuristic searches were carried out with 100 random addition sequence replicates, tree bisection reconnection (TBR) branch wrapping and MULTREES option. Bootstrap analysis was carried out by 1000 replicates of heuristic search with TBR branch swapping, ACCTRAN optimization, and random taxon addition.

1.5 Analysis of AFLP data

For clonal identification, individuals showing identical AFLP band patterns were considered as the ramets from one genotype. The genotypic diversity was evaluated by Simpson's index of diversity, which was developed to estimate the probability that two sample units chosen at random from the sample population would belong to the same clonal lineage (Arnaud-Haond et al., 2007). The index of diversity was given by $D = 1 - \{[\sum \mathrm{ni}\ (\mathrm{ni} - 1)] / [\mathrm{N}(\mathrm{N} - 1)]\}$, where ni is the number of samples of genotype i and N is the total number of samples (Pielou, 1969); D ranges from 0 (where the population is composed of merely one genotype) to 1 (where every sample is a different genotype). The genetic diversity of the Chinese *Wolffia* at the genet level was estimated by the percentage of polymorphic fragments (P) and Nei's gene diversity (H) using POPGENE 3.2 (Yeh et al., 1999).

The genetic relationship among different AFLP genotypes was estimated by a UPGMA tree using PAUP* version 4.0b10 (Swofford, 2002). STRUCTURE 2.2 (Pritchard et al., 2000) was used to estimate the number of genetic clusters (K) based on Bayesian cluster analysis. For each value of K from 1 to 13, we ran the program 10 times using a burn-in length of 50 000, followed by 50 000 iterations. All simulations were run using the admixture model without prior population information and the correlated allele frequencies. The distribution of posterior probabilities ln Pr (X/K) was used as a pointer in selecting the "correct" value of K.

Nei's genetic distance and pairwise Φst distance between sampling groups were also calculated, and genetic relationships between the sampling groups were also estimated by UPGMA. With the use of genetic and geographic distance matrices, we carried out a spatial autocorrelation analysis using GenAlEx version 6.1 (Peakall & Smouse, 2006). The autocorrelation coefficient (r) ranges from −1 to 1, with 0 indicating that autocorrelation does not differ from random. A random permutation calculation was executed (N = 999) to generate a 95% confidence interval for all distance class intervals. We present the autocorrelograms with variable distance classes that span the full range of geographic distance among our samples.

2 Results

2.1 Analysis of chloroplast sequences

The analysis of five chloroplast DNA sequences gave the result that only two haplotypes (*glo-cp* and *un-cp*) were found after screening all collected *Wolffia* populations in China. Geographical distribution and frequencies of two chloroplast haplotypes are also shown (Fig. 1).

By aligning the two haplotypes (GenBank Accession Nos. of cpDNA sequences GU722161–GU722168 and GU983039) and haplotypes of *Wolffia* species, the *glo-cp* was identified to be exactly the same as the haplotype of *W. globosa*, whereas the *un-cp* was a new haplotype. The aligned chloroplast DNA sequence matrix has 3498 characters, containing 366 variable sites (10.5%) with 176 parsimony informative sites (5%). The *un-cp* and three *Wolffia* species, *W. globosa*, *W. neglecta*, and *W. angusta* Landolt, formed a clade with strong bootstrap support (100%), but the phylogenetic relationship between *un-cp* and the three species was not resolved (Fig. 2).

2.2 Analysis of genotypic and genetic diversity

Of 247 Chinese *Wolffia* samples, 66 distinct genotypes were identified based on AFLP fingerprints. The overall Simpson's index of diversity was $D = 0.97$, and Nei's diversity $H = 0.1835$ is shown at the genet level (Table 2). Genotypic and genetic diversity of *Wolffia* species with two different cpDNA haplotypes showed similar levels.

Table 2 Statistical analysis of genotypic and genetic diversity of *Wolffia* species with two cpDNA haplotypes, *glo-cp* and *un-cp*

Group	Genotypic diversity			Genetic diversity	
	N	G	*D*	*P*	*H*
glo-cp	161	45	0.97	0.50	0.1599
un-cp	86	21	0.94	0.55	0.1243
Total	247	66	0.97	0.652	0.1835

D, Simpson's index of diversity; G, Number of unique genotypes identified; *H*, Nei's gene diversity; N, Number of samples; *P*, percentage of polymorphic fragments.

The AFLP selective primer pairs amplified a variable number of bands, from 28 to 50. The percentage of polymorphic bands between primer pairs varied from 53.1% to 79.1%. The total number of bands was 296, of which 193 were polymorphic (65.2%). Additionally, AFLP band patterns (Fig. 3) of each primer pair showed that the *un-cp* individuals were much more similar to

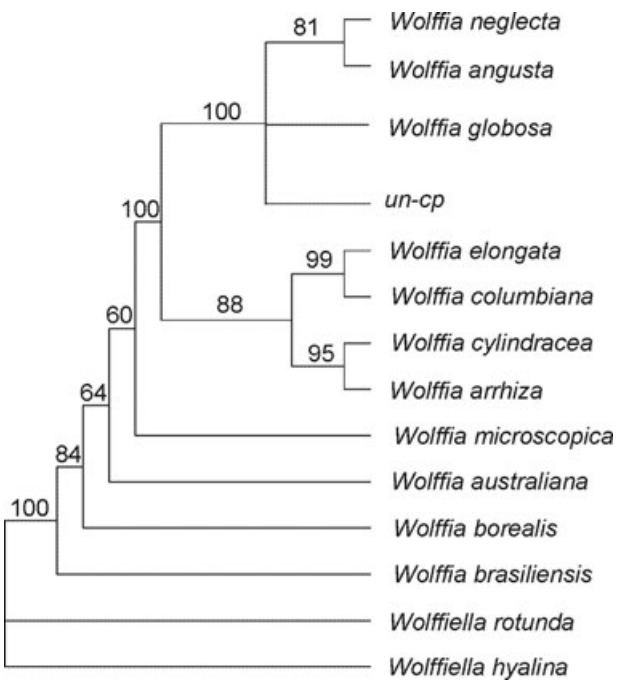

Fig. 2. Maximum parsimony cladogram of *Wolffia* species derived from chloroplast sequence (strict consensus trees shown). Bootstrap support for nodes is indicated above branches.

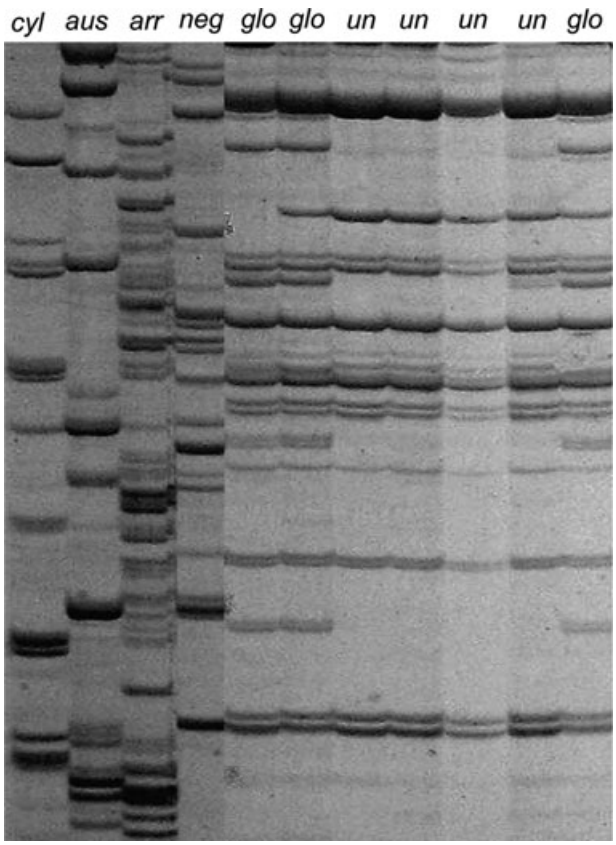

Fig. 3. Examples of amplified fragment length polymorphism fingerprints with primer pair *Eco*RI-AAG/*Mse*I-CAA. *arr*, *Wolffia arrhiza*; *aus*, *W. australiana*; *cyl*, *W. cylindracea*; *glo*, *W. globosa*; *neg*, *W. neglecta*; *un*, *un-cp Wolffia*.

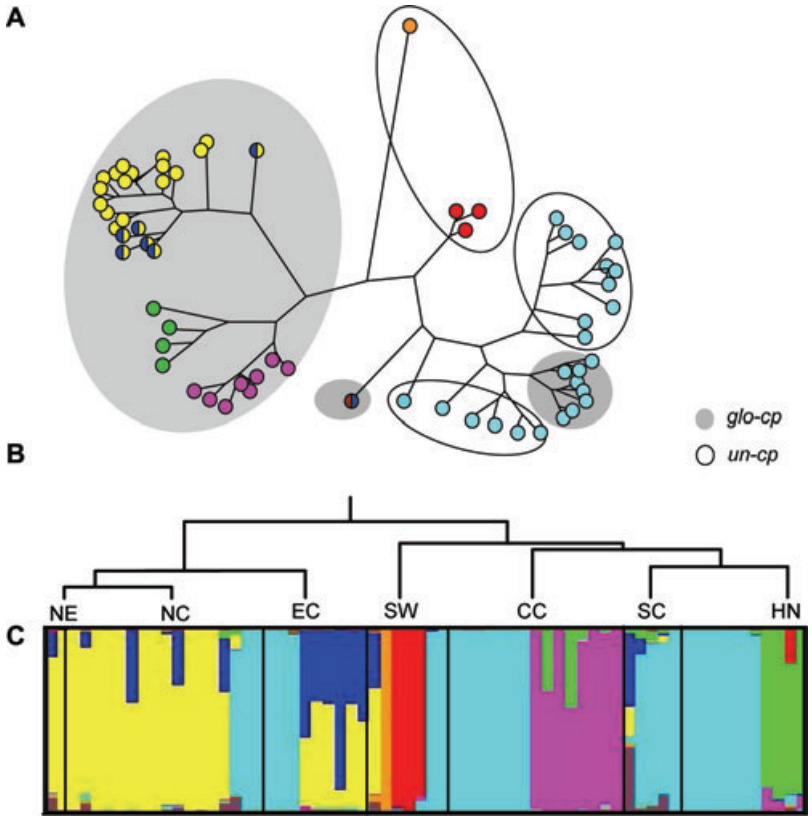

Fig. 4. Genetic differentiation of *Wolffia* in China. **A,** Genetic relationships among 66 amplified fragment length polymorphism genotypes estimated by UPGMA. **B,** Genetic relationships among seven sampling groups of *Wolffia* estimated by UPGMA. **C,** Clustering analysis of the amplified fragment length polymorphism genotypes using STRUCTURE. Different colors represent ancestry from hypothetical genetic populations (K = 8). CC, Central sampling group; EC, East group; HN, Hainan group; NC, North group; NE, Northeast group; SC, South group; SW, Southwest group.

W. globosa (*glo-cp* individuals) than to other species (*W. neglecta*, *W. arrhiza*, *W. australiana*, and *W. cylindracea*).

2.3 Analysis of genetic differentiation

In order to evaluate the genetic differentiation of all Chinese *Wolffia* genotypes, the UPGMA tree and STRUCTURE were built based on AFLP data (Fig.4). The genetic relationship between the sampling groups was visualized by the UPGMA dendrogram (Fig. 4: B). The dendrogram was divided into two clades. Clade I consists of sampling groups in the north and east of China (NE, NC, and EC groups), and Clade II contained the remaining four sampling groups of southern China. The genetic relationship between the group pairs was also supported by Nei's genetic distances and Φst distances (Table 3).

Using STRUCTURE analysis, we estimated the number of natural genetic clusters as K = 8 (Fig. 4: C). These clusters have different geographic distributions. The yellow and blue clusters concentrated in the NE, NC, and EC groups. The light-blue cluster spread widely to all groups but distributed mainly in southern China (SW, CC, SC, and HN). The remaining five clusters were endemic in four groups of southern China. Among them, the SW group was found to have two endemic clusters, although we only collected small samples.

The genetic relationship between the genotypes, visualized by the UPGMA dendrogram, was consistent with the structure cluster (Fig. 4: A). Individuals of the

Table 3 Nei's genetic distance matrix and pairwise Φ_{st} distance between seven sampling groups of *Wolffia* species in China (Φ_{st} above diagonal and Nei's genetic distance below diagonal)

	NE	NC	EC	CC	SW	SC	HN
NE		0.002	0.020	0.848	0.618	0.763	1.265
NC	0.0142		0.159	0.805	0.761	0.849	1.095
EC	0.049	0.0226		0.0857	0.0664	0.174	0.416
CC	0.1406	0.1122	0.335		0.0782	0.316	0.330
SW	0.1395	0.0991	0.186	0.283		0.121	0.207
SC	0.1346	0.0902	0.0524	0.0747	0.0686		0.089
HN	0.1892	0.1397	0.1014	0.0620	0.0678	0.0374	

Sampling groups: CC, Central China; EC, East China; HN, Hainan; NC, North China; NE, Northeast China; SC, South China; SW, Southwest China.

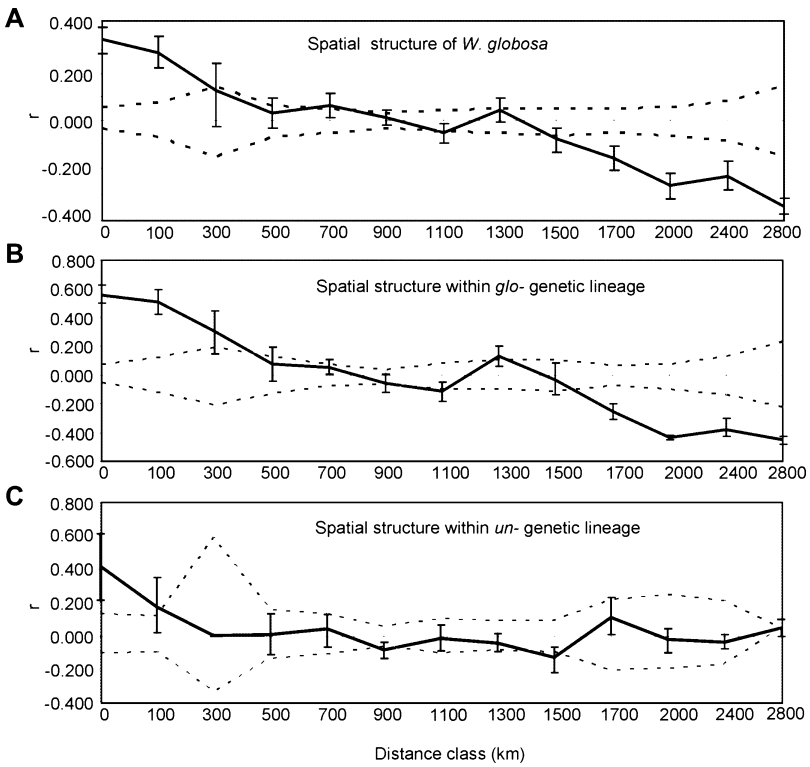

Fig. 5. GenAlEx spatial autocorrelagrams of *Wolffia globosa*. **A,** Spatial genetic structure of overall *W. globosa* samples in China. **B,** Spatial genetic structure within *glo-* genetic lineage. **C,** Spatial genetic structure within *un-* genetic lineage.

yellow, yellow-blue, purple, and green clusters carried the *glo-cp* haplotype, and were more closely related than others. Of the rest, genotypes of the light-blue cluster were predominant, and chloroplast haplotypes of major genotypes belonged to the *un-cp*. We designated them the *glo-* and *un-* genetic lineages; genetic differentiation between *glo-* and *un-* lineages was $\Phi st = 0.417$. Additionally, nine genotypes, collected from the SC and HN groups, were found to be incongruent between nuclear and chloroplast datasets.

2.4 Analysis of spatial genetic structure

The spatial autocorrelation over all samples (Fig. 5: A) was significant within the 300 km distance as well as distance classes beyond 1500 km. To estimate whether the two different genetic lineages differ in spatial structure, patterns of spatial genetic structure within *glo-* and *un-* genetic lineages were analyzed separately. In the *glo*-lineage, there was a similar pattern to the overall spatial structure (Fig. 5: B), whereas in the *un*-lineage, significant genetic structure was observed only within the 100 km distance class, and the points at 900 km and 1500 km (Fig. 5: C).

3 Discussion

3.1 *Wolffia* species in China

Historically, fixed morphological differences have been used to distinguish species. But the deficiency of distinguishing morphological characteristics for some species always put the taxonomical identification in a dilemma. Nowadays, molecular marker can provide insights into the genetic divergence underlying morphologically indistinguishable taxa (Lee, 2000; Whittall et al., 2004; McKinnon et al., 2008; Dasmahapatra et al., 2010). Previous analysis of the *matK* sequence variation found two *matK* haplotypes of the Chinese *Wolffia* species, one identical to the published sequence of *W. globosa* by Les et al. (2002), and the other represented a newly found haplotype (Huang, 2007). By sampling more populations and using more cpDNA markers, our survey also showed two distinct lineages (Figs. 1, 4: A), the *glo*-lineage (=*W. globosa*) and the *un*-lineage. Phylogenetic analysis of cpDNA sequences was used to ascertain the phylogenetic position of the *un*-lineage, and it revealed that this lineage is closely related to *W. globosa* and to a pair of sister species, *W. neglecta* and *W. angusta*.

The AFLP method further proved to be an effective tool for distinguishing genetically closely related species (Lamote et al., 2005; Bog et al., 2010; Chen et al., 2010). In the present study, the AFLP band patterns (Fig. 3) clearly showed that few AFLP bands were shared among the *un*-lineage and several related species (*W. neglecta, W. arrhiza*, *W. australiana*, and *W. cylindracea*), whereas 103 monomorphic bands (34.8% of all AFLP bands) were shared between *W. globosa* and the *un*-lineage, indicating that the *un*- and *glo*- lineages are genetically close to each other, and genetic divergence between *glo-cp* and *un-cp* genotypes is lower than that between *glo-cp* and its closely related species.

Considering the contrasted patterns of inheritance of nuclear and chloroplast genomes of angiosperms, we combined the data of chloroplast and nuclear markers in order to unravel the complexity of gene flow in plants (Petit et al., 2005). We found that genetic differentiation in the nuclear genome showed general congruence with chloroplast lineage differences. However, 9 of 66 genotypes appeared incongruent (Fig. 4: A), which may potentially be due to hybridization (Sang & Zhong, 2000; Nishimoto et al., 2003). Although how the hybrid is proceeding remains a mystery, molecular evidence of gene flow between the *glo*-lineage and the *un*-lineage suggests the absence of sufficient reproductive barriers.

Although distinct genetic divergence does exist between the *glo*- and *un*- lineages (Fig. 4: A), significant differentiation was not found between them in morphologic characters, including the keys for species identification in the genus, such as frond length/width ratio and height/width ratio (data not shown). Considering the cryptic morphological difference, the presence of genetic exchange and the sympatric distribution of two lineages (Fig. 1), we thought that differentiation between *glo*- and *un*-lineage genotypes does not reach to species level, but does reach to varieties within *W. globosa.*

3.2 Genotypic and genetic diversity

As the smallest flowering plants, fruit and seed-setting of *Wolffia* are rarely observed. It has long been considered that the propagation of *Wolffia* species is nearly exclusively vegetative (Vasseur et al., 1993; Crawford et al., 1997; Les et al., 2002; Crawford et al., 2005; Bog et al., 2010). The predominant clonal reproduction may reduce the level of genotypic and genetic diversity (Vasseur et al., 1993; Santamaria, 2002), but these results are somewhat surprising in the present study. Simpson's diversity index (*D*) of either *glo*-lineage or *un*-lineage was higher than 0.62–0.75 of other clonal plants, as summarized in previous reviews (Ellstrand & Roose, 1987; Widen et al.,1994). The level of genetic diversity of these two lineages was also considerably higher than other clonal plants, but similar to outcrossing non-clonal plants (Nybom & Bartish, 2000). The observed genotypic and genetic diversity might be correlated with the following factors. (i) Somatic mutation may provide an important source of genetic variation, particularly in clonal species with rare periods of sexual reproduction (Orive, 2001). (ii) Effective sexual recruitment, together with gene flow and genetic recombination, may also play a role in generating genotypic and genetic diversity. In the present study, the AFLP data and chloroplast haplotypes (Fig. 4: A) showed the phylogenetic incongruence of nine genotypes. The incongruence hinted that genetic exchange occurred among individuals with two chloroplast haplotypes. Regrettably, how the sexual process is proceeding, and what the relative ratio is between clonal and sexual reproduction for species maintenance, have remained mysterious. (iii) The influx of new genotype is also a potential source of genotypic and genetic diversity. The small size of *Wolffia* allow them to be readily spread by zoochory, which can effectively increase genetic and genotypic diversity within a group, without directly invoking the potential importance of sexual reproduction (Cole & Voskuil, 1996; Silvertown, 2008).

3.3 Spatial genetic structure

Patterns of spatial genetic structure might be shaped by complex interactions among gene flow, various natural selection pressures, and genetic drift (Epperson, 1990; England et al., 1999; Wang et al., 2009). Focusing on the spatial structure of *W. globosa* overall (Fig. 5: A), significant autocorrelation within a short distance was detected, indicating that gene flow is most probably restricted, which is congruent to the rare occurrence of flowering and seed-setting in the genus.

Furthermore, spatial genetic analysis revealed difference patterns between two genetic lineages (*glo*- and *un*-): the positive autocorrelation at short distances and slightly negative autocorrelations at very large distances were detected within *glo*-lineage; whereas no significant spatial genetic structure was detected beyond 100 km within *un*-lineage (Fig. 5: B, C). The distinct patterns of *glo*- and *un*- lineages were associated with their different geographic patterns. For the *glo*- genetic lineage, the yellow, yellow-blue, purple, and green clusters (Fig. 4: C) were geographically specific and formed four local pedigree structures in NC, EC, CC, and HN sampling groups, respectively, whereas the absence of spatial genetic structure within the *un*-lineage could be attributed to the present prevalent distribution that the predominant light-blue cluster was widespread from the northern NC group to the southern HN group (Fig. 4: C). The present geographic patterns were probably caused by

multiple factors, such as reproductive mode, ecological factors, and genetic drift. However, more biological evidence is needed to further study and explain the distinct patterns between *glo-* and *un-* lineages.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1 Primers used in amplification and sequencing of chloroplast markers in this study

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