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Genetic diversity and geographic differentiation analysis of duckweed using inter-simple sequence repeat markers

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Abstract Duckweed, with rapid growth rate and high starch content, is a new alternate feedstock for bioethanol production. The genetic diversity among 27 duckweed populations of seven species in genus *Lemna* and *Spirodela* from China and Vietnam was analyzed by ISSR-PCR. Eight ISSR primers generating a reproducible amplification banding pattern had been screened. 89 polymorphic bands were scored out of the 92 banding patterns of 16 *Lemna* populations, accounting for 96.74% of the polymorphism. 98 polymorphic bands of 11 *Spirodela* populations were scored out of 99 banding patterns, and the polymorphism was 98.43%. The genetic distance of *Lemna* varied from 0.127 to 0.784, and from 0.138 to 0.902 for *Spirodela*, which indicated a high level of genetic variation among the populations studied. The unweighted pair group method with arithmetic average (UPGMA) cluster analysis corresponded well with the genetic distance. Populations from Sichuan China grouped together and so did the populations from Vietnam, which illuminated populations collected from the same region clustered into one group. Especially, the only one population from Tibet was included in subgroup A2 alone. Clustering analysis indicated that the geographic differentiation of collected sites correlated closely with the genetic differentiation of duckweeds. The results suggested that geographic differentiation had great influence on genetic diversity of duckweed in China and Vietnam at the regional scale. This study provided primary guidelines for collection, conservation, characterization of duckweed resources for bioethanol production etc.

Keywords ISSR-PCR · Duckweed · Genetic diversity · Bioethanol · Geographic differentiation

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Introduction

Lemnaceae (Duckweed) is a stemless, aquatic, flowering plant. It has 38 species of four genuses [1–3], which grows usually on the surface of still or slow moving water in carpet-like groups [4]. Duckweed is able to grow in many parts of the world except for very cold regions. With a longer growing period than most plants, duckweed produces a continual biomass supply for 9–12 months every year depending on the agricultural zone. Duckweed reproduces quickly, doubling its biomass in only 2–7 days. The growth of duckweed is dependant on the ability of the roots to recover nutrients from the water [5, 6]. It consumes carbon dioxide from the atmosphere by photosynthesis, which is beneficial for reducing the greenhouse effect. Duckweed grows 28 times faster than corn and accumulates biomass at a greater rate than most other plants including field crops. The rate of biomass accumulation is 2.3 g of dry weight produced per original unit (g) of dry weight per week for corn [7] and up to 64 g/g/week for the duckweed species *Lemna paucicostata* [8]. Oron reported that the annual biomass yield for the duckweed *Lemna gibba* was about 55 tons of dry weight per hectare using domestic wastewater [9]. The starch content of duckweed

is highly variable, ranging from 3 to 75% of the dry weight. Chen et al. reported that the starch in duckweed could be transformed into ethanol by yeast fermentation [10].

The duckweed represents potential sources for bioethanol production, wastewater treatment and model plant gene expression research as members posses many rare and beneficial characteristics [8, 11, 12]. The selection of high starch varieties of duckweed is very important. Because genetic variability is a prerequisite for a selection program, it is necessary to detect and document the amount of variation existing within and among the populations. Traditionally, morphological observations and progeny tests have been used as descriptors of genetic diversity; however, they failed to reveal the exact taxonomic relationships because most of the morphological characters are plastic and influenced by environmental factors [13]. During the past two decades, levels and patterns of genetic diversity were evaluated by DNA marker tools that were used in the study of genetic diversity in aquatic plant species and provided an increasingly accurate assessment of the taxonomic relationships [14–16]. Furthermore, DNA-based fingerprinting can provide reliable information on their phylogenetic relationships.

Inter-simple sequence repeat (ISSR) was a molecular marker technique developed in 1994 [17]. These semi-arbitrary markers can be amplified by polymerase chain reaction (PCR) in the presence of one primer complementary to a target microsatellite. Such amplification does not require genome sequence information and produces multilocus and highly polymorphous patterns [17–19]. ISSR primers anneal directly to SSRs that are abundant and are able to evolve rapidly throughout the eukaryotic genome [20]. ISSR has a few advantages over other DNA marker techniques. It has been shown to provide a powerful, rapid, simple, reproducible and inexpensive means of assessing genetic diversity and identify closely related cultivars in many species [16, 21–23]. Consequently, ISSR may reveal more polymorphic fragments with each primer than are revealed by random amplified polymorphic DNA (RAPD) [24, 25].

So far, there are no reports on the genetic diversity of the duckweed species using ISSR markers. The aim of this study was to assess the genetic relationships between 27 populations from Vietnam and West China based on ISSR-PCR.

Materials and methods

Plant materials

In this study, 81 accessions of 27 populations from seven duckweed species of genus *Lemna* and *Spirodela* were collected from different regions of Vietnam and West China. Systematics of duckweed was classified according to the method of E. Landolt et al. [3] and the number of populations and accessions was as following [23, 26–30]: 16 populations were from five species of genus *Lemna* and 11 populations from two species of genus *Spirodela*. Five *Lemna* species, *Lemna minuta*, *Lemna minor*, *Lemna aequinoctialis, L. gibba, Lemna perpusilla,* had 3, 5, 3, 3, 2 populations, respectively. Each population was from a different original place and had three accessions in the experiment. 16 populations were used to analyze genetic diversity and geographic influence inter populations and five species through ISSR maker, and so were the 11 populations being used from species *Spirodela punctata* and *Spirodela polyrhiza* of genus *Spirodela*, five populations from species *S. punctata* and six from *S. polyrhiza* were assessed. The populations and their collection sites are listed in Table 1. After collection duckweeds were cultured in artificial wastewater.

DNA extraction and ISSR-PCR

The young fronds of duckweeds were collected and washed with distilled water. Genomic DNA was extracted from freshly harvested fronds by Axyprep^{TM} Multisource Genomic DNA Miniprep kit (Axygen Scientific, Inc USA).

One hundred primers designed by the biotechnology laboratory of the University of British Columbia (UBC set no. 9) were synthesized by the Invitrogen Company and initially screened for their repeatable amplification. Three duckweed genomic DNA was used as a template for screening primers, one genomic DNA of *S. polyrhiza* and two from *L. minor* and *L. gibba*. Primers were selected for further analysis based on their ability to detect distinct polymorphic amplified products across the accessions. To ensure reproducibility, the primers generating weak products were discarded. The lists of primers used in this study are presented in Tables 2 and 3.

ISSR-PCRs were performed in a 25 μl mixture containing 10 ng genomic DNA, 15.8 μl ddH_2O, 2.5 μl 10× buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1U *Taq* DNA polymerase and 0.8 μM ISSR primers (Takara Biotechnology CO., LTD, Dalian, China).

The PCR amplification was conducted using a Bio-Rad cycler machine DY003401 (Bio-Rad Company, USA) and performed as follows: initial denaturation at 94°C for 5 min, 40 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min followed by an extension for 5 min at 72°C. After the reaction was completed, 5 μl of the amplified PCR products were subjected to electrophoresis in 1.8% agarose gels (Biowest agarose, Spain, Distributed by GENE TECH Shanghai Company limited) in 0.5× TBE

Table 1 Duckweeds used in this study and their collection sites

Genus	S. no.	Population name	Scientific name	Place of collection	Latitude and longitude	Altitude (m)
Lemna	1	P1	*L. minuta*	Pujing Chengdu	30°11′49.66″N, 103°30′23.35″E	515
	2	S2	*L. minor*	BridgeCun Chengdu	30°36′40.00″N, 104°19′17.76″E	507
	3	S3-1	*L. minuta*	BridgeCun Chengdu	30°36′39.18″N, 104°09′13.01″E	506
	4	S4	*L. minor*	BridgeCun Chengdu	30°36′35.47″N, 104°09′21.96″E	509
	5	S7	*L. minor*	BridgeCun Chengdu	30°36′46.80″N, 104°09′08.15″E	504
	6	Q2	*L. aequinoctialis*	Qionglai Sichuan	30°24′54.48″N, 103°27′45.71″ E	504
	7	G	*L. gibba*	Guanghan Sichuan	30°58′34.25″N, 104°16′56.75E	478
	8	4#	*L. minuta*	Chengdu	30°39′30.97″N, 104°03′53.49″E	498
	9	2#	*L. aequinoctialis*	Guangyuan Sichuan	32°13′29.43″N, 106°17′44.88″E	598
	10	Z	*L. gibba*	ZiZhong Sichuan	29°46′31.70″N, 104°51′03.82″E	338
	11	5#	*L. aequinoctialis*	Tibet	29°38′56.86″N, 94°21′41.36″E	4314
	12	V1	*L. minor*	Hanoi Vietnam	21°03′21.09″N, 105°49′44.51″E	9
	13	V4	*L. perpusilla*	Hathin Vietnam	10°47′35.01″N, 106°37′44.87″E	2
	14	V7-1	*L. gibba*	Hanoi Vietnam	21°17′15.12″N, 106°09′25.46″E	59
	15	V8	*L. perpusilla*	Hochiminh city Vietnam	10°47′09.85″N, 106°40′29.93″E	1
	16	V9	*L. minor*	Hanoi Vietnam	21°04′22.34″N, 105°49′07.26″E	10
Spirodela	1	1#	*S. punctata*	Living Water Garden Chengdu	30°46′08.48″N, 104°05′27.76″E	496
	2	3#	*S. punctata*	Chengdu	30°39′30.97″N, 104°03′53.49″E	498
	3	S1	*S. punctata*	BridgeCun Chengdu	30°36′39.18″N, 104°09′13.01″E	506
	4	S3	*S. punctata*	BridgeCun Chengdu	30°36′39.18″N, 104°09′13.01″E	506
	5	V5	*S. punctata*	Hochiminh city Vietnam	10°47′09.64″N, 106°40′52.77″E	5
	6	V1-2	*S. polyrhiza*	Hanoi Vietnam	21°03′21.09″N, 105°49′44.51″E	9
	7	V4-1	*S. polyrhiza*	Hathin Vietnam	10°47′35.01″N, 106°37′44.87″E	2
	8	V5-1	*S. polyrhiza*	Hochiminh city Vietnam	10°47′09.64″N, 106°40′52.77″E	5
	9	V7	*S. polyrhiza*	Hanoi Vietnam	21°17′15.12″N, 106°09′25.46″E	59
	10	V7′	*S. polyrhiza*	Hanoi Vietnam	21°17′15.12″N, 106°09′25.46″E	59
	11	V9-1	*S. polyrhiza*	Hanoi Vietnam	21°04′22.34″N, 105°49′07.26″E	10

Table 2 List of primers used for ISSR amplification

S. no.	Primer	Sequence (5′–3′)
1	UBC856	ACA CAC ACA CAC ACA CCA
3	UBC855	ACA CAC ACA CAC ACA CCT
4	UBC861	ACC ACC ACC ACC ACC ACC
10	UBC857	ACA CAC ACA CAC ACA CTG
21	UBC811	GAG AGA GAG AGA GAG AC
35	UBC827	ACA CAC ACA CAC ACA CG
51	UBC845	CTC TCT CTC TCT CTC TGG
94	UBC849	GTG TGT GTG TGT GTG TCA

buffer at 140 V for 1 h. The electronic image of the ethidium bromide stained gel was captured and documented using the gel documentation system G: BOX (Gene Company limited, USA).

Data analysis

Only the ISSR primers that gave consistent profiles across the populations were used. The presence or absence of bands was scored as a 1 or a 0, respectively. Faint bands were not recorded for analysis. By comparing the banding patterns of genotypes for a specific primer, genotype-specific bands were identified. The binary data that were generated were used to estimate the levels of polymorphism by dividing the polymorphic bands by the total number of scored bands.

Genetic identity and genetic distance was calculated on the basis of Nei's original measures [31]. Dendrogram was constructed based on the resulting matrix of genetic distance through the unweighted pair group method with arithmetic mean (UPGMA) of the statistical package popgene.

Table 3 ISSR primer characteristics in duckweed population polymorphisms

Genus	S. no.	Primer	Number of amplification products		Polymorphism %
			Total	Polymorphic	
Lemna	1	UBC856	17	17	100
	3	UBC855	15	15	100
	4	UBC861	10	10	100
	10	UBC857	17	17	100
	21	UBC811	5	4	80
	35	UBC827	9	8	88.89
	51	UBC845	11	11	100
	94	UBC849	8	7	87.5
	Total		92	89	–
	Average		11.50	11.13	96.74
Spirodela	1	UBC856	18	18	100
	3	UBC855	11	11	100
	4	UBC861	9	9	100
	10	UBC857	19	19	100
	21	UBC811	9	9	100
	35	UBC827	12	12	100
	51	UBC845	12	12	100
	94	UBC849	9	8	88.89
	Total		99	98	–
	Average		12.38	12.25	98.43

Results and discussion

Primer selection and amplification

100 ISSR primers were used, from which eight primers produced clear banding patterns with three duckweed genomic DNA as template.

From the 16 populations of *Lemna*, the eight primers amplified a total of 92 bands, of which 89 bands were polymorphic, polymorphism was 96.74%. For genus *Lemna*, the primers UBC 845, 855, 856, 857 and 861 generated 100% of the polymorphic patterns. The number of bands generated per primer varied from 5 to 17 (Table 3; Fig. 1). The minimum of five bands was generated by primer UBC811, and the maximum of 17 bands was observed with primers 856 and 857 (Fig. 1). Each of the 16 *Lemna* populations presented a unique ISSR genotype, indicating extensive genetic variation in the populations studied. Some homologous loci were present of population 1–10, same in populations 12, 14, 15 and 16. Especially, the genotype of 11 and 13 were very different from other populations (Fig. 1, the results were similar in the images of gels of other six primers). It corresponded to the collection region and growth characteristic of 16 populations. Populations 1–10 were from Sichuan China, population 11 was the only one sample collected from Tibet, populations 12–16 from Vietnam,

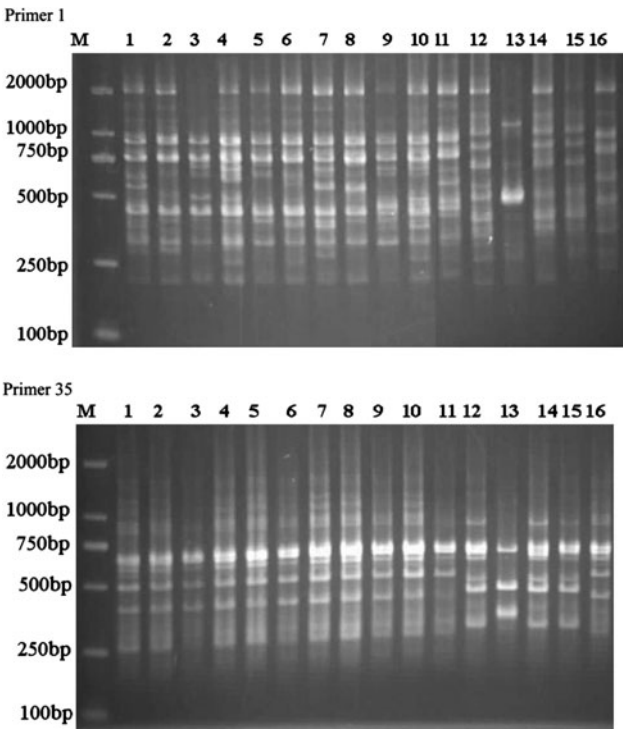

Fig. 1 ISSR profile of the primer I and 35

and population 13 had a faster growth rate and its endurance under threatening environment was better than other *Lemna*.

From the 11 populations of *Spirodela*, 99 bands were amplified and 98 were polymorphic, polymorphism was 98.43%. Only primer UBC849 didn't have a 100% polymorphism. The average number of bands was 12.25. There were more bands in genotype of *Spirodela*, 11 populations always had some collective loci. Their correlation would be showed in dendrogram in Fig. 3.

Genetic diversity

Genetic identity and genetic distance were showed in Tables 4 and 5. Genetic distance of 16 populations of *Lemna* indicated that the maximum value was 0.784 and the minimum value was 0.127. The mean value of 5 species *L. minuta*, *L. minor*, *L. aequinoctialis, L. gibba, L. perpusilla* were 0.192, 0.347, 0.343, 0.430, 0.379, respectively. China and Vietnam populations were 0.251 and 0.330, while the value of 16 populations was 0.364. Genetic distance of different populations inter each specie, *L. minor*, *L. aequinoctialis, L. gibba, L. perpusilla,* were higher than that among different species, it meant that different geographic distance had higher influence than specie did. Genetic diversity of Vietnam populations was higher than China population. Genetic distance between population 11 and other populations of Sichuan China was 0.414, and 0.452 between population 11 and Vietnam populations. Collected from Tibet may be the main reason coursed the genetic distance were far beyond the mean value.

Table 4 Genetic identity and genetic distance of 16 *Lemna* populations[a]

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1		0.880	0.859	0.815	0.837	0.804	0.826	0.848	0.761	0.772	0.652	0.641	0.576	0.576	0.674	0.674
2	0.127		0.826	0.783	0.848	0.794	0.837	0.815	0.750	0.739	0.707	0.630	0.587	0.587	0.641	0.663
3	0.152	0.191		0.783	0.826	0.794	0.772	0.772	0.794	0.739	0.620	0.630	0.565	0.544	0.620	0.685
4	0.204	0.245	0.245		0.804	0.772	0.750	0.794	0.728	0.783	0.576	0.609	0.522	0.457	0.554	0.641
5	0.178	0.165	0.191	0.218		0.837	0.880	0.859	0.837	0.804	0.685	0.652	0.565	0.544	0.598	0.750
6	0.218	0.231	0.231	0.259	0.178		0.826	0.804	0.761	0.794	0.674	0.685	0.554	0.598	0.652	0.674
7	0.191	0.178	0.259	0.288	0.127	0.191		0.870	0.826	0.815	0.652	0.641	0.511	0.554	0.609	0.717
8	0.165	0.204	0.259	0.231	0.152	0.218	0.140		0.848	0.859	0.696	0.663	0.576	0.554	0.630	0.717
9	0.273	0.288	0.231	0.317	0.178	0.273	0.191	0.165		0.815	0.696	0.641	0.554	0.533	0.630	0.783
10	0.259	0.302	0.302	0.245	0.218	0.231	0.204	0.152	0.204		0.663	0.674	0.609	0.609	0.641	0.728
11	0.427	0.347	0.479	0.552	0.379	0.395	0.427	0.363	0.363	0.411		0.641	0.598	0.663	0.630	0.652
12	0.444	0.461	0.461	0.496	0.427	0.379	0.444	0.411	0.444	0.395	0.444		0.674	0.783	0.859	0.728
13	0.552	0.533	0.571	0.651	0.571	0.590	0.672	0.552	0.590	0.496	0.515	0.395		0.630	0.685	0.685
14	0.552	0.533	0.610	0.784	0.610	0.515	0.590	0.590	0.630	0.496	0.411	0.245	0.461		0.794	0.663
15	0.395	0.444	0.479	0.590	0.515	0.427	0.496	0.461	0.461	0.444	0.461	0.152	0.379	0.231		0.717
16	0.395	0.411	0.379	0.444	0.288	0.395	0.332	0.332	0.245	0.317	0.427	0.317	0.379	0.411	0.332	

[a] Obtained by Nei's original measures, Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Table 5 Genetic identity and genetic distance of 11 *Spirodela* populations[a]

Population	1	2	3	4	5	6	7	8	9	10	11
1		0.762	0.772	0.723	0.644	0.555	0.564	0.525	0.545	0.555	0.465
2	0.271		0.852	0.782	0.584	0.535	0.545	0.505	0.485	0.495	0.406
3	0.258	0.161		0.753	0.594	0.564	0.555	0.535	0.535	0.525	0.436
4	0.325	0.246	0.284		0.663	0.594	0.604	0.564	0.584	0.574	0.525
5	0.441	0.538	0.521	0.41		0.634	0.624	0.604	0.703	0.654	0.644
6	0.59	0.626	0.572	0.521	0.456		0.871	0.772	0.713	0.762	0.673
7	0.572	0.608	0.59	0.504	0.472	0.138		0.762	0.723	0.832	0.703
8	0.645	0.683	0.626	0.572	0.504	0.258	0.271		0.624	0.654	0.584
9	0.608	0.723	0.626	0.538	0.352	0.339	0.325	0.472		0.852	0.802
10	0.59	0.703	0.645	0.555	0.426	0.271	0.184	0.426	0.161		0.852
11	0.765	0.902	0.831	0.645	0.441	0.396	0.352	0.538	0.221	0.161	

[a] Obtained by Nei's original measures, Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

The result of genus *Spirodela* also showed genetic distance was correlated with geographic distance, from 0.138 to 0.902. The mean value of genetic distance of 11 *Spirodela* populations was 0.463, which was higher than *Lemna* populations. The mean value of 4 population of Sichuan China was o.258, and 0.341 of Vietnam, so *Spirodela* populations of Vietnam had higher genetic diversity in the same way. It was 0.346 and 0.301 for species *S. punctata* and *S. polyrhiza*. Among them, population 5 of *Spirodela* was very typical. Although both of them were *S. punctata,* the value between it and Sichuan China samples was 0.477, but 0.442 between it and anther 6 *S. polyrhiza* populations from Vietnam. Those values indicated that geographic region had significant influence on the populations' genetic diversity.

The UPGMA dendrogram was identified with the results of genetic distance (Figs. 2, 3). Most of duckweeds did not group according to the species only, and the collected sites had obvious influence on the populations' genetic diversity. 16 populations of *Lemna* were clustered into two groups, group A and B, furthermore the group A were divided into two groups, population 11 (5#, *L. aequinoctialis*) was group alone in A2 obviously. Group A1, A2 and B were interesting for their collection sites. All populations of group A1 were from Sichuan of West China, A2 from Tibet and group B were all from Vietnam. Geographic difference was particularly clear in the dendrogram of *Spirodela* (Fig. 3).

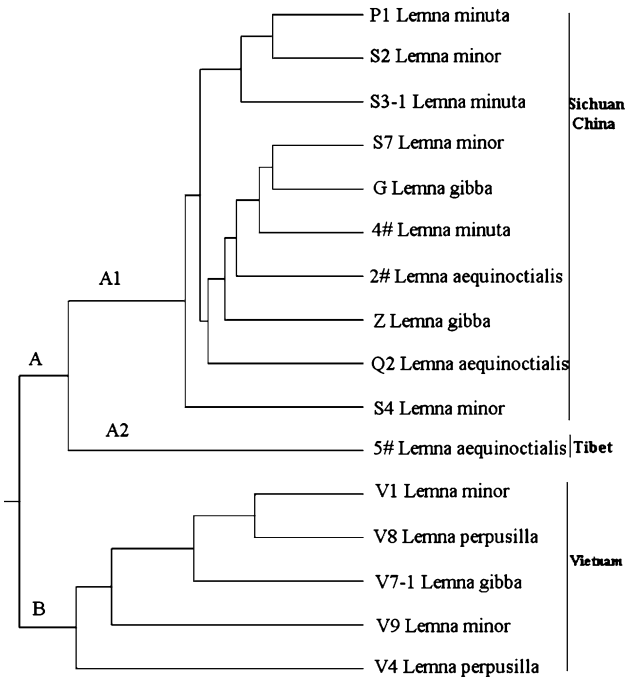

Fig. 2 Dendrogram of 16 populations of *Lemna* constructed with UPGMA method

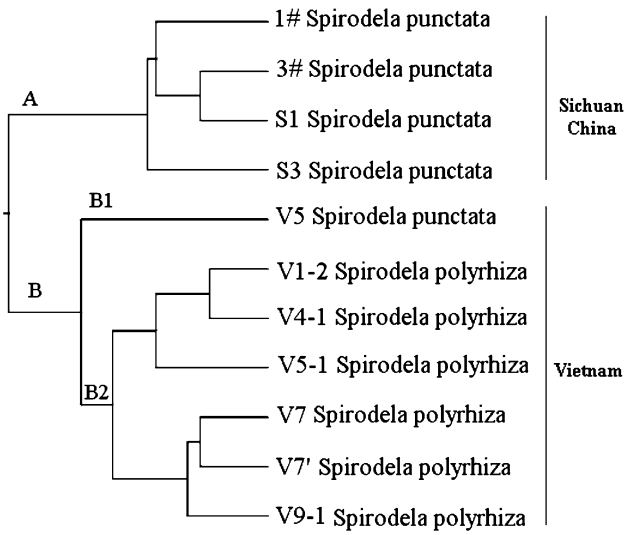

Fig. 3 Dendrogram of 11 populations of *Spirodela* constructed with UPGMA method

11 populations of *Spirodela* was divided into two group, A and B. Group A belonged to Sichuan China, and group B all from Vietnam. Especially, Population 5 (V5, *S. punctata*) grouped with the populations of same collection region but not the same species.

The specific ISSR markers of these populations could potentially be used to provide a comprehensive molecular background of duckweed species. This study provides guidelines for the future analysis of genetic diversity in duckweed with even more reliable molecular markers to facilitate duckweed application and conservation. It also has the potential to develop authentic genetic fingerprints of all available duckweed using a combination of molecular markers.

Geographic differentiation of duckweed

We found that geographic distances affected the differentiation of duckweed populations and that isolation by distance among these populations was significant. *L. aequinoctialis* 5# was collected in Tibet and 2# was collected from Guanghan, Sichuan province, while *L. gibba* Z was from Zizhong, Sichuan province. These three duckweeds had a greater geographic distance than the other *Lemna* populations of China. In addition, *L. aequinoctialis* Q2 and *L. minuta* P1 were from Qionglai and Pujiang, Sichuan province, respectively. Among 11 populations of genus *Spirodela*, *S. punctata* population 5 didn't group with the four populations of same specie from Sichan China, but grouped with six *S. polyrhiza* populations from same collection place. The results indicated that geographic region had significant influence on the populations' genetic diversity.

These results correspond with the report of Bergmann et al. that large differences among geographic isolates of duckweeds were found when they selected superior duckweed (Lemnaceae) genotypes for the utilization of nutrients in animal wastes [32], and the geographic differentiation of some other plants [33–36]. Different communities may have resulted in the significant genetic differentiation between duckweed populations; local selective forces may greatly affect genetic diversity within duckweed populations.

The biological diversity is due to its special geographic environment. Geography, climate, landscape, and soil comprise the main selection pressures of the natural environments of different geographic regions and seem to be complex and far from constant across space and time. Furthermore, duckweed has the smallest flower in the world [3, 27, 37]; its flowering behavior (photoperiodism) is regulated by the phytochrome system and the time between summer and autumn was short [12]. Although duckweed can set seed and produce fruit like other flowering plants, they mostly reproduce vegetatively. Therefore, it is difficult for duckweeds to transfer genes from one place to another and evolutionary processes are greatly affected by geographic isolation.

In this study, the ISSR markers have been used to group duckweed accessions at intra and inter specific levels of two genuses. Across species of duckweeds has revealed a high level of genetic diversity among the species. Populations of different species collected from same region usually had nearer genetic distance. Geographic difference of collected sites had obvious influence on the genetic diversity of duckweeds. Until now, no general biogeographic pattern has ever been proposed for explaining the diversity of duckweed. The exploration and evaluation of duckweed not only provides enhanced information for genetic diversity but also provides valid guidelines for collection, conservation and characterization of duckweed genetic resources, such as those for bioethanol, animal feed and wastewater treatment.

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