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PRIMARY RESEARCH PAPER

Species distribution, genetic diversity and barcoding in the duckweed family (Lemnaceae)

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Abstract Duckweeds are a family of aquatic flowering plants that have a high potential for environmental remediation and biofuel manufacture. Two hundred and twenty clones of duckweeds were collected in Hainan Island, China. Based on morphological and phylogenetic analyses of the chloroplast ribosomal protein S16 intron (*rps16*) and *atpF-atpH* intergenic spacer sequences, these clones were classified into four species belonging to four genera: *Lemna aequinoctialis*, *Spirodela polyrhiza*, *Wolffia globosa*, and *Landoltia punctata*. Eight community types including single-, bi-, and/or tri-species communities were observed. *L. aequinoctialis* was the most widely distributed of the four species. *W. globosa* has the highest genetic diversity followed by *L. aequinoctialis*, whereas *S. polyrhiza* and *L. punctata* did not show any significant diversity. Duckweeds collected from the south of Hainan had higher diversity than those from the north. Moreover, very high rates of transversional nucleotide substitutions were found in the *rps16* sequences of *L. aequinoctialis* and *W. globosa*, which make these duckweeds special with respect to nucleotide substitutions.

Keywords Duckweed · Lemnaceae · Single nucleotide polymorphism · Haplotype diversity · Transversion · Hainan

Introduction

The duckweed family comprises the smallest angiosperms that float on the surface of fresh water bodies such as lakes, ponds, ditches, paddy fields and wetlands. Because of their high protein content, they

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serve as an important source of feed for waterfowl, fish and other animals, and even as food supplement for humans in some parts of the world. Some of the duckweed clones have been proven very effective in municipal and agricultural wastewater treatment (Nhapi et al., 2003; Ozengin & Elmaci, 2007; Short et al., 2007), since they grow rapidly and absorb mineral nutrients, particularly nitrogen (nitrate and ammonium) and phosphate. They are also effective in remediation of water bodies polluted with heavy metals (Zhang et al., 2009). Complete cover of water surface by duckweed mat prevents algal growth (Debusk et al., 1981; Landolt & Kandeler, 1987), and keeps water free of mosquito larvae viz., *Anopheles*, *Culex* (Landolt & Kandeler, 1987; Eid et al., 1992; Iqbal, 1999). Recently, their potential use in biofuel production was emphasized because of their ability to accumulate high amounts of starch under certain conditions (Cheng & Stomp, 2009; Ge et al., 2012; Xu et al., 2012; Zhao et al., 2012; Sree & Appenroth, 2014).

Among duckweeds, there are significant inter- and intra-species differences in their potential for water remediation and in their starch, protein, and oil contents (Mkandawire & Dudel, 2005; Hou et al., 2007; Alvarado et al., 2008; Zhang et al., 2009; Yamaga et al., 2010; Yan et al., 2013). Thus, collection and screening of duckweed ecotypes for specific applications is important, especially local ecotypes for local applications. Carl von Linné (1707–1778) was the first botanist to collect duckweed and he described four species belonging to the genus *Lemna* (Les et al., 2002). Over the years, many researchers have been involved in duckweed research, including the identification of new duckweed species (for reviews cf. Landolt, 1986; Les et al., 2002; Appenroth et al., 2013). Elias Landolt spent a large portion of his scientific career collecting duckweeds and classifying them systematically. He categorized the duckweed family into five genera and 37 species mainly on the basis of morphology (Landolt, 1986; Appenroth et al., 2013). Because of extreme reduction of morphological structures, classification of duckweeds based exclusively on morphology is unreliable to a certain extent, especially for genera with a large number of related species, i.e. *Lemna*, *Wolffia* and *Wolffiella*. Consequently, the classification of duckweeds was conflicted in different studies (Les et al., 2002; Shaw et al., 2005). Much of the classification of duckweeds is based on integrative methods including morphological, flavonoid, isozyme, and DNA markers; however, the resolution between closely related species is still not very clear with very low bootstrap support values (Les et al., 2002; Appenroth et al., 2013). DNA barcoding based on diverse number of loci was investigated to resolve the shortcomings (Shaw et al., 2005; Wang et al., 2010; Bog et al., 2013). Currently, five genera and 37 species have been identified and this is the most widely accepted classification of duckweed family, Lemnaceae (Appenroth et al., 2013).

Duckweed biodiversity in China has not yet been systematically investigated and the number of duckweed species present is still unclear, except for the Jiangshu and Zhejiang Provinces where the duckweed species have been more systematically studied (Shen et al., 2004; Li, 2010; Wang et al., 2012; Wu et al., 2012). Our analysis of the published records suggested that four genera and 12 species are likely to occur in China (Diao, 1990; Li, 2010; Wang et al., 2012; Wu et al., 2012; Tang et al., 2014). These included *Spirodela polyrhiza* (L.) Schleid., *Landoltia punctata* (G. Meyer) Les & Crawford (synonym *Spirodela oligorrhiza*), *Lemna japonica* Landolt, *Lemna trisulca* L., *Lemna perpusilla* Torr., *Lemna aequinoctialis* Welw., *Lemna turionifera* Landolt, *Wolffia arrhiza* (L.) Horkel ex Wimm., and *Wolffia globosa* (Roxb.) Hartog.

Hainan Island is located in the northern part of the South China Sea, and is isolated from Guangdong Province of mainland China by the Qiongzhou Strait (Fig. 1). This island has a land area of 35,400 km^2, and a tropical climate with annual average temperature between 23 and 26°C suitable for the growth of duckweeds throughout the year. The isolated ecosystem of the island, suitable growth conditions for duckweeds and the lack of systematic investigations on duckweed germplasms in the island make it interesting to carry out the following study.

In the present paper, we report about the distribution and biodiversity of duckweed species on Hainan Island. We investigated the phosphate and nitrate content of the water bodies inhabited by duckweeds and the presence of different types of duckweed communities. Duckweed samples were collected from different locations in Hainan and

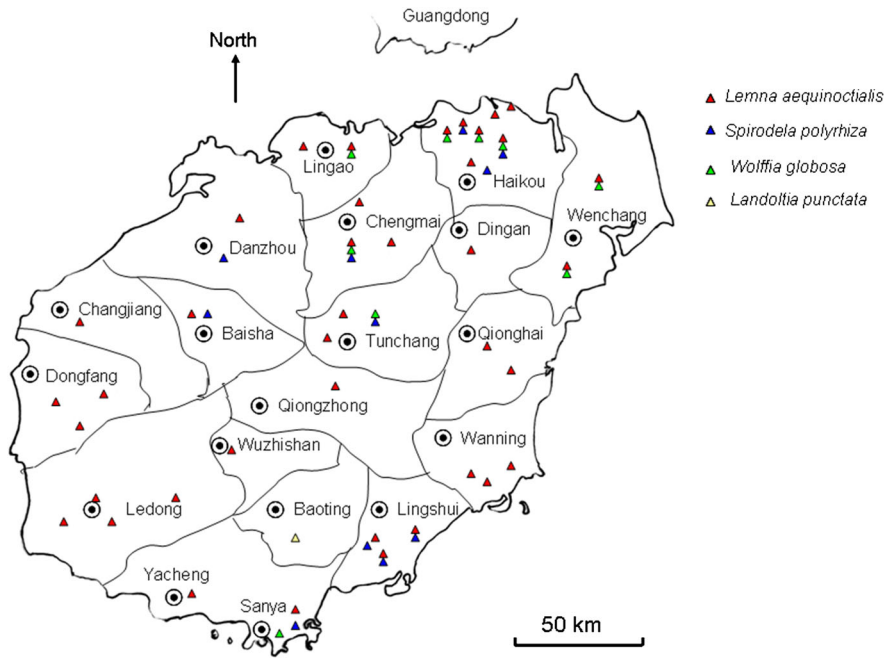

Fig. 1 Locations of duckweed samples on the Hainan Island, China. Scale bar represents 50 km

were cultivated under axenic conditions in vitro. The genotypes were characterized by sequencing and analysing two of the chloroplast markers: ribosomal protein S16 (*rps16*) intron and *atpF-atpH* intergenic spacer region.

Materials and methods

Collection and cultivation of duckweed clones

Duckweeds and the waters inhabited by them were sampled from various parts of Hainan Island, and the locations of representative samples are listed in Table 1. The plants were rinsed in clean water, preliminarily separated into ecotypes according to their morphology, and cultivated in Hoagland medium (pH 5.8) (Hoagland & Arnon, 1950) for 1 week. They were then sterilized with 1.5% sodium hypochlorite solution for 5 min. A single sterile frond from each culture was isolated and cultured on agar-solidified Hoagland medium at 28°C, 14-h light per day, and 150 µmol m^{-2} s^{-1} light intensity. These clones were maintained in Hoagland medium at the above-mentioned conditions and transferred to fresh medium once every month.

Morphological and molecular analysis

Morphological classification was performed using Landolt's key (1980) based on size, shape, colour, number of veins in a frond and the number of roots. To characterize duckweed clones using molecular methods, total DNA was extracted with a Rapid Plant Genomic DNA isolation kit (TianGen, Beijing, China). The chloroplast ribosomal protein S16 gene intron was amplified using the primers *rps16* F (5′AAA CGA TGT GGT ARA AAG CAA C 3́) and *rps16* R (5′AAC ATC WAT TGC AAS GAT TCG ATA 3́) as described previously (Shaw et al., 2005). The PCR conditions were pre-denatured at 94°C for 4 min, followed by 30 cycles at 94°C, 30 s; 58°C, 45 s; 72°C, 1 min, and a further extension at 72°C for 7 min. The primer set used to amplify the non-coding spacer *atpF-atpH* was HNP307: 5′-ACT CGC ACA CAC TCC CTT TCC-3′ and HNP308: 5′-GCT TTT ATG GAA GCT TTA ACA AT-3′ as described previously (Wang et al., 2010). The PCR conditions were pre-denatured at 94°C for 4 min, followed by 30 cycles of 94°C, 30 s; 53°C, 45 s; 72°C, 1 min, and a further extension at 72°C for 7 min. The PCR fragments were sequenced on both strands at BGI (Beijing Genomic Institute), Shenzhen, China. The GenBank accession numbers of the *rps16* and *atpF-atpH* sequences are listed in Table 1.

Table 1 Representative strains isolated in Hainan and their *rps16* and *atpF-atpH* sequences

Strain	Species	Location	GenBank accession number	
			rps16	*atpF-atpH*
DW0101-3	*Lemna aequinoctialis*	Haikou	KJ503283	KJ630511
DW0201-3	*Lemna aequinoctialis*	Haikou	KJ503284	KJ630512
DW0202-3	*Spirodela polyrhiza*	Haikou	KJ503285	KJ630513
DW0301-3	*Lemna aequinoctialis*	Haikou	KJ503286	KJ630514
DW0401-3	*Wolffia globosa*	Haikou	KJ503287	KJ630515
DW0402-3	*Lemna aequinoctialis*	Haikou	KJ503288	KJ630516
DW0501-3	*Wolffia globosa*	Haikou	KJ503289	KJ630517
DW0502-3	*Lemna aequinoctialis*	Haikou	KJ503290	KJ630518
DW0503-3	*Spirodela polyrhiza*	Haikou	KJ503291	KJ630519
DW0601-3	*Wolffia globosa*	Haikou	KJ503292	KJ630520
DW0602-3	*Lemna aequinoctialis*	Haikou	KJ503293	KJ630521
DW0701-3	*Lemna aequinoctialis*	Haikou	KJ503294	KJ630522
DW0801-3	*Spirodela polyrhiza*	Haikou	KJ503295	KJ630523
DW0901-3	*Lemna aequinoctialis*	Wanning	KJ503296	KJ630524
DW1001-3	*Lemna aequinoctialis*	Wanning	KJ503297	KJ630525
DW1101-3	*Lemna aequinoctialis*	Wanning	KJ503298	KJ630526
DW1201-5	*Lemna aequinoctialis*	Dingan	KJ503299	KJ638709
DW1301-3	*Lemna aequinoctialis*	Chengmai	KJ503300	KJ630527
DW1301-4	*Lemna aequinoctialis*	Chengmai	KJ503301	KJ630528
DW1401-1	*Lemna aequinoctialis*	Chengmai	KJ503302	KJ630529
DW1502-4	*Lemna aequinoctialis*	Chengmai	KJ503303	KJ630530
DW1502-5	*Lemna aequinoctialis*	Chengmai	KJ503304	KJ630531
DW1503-2	*Spirodela polyrhiza*	Chengmai	KJ503305	KJ630532
DW1601-1	*Lemna aequinoctialis*	Lingao	KJ503306	KJ630533
DW1601-4	*Lemna aequinoctialis*	Lingao	KJ503307	KJ630534
DW1701-3	*Wolffia globosa*	Lingao	KJ503308	KJ630535
DW1702-1	*Lemna aequinoctialis*	Lingao	KJ503309	KJ630536
DW1702-4	*Lemna aequinoctialis*	Lingao	KJ503310	KJ630537
DW1801-1	*Lemna aequinoctialis*	Danzhou	KJ503311	KJ630538
DW1801-4	*Lemna aequinoctialis*	Danzhou	KJ503312	KJ630539
DW1901-3	*Lemna aequinoctialis*	Qiongzhong	KJ503313	KJ630540
DW1901-4	*Lemna aequinoctialis*	Qiongzhong	KJ503314	KJ630541
DW2001-3	*Lemna aequinoctialis*	Tunchang	KJ503315	KJ630542
DW2001-4	*Lemna aequinoctialis*	Tunchang	KJ503316	KJ630543
DW2101-4	*Wolffia globosa*	Tunchang	KJ503317	KJ630544
DW2102-4	*Spirodela polyrhiza*	Tunchang	KJ503318	KJ630545
DW2201-3	*Lemna aequinoctialis*	Tunchang	KJ503319	KJ630546
DW2201-4	*Lemna aequinoctialis*	Tunchang	KJ503320	KJ630547
DW2301-5	*Lemna aequinoctialis*	Lingshui	KJ503321	KJ630548
DW2401-2	*Lemna aequinoctialis*	Lingshui	KJ503322	KJ630549
DW2402-5	*Spirodela polyrhiza*	Lingshui	KJ503323	KJ630550
DW2501-5	*Spirodela polyrhiza*	Lingshui	KJ503324	KJ630551
DW2601-1	*Lemna aequinoctialis*	Lingshui	KJ503325	KJ630552

Table 1 continued

Strain	Species	Location	GenBank accession number	
			rps16	*atpF-atpH*
DW2602-5	*Spirodela polyrhiza*	Lingshui	KJ503326	KJ630553
DW2701-1	*Landoltia punctata*	Baoting	KJ503327	KJ630554
DW2701-4	*Landoltia punctata*	Baoting	KJ503328	KJ630555
DW2801-3	*Wolffia globosa*	Sanya	KJ503329	KJ630556
DW2901-4	*Lemna aequinoctialis*	Sanya	KJ503330	KJ630557
DW2901-5	*Lemna aequinoctialis*	Sanya	KJ503331	KJ630558
DW3001-1	*Lemna aequinoctialis*	Ledong	KJ503332	KJ630559
DW3101-1	*Lemna aequinoctialis*	Ledong	KJ503333	KJ630560
DW3101-5	*Lemna aequinoctialis*	Ledong	KJ503334	KJ630561
DW3201-3	*Lemna aequinoctialis*	Dongfang	KJ503335	KJ630562
DW3201-4	*Lemna aequinoctialis*	Dongfang	KJ503336	KJ630563
DW3301	*Spirodela polyrhiza*	Sanya	KJ503337	KJ630564
DW3401-4	*Lemna aequinoctialis*	Sanya	KJ503338	KJ630565

To analyze the genetic diversity, *rps16* and *atpF-atpH* sequences were aligned using ClustalX (Thompson et al., 1997). The alignment results were exported into DnSP 5.10.01 software (Librado & Rozas, 2009). The haplotype diversity, nucleotide diversity and the population mutation rate (Watterson estimator θw, Watterson, 1975) were estimated using the same default parameters of the software in all cases (Librado & Rozas, 2009).

DNA barcoding analysis

Reference *rps16* and *atpF-atpH* sequences for DNA barcoding using tree-based methods were retrieved from the GenBank database. Preliminary trees were built, and the redundant sequences were removed to make the tree more precise. The final trees included 26 reference *rps16* sequences and/or 12 *atpF-atpH* sequences. Their GenBank accession numbers are listed in Supplementary Table 1. To build the final trees, primer regions and uneven 5′ and 3′ ends were removed from all sequences. The edited sequences were aligned with the help of ClustalX (Thompson et al., 1997), and the alignment results were imported to Mega6.0 (Tamura et al., 2013). The genetic diversity was inferred using the Maximum Likelihood method based on the Tamura–Nei model (Tamura & Nei, 1993). The tree was drawn to scale, with branch lengths measured as the number of substitutions per site. All positions containing gaps and missing data were eliminated automatically by the software, with a total of 717 positions remaining in the final *rps16* dataset, and 647 positions in the final *atpF-atpH* dataset. Analyses were conducted in MEGA6 (Tamura et al., 2013). Cluster analyses were done using Maximum Likelihood (ML), Minimum Evolution (ME), and Neighbour-Joining (NJ) algorithms and were tested for robustness by bootstrap analysis using 1,000 replicates.

Nutritional status of the water samples

Total nitrogen in the water was measured according to the alkaline potassium persulfate digestion method GB11894-89 published by the State Bureau of Technological Supervision of China (SBTSC). The total phosphorus in the water samples was measured according to the ammonium molybdate spectrophotometric method GB11893-89 also published by the SBTSC. To test the possible relation between nutrient content of the water bodies and the occurrence of duckweed species, one-way ANOVA and multivariate discriminant analysis with step-wise selection of variables were performed.

Results

Distribution of duckweed species on Hainan Island

A total of 220 duckweed clones were isolated as pure clones from 18 districts of Hainan Island (Fig. 1). These clones were morphologically identified as four

Table 2 Morphological identification of duckweed strains

	Morphology	Locations	Number of strains
Lemna aequinoctialis	One root. Fronds flattened, 2–3.5 mm long, obviate in outline, asymmetrical at basal end, floating on water surface; dorsal surface with a media series of papillae and three main veins; 2 lateral pouches on either side of basal end	Haikou, Wenchang, Qionghai, Wanning, Sanya, Lingshui, Baoting, Dongfan, Ledong, Changjiang, Danzhou, Lingao, Chengmai, Dingan, Dunchang, Qiongzhong, Wuzhishan, Baisha	140
Spirodela polyrhiza	Roots 7–21 roots, 1 or rarely 2 perforating the prophyllum, fronds flattened, wide obovate, 5–8 mm long and 4–6 mm wide, above, below purple. 2 lateral pouches on either side of basal end	Haikou, Sanya, Dunchang, Lingshui, Chengmai, Danzhou, Baisha	45
Landoltia punctata	Roots 2–7 roots (rarely 1–12), all perforating prophyllum. Fronds ovate to lanceolate, 1.5–2 times longer than wide; above green with 3–7 veins, and a clear series of papillae in the middle, bellow red. 2 lateral pouches on either side of basal end	Baoting	5
Wolffia globosa	Rootless. Fronds globoid or ovoid and flat-topped, with a diameter of 0.5–1.5 mm. Only one pouch on the side and one daughter plant produced once	Haikou, Wenchang, Chengmai, Dunchang, Shangya	30

species belonging to four genera of Lemnaceae, viz., *L. aequinoctialis*, *S. polyrhiza*, *W. globosa* and *L. punctata* (Table 2). A total of eight community types were found in Hainan Island (Fig. 2). All four species were able to live as single-species communities. However, 29% of duckweed communities were composed of two or more genera (Fig. 2), including combinations of *S. polyrhiza* and *L. aequinoctialis*, *S. polyrhiza* and *W. globosa*, *L. aequinoctialis* and *W. globosa*, and a combination of the above three species. *L. punctata* was observed only as a single-species community.

Lemna aequinoctialis was the most widely distributed of the four species: 140 clones out of 220 were *L. aequinoctialis*. It was distributed all over Hainan and was found in a wide range of water bodies with respect to nutrient content and pH values, including waters with lower nitrogen and phosphorous concentrations (Table 3). *S. polyrhiza* was also common in Hainan, but was less frequently found than *L. aequinoctialis*. It was found in both single- and multiple-species communities (Fig. 2). *W. globosa* was often observed in double-species community together with *S. polyrhiza* or *L. aequinoctialis*, or in tri-species communities with both *L. aequinoctialis* and *S. polyrhiza. Wolffia* species were not reported to exist in a monoculture in the environment in China (Wu et al., 2012). However, we observed one site in Sanya in the south of Hainan, where *W. globosa* flourished as a single-species community (Fig. 2). *L. punctata* was discovered only in a single pond in Baoting, on the south hillside of the Wuzhisan Mountains.

In general, distribution of duckweed depends on the availability of nitrate and especially phosphate (Lüönd, 1983). Therefore, it was tested whether the occurrence of the three frequently existing species (*S. polyrhiza*, *L. aequinoctialis*, *W. globosa*) correlated with distinct nitrate and/or phosphate concentrations

Fig. 2 Duckweed community types. *Scale bars* represent 5 mm

at the place of collection. Tests with single factors (ANOVA) did not show any significant relation: nitrate ($F = 1.9089$, $P = 0.1626$); phosphate ($F = 2.2363$, $P = 0.1211$); pH ($F = 0.7698$, $P = 0.4758$). A multivariate discriminant analysis with step-wise selection of variables also did not show any significant relation: nitrate and phosphate (40% of the clones were misclassified), nitrate, phosphate and pH (58% of the clones were grouped incorrectly). Thus, there was no specific influence of these two nutrients on the relative frequency of occurrence of these duckweed species.

Genetic diversity of duckweed clones based on *rps16* sequences

The chloroplast *rps16* intron fragments of 56 representative clones, including 39 clones of *L. aequinoctialis*, 9 clones of *S. polyrhiza*, 6 clones of *W. globosa* and 2 clones of *L. punctata* (collected at two different locations in the same lake), were amplified and sequenced.

Three haplotypes were identified in the 39 *rps16* sequences of *L. aequinoctialis*. Type I was dominant (32 out of 39) and had a length of 1,054 bp, type II (4 clones) and type III (3 clones) had a length of 1,052 bp. The overall sequence identity was 99%, with an Indel and eight single nucleotide polymorphism (SNP) sites. The haplotype diversity (Hd) of *L. aequinoctialis* was 0.318 ± 0.090, the nucleotide diversity (Pi) was 0.00200 and the θw was 0.00188. Moreover, amongst the SNPs, six out of seven between type I and II, seven out of eight between type I and type III were transverse substitutions, and the one SNP between type II and III was also transverse. The rate of transverse substitution in *L. aequinoctialis* was 87.5%. This high rate of transversional substitutions is unusual, because many of the SNPs are normally transition substitutions (Collins and Jukes, 1994; Zhang et al., 2013).

The length of the amplified *rps16* sequences from all six *W. globosa* clones was 1,061 bp. Alignment analysis revealed two haplotypes with eight SNP sites. Type I was dominant (five clones), and type II had a single clone. Transversional substitutions were also dominant in the SNPs in *W. globosa* clones, 75% of the SNPs in *W. globosa* were transversional. The haplotype diversity (Hd) of *W. globosa* as calculated by DnSP 5.10.01 was 0.333, nucleotide diversity (Pi) was 0.00263 and θw was 0.00345. All the diversity parameters of *W. globosa* were higher than that of *L. aequinoctialis* (Table 4).

The sequenced *S. polyrhiza* clones included four representatives from the north (three clones from Haikou, one clone from Chengmai), one representative from the central part of the island (Tunchang) and four clones from the southern part of the island (three clones from Lingshui, one clone

Table 3 Information of representative duckweed communities and their residing waters

Communities	Locations	N (mg/l)	P (mg/l)	pH	Community types
1	Haikou	6.41	0.68	7.10	L
2	Haikou	–	–	7.10	L + S
3	Haikou	41.93	42.08	7.20	L
4	Haikou	40.78	41.08	7.20	L + W
5	Haikou	41.12	40.24	7.20	L + S + W
6	Haikou	41.85	42.08	7.20	L + W
7	Haikou	5.85	0.43	7.50	L
8	Haikou	6.13	1.27	6.80	S
9	Wanning	1.90	0.82	6.42	L
10	Wanning	12.71	1.17	6.83	L
11	Wanning	6.48	1.20	8.01	L
12	Dingan	2.61	0.15	6.60	L
13	Chengmai	1.06	0.78	6.50	L
14	Chengmai	10.10	5.20	6.50	S
15	Chengmai	24.56	8.33	6.50	L + S + W
16	Lingao	5.72	2.49	6.80	L
17	Lingao	3.24	1.33	6.81	L + W
18	Danzhou	0.65	1.27	6.83	L
19	Qiongzhong	0.23	1.00	7.01	L
20	Tunchang	8.85	0.84	6.52	L
21	Tunchang	13.80	4.69	7.52	L + S + W
22	Tunchang	24.37	9.99	8.15	L
23	Lingshui	10.12	1.90	6.80	L
24	Lingshui	10.95	0.85	6.98	L + S
25	Lingshui	30.74	10.10	6.38	S
26	Lingshui	13.78	1.13	7.01	L + S
27	Baoting	2.93	0.98	6.78	*L. punctata*
28	Sanya	–	–	–	W
29	Sanya	10.72	6.64	6.32	L
30	Ledong	48.07	6.64	7.10	L
31	Ledong	4.97	1.93	7.12	L
32	Dongfang	3.16	3.19	7.02	L
33	Sanya	–	–	–	S
34	Sanya	–	–	–	L
35	Wenchang	–	–	–	L + W

'–' indicates not measured

The letter 'L', 'S', 'W' stands for *L. aequinoctialis*, *S. polyrhiza*, and *W. globosa* respectively

from Sanya). The amplified length of the *rps16* fragment of all nine clones was 1,019 bp, and their sequences were identical, which suggested that the genetic diversity of *S. polyrhiza* was low. The *rps16* sequences of the two *L. punctata* clones were also identical to each other with a length of 1,056 bp, and no genetic diversity between the two clones was observed.

Genetic diversity based on the noncoding *atpF-atpH* region

Genetic diversity of duckweeds based on the *atpF-atpH* region was similar to that based on the *rps16* intron sequences. Three haplotypes were identified in the 39 *atpF-atpH* sequences of *L. aequinoctialis*, with lengths of 686, 688 and 684 bp, for type I, type II and

Table 4 Genetic diversities of *L. aequinoctialis* and *W. globosa* in Hainan Island

	L. aequinoctialis		*W. globosa*	
	rpS16	*atpF-atpH*	*rpS16*	*atpF-atpH*
InDels	1	4	0	2
SNPs	8	3	8	3
Haplotype number	3	3	2	2
Haplotype diversity	0.318	0.309	0.333	0.333
Nucleotide diversity	0.00200	0.00135	0.00263	0.00140
Theta-W per site	0.00188	0.00104	0.00345	0.00184

type III, respectively. All clones were grouped by the *atpF-atpH* sequences into the same haplotypes as by the *rps16* sequences. In other words, Type I was still the dominant (32 out of 39). A total of 4 Indels and 3 SNP sites were found in *atpF-atpH* region. The haplotype diversity (Hd) of *L. aequinoctialis* was 0.309, the nucleotide diversity (Pi) was 0.00135 and the θw per site was 0.00104.

Two haplotypes of *W. globosa* clones were either 715 bp or 717 bp in length with 2 Indels and 3 SNPs. The separation of the haplotypes based on the *atpF-atpH* sequences was also the same as in the case of *rps16* sequences. The haplotype diversity (Hd), nucleotide diversity and θw per site of *W. globosa* were 0.333, 0.00140 and 0.00184, respectively.

The *atpF-atpH* region in *S. polyrhiza* (707 bp) and *L. punctata* (727 bp) displayed no genetic diversity, in consistence with the results of *rps16* sequences

Geographical differences of duckweed diversity

Duckweed populations in south Hainan (Sanya, Lingshui and Ledong) contain more genetic diversity than that in the north (Haikou, Chengmai and Wenchang), especially in the case of *L. aequinoctialis*. The haplotype diversity of the *rps16* and the *atpF-atpH* sequences of *L. aequinoctialis* populations in the southern part of the island was 0.679 and 0.556, respectively; whereas the haplotype diversity in the northern part of the island was 0.167 and 0.167 for the *rps16* and the *atpF-atpH*, respectively. The nucleotide diversity, θw, and average number of nucleotide differences as calculated with the *rps16* and the *atpF-atpH* sequences in the south are all higher than that in the north (Table 5). As calculated with all *rps16* and *atpF-atpH* sequences on family level, the haplotype diversity, nucleotide diversity, θw and average number of nucleotide differences were higher for clones collected from the south than from the north (Table 6).

Table 5 Comparison of genetic diversities of *L. aequinoctialis* populations in the southern and northern parts of Hainan Island

	rps16		*atpF-atpH*	
	South	North	South	North
Haplotype diversity	0.679	0.167	0.556	0.167
Nucleotide diversity	0.00451	0.00116	0.00244	0.00073
Theta-W per site	0.00307	0.00230	0.00161	0.00145
Nucleotide differences per kb	4.536	1.167	1.667	0.500

Table 6 Comparison of genetic diversities between the duckweed populations in the southern and northern parts of Hainan Island on family level

	rpS16		*atpF-atpH*	
	South	North	South	North
Haplotype diversity	0.859	0.561	0.833	0.626
Nucleotide diversity	0.084	0.076	0.053	0.042
Theta-W per site	0.072	0.060	0.048	0.033
Nucleotide differences per kb	77.500	70.316	35.455	28.094

Tree-based identification of species considering *rps16* and *atpF-atpH* sequences

Cluster analyses based on the *rps16* and *atpF-atpH* sequences resulted in topologically similar trees for all three cluster algorithms used (Fig. 3). A total of 39 clones were clustered in the clade of *L. aequinoctialis* with 100% bootstrap support for all three cluster algorithms (ML, ME, and NJ) and for both the chloroplast markers used in the present study (Fig. 3A, B). These clones were divided into three haplotypes, as represented by the clones DW0101-3, DW1301-3 and DW2601-1 in Fig. 3A, consistent with the DnSP analysis. The positions of *S. polyrhiza* and *L. punctata* clones were all supported by 100% bootstrap values for ML, ME, and NJ analyses.

The position of six *W. globosa* clones in the *W. globosa* lineage was supported by bootstrap values of

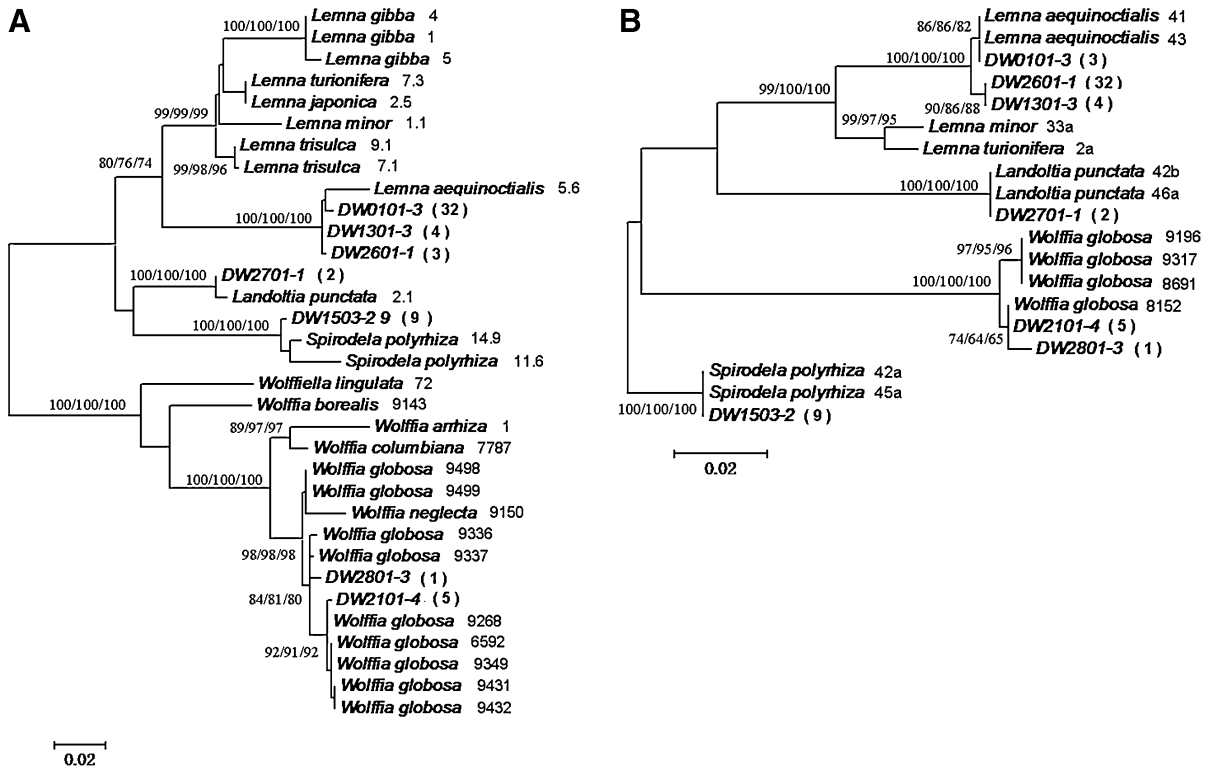

Fig. 3 Maximum Likelihood (ML) trees built on duckweed chloroplast *rps16* (**A**) and *atpF-atpH* (**B**) sequences. The tree with the highest log likelihood is shown and drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar represents 0.02 substitutions. All positions containing gaps and missing data were eliminated, so that a total of 717 positions (*rps16*) and 647 positions (*atpF-atpH*) remained in the final dataset. Analyses were conducted in MEGA6 (Tamura et al., 2013). Trees built with Neighbour-Joining (NJ) and Minimum Evolution (ME) methods were topologically similar and not shown. Bootstrap values (ML, ME, and NJ) are presented above or below the branches. The *numbers in brackets* indicate number of strains represented by the taxa

98% based on the *rps16* sequences, and by bootstrap support of 100% based on the *atpF-atpH* sequences as calculated with ML, ME and NJ, respectively (Fig. 3). In a previous report (Bog et al., 2013), clones of the species *W. globosa* were separated into two different groups based on *rps16* sequences: One group was termed "*W. globosa*" as it consisted of only *W. globosa* clones which were classified on the basis of morphological markers. The other group was a mixture of *W. globosa* and *W. neglecta*, also classified on the basis of morphological markers. In the present study, all the clones collected from Hainan belonged to the "*W. globosa*" group proving their identity.

Discussion

In our extensive survey of the occurrence of duckweeds on Hainan Island, China, 220 clones of duckweeds were collected from more than 50 ponds and/or lakes. In this collection, four species were identified, in the first attempt at identification based on morphological markers (Landolt, 1980): *L. aequinoctialis*, *W. globosa*, *S. polyrhiza*, and *L. punctata*. These four species belonged to four of the five known duckweed genera (Appenroth et al., 2013), leaving only *Wolffiella* out. The frequency of occurrence of these species is very different. For example, *L. aequinoctialis* was often seen in the southwest part of Hainan, where the other three species were rarely found (Fig. 1). *L. aequinoctialis* was the most common duckweed on the island (140 out of 220 clones). Although *S. polyrhiza* and *W. globosa* were less common than *L. aequinoctialis*, they were still frequently occurring. This was not the case with *L. punctata*. The rare presence of *L. punctata* could be due to its recent introduction to Hainan Island. The dispersion of duckweeds is likely to be mediated either

by flowing waters, wind, and/or human and animal activities, out of which human activities may be expected to have a great influence on the migration of duckweed especially to an island. During our field trips, duckweed showed up more often in municipal wastewater treatment ponds, as well as in water bodies polluted with wastewater from animal farms. In larger reservoirs and natural rivers, duckweeds were only occasionally observed. According to our observations, *L. aequinoctialis* is the most suitable and may be the best candidate for future applications in wastewater remediation in Hainan.

Many waterbodies on Hainan supported monocultures of the three duckweed species, *S. polyrhiza*, *L. aequinoctialis* or *W. globosa*. The reason for this is not clear because no preferences could be detected among the three species for particular nutrient compositions. Accordingly, one may speculate that single species monocultures may establish through chance transfer of some fronds of a species to a water body, with the fronds becoming starter culture for mass propagation of that species. Approximately one third of water bodies containing duckweeds supported two or three species. This demonstrated that the general growth requirements of these three species are very similar.

Although a detailed key for species identification based on morphological characters is available (Landolt, 1980), lack of experience and expertise in the field of morphological taxonomy restricts the use of this key for identification of duckweed species around the globe. Because of this very limited usage of morphological markers, two of the molecular markers, *rps16* and *atpF-atpH*, were used for the identification of duckweed species existing in Hainan. The present data show that *rps16* and *atpF-atpH* are very useful for barcoding of duckweed at the species level (Appenroth et al., 2013). However, these markers alone do not distinguish all clones of the same species. For this purpose, either several molecular markers from chloroplast and mitochondria may be used (Wang et al., 2010; Wang & Messing, 2011; Bog et al., 2013) or more global markers like amplified fragment length polymorphism (Bog et al., 2010), or even whole chloroplast genome, may be considered as next generation sequencing becomes cheaper. The recent progress on organellar (Wang et al., 2010) and nuclear genome sequencing (Wang et al., 2014) could be used to mine the potential markers in future. Nevertheless, it was possible to confirm the identifications of the species on Hainan using only the *rps16* and *atpF-atpH* markers. Using the *rps16* data of Bog et al. (2013) all sequenced (6) clones of the species *W. globosa* from Hainan were clustered in the group of "*W. globosa*" which may also be called "true *Wolffia globosa*" group.

Although genetic diversity in particular duckweed species is rather low in a cosmopolitan context (Jordan et al., 1996), the plastidic markers *rps16* and *atpF-atpH* revealed genetic differences among clones of *L. aequinoctialis* and *W. globosa*, and it is questionable if sequencing of a higher number of clones would significantly increase this genetic diversity. Our results showed that the clones of *W. globosa* have the largest genetic diversity among the four species, followed by *L. aequinoctialis* (Table 4). Although *S. polyrhiza* was also widely distributed, the genetic background of these clones was more uniform. Moreover, there seems to be a geographical influence on the genetic diversity as the variation was found to be higher in all the duckweed clones collected from the southern Hainan than those from the north (Table 6), especially in the species *L. aequinoctialis* (Table 5). This is possibly due to the lower latitude, higher temperature and ultraviolet irradiation in the south of the island (Li, 2002). This phenomenon was also observed in a previous report (Les et al., 2002), in which duckweed clones from tropical Africa showed higher genetic diversity than those from other regions, possibly due to higher temperature and irradiation in the tropical region.

Eighty-eight per cent of genetic variation in *L. aequinoctialis* was based on transverse substitution and not on transition. This makes *L. aequinoctialis* special in terms of nucleotide substitution. High rate of transversions (61%) were also observed in an invasive species *Bemisia tabaci* (Lu et al., 2013), and its highly variable genome may be essential for its adaptability to different environments. The high proportion of transverse substitutions in *L. aequinoctialis* might also contribute to its widespread distribution and its adaptability to varying environmental conditions. Further investigation of the adaptability of different clones of *L. aequinoctialis* might suggest its use in bioremediation of waste waters in Hainan Island and elsewhere. The present study depicts an overview of the different duckweed species and communities existing on the island which were also collected and are maintained in the laboratory in Hainan. This will help in making use of the native duckweed clones for

any future application as these clones have already been adapted to the specific climate of Hainan Island.

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