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Morphological variation, chromosome number, and DNA barcoding of Giant Duckweed (*Spirodela polyrhiza*) in Viet Nam

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ABSTRACT

Our present study is the first systematic survey on duckweed biodiversity in Viet Nam. More than 100 samples of Spirodela, Lemna, and Wolffia were collected throughout Viet Nam and maintained under laboratory conditions. In this paper, the morphological variation, chromosome number, and DNA barcoding on Spirodela samples were investigated. S. polyrhiza and S. intermedia are the only two species of Spirodela genus - the most ancient genus among the five duckweed genera. The obtained DNA sequences of atpF- atpH and psbK – psbI regions showed that all 29 Spirodela samples collected from different regions along Viet Nam are S. polyrhiza. Specific SNPs of individual S. polyrhiza clones were identified in the psbK-psbI region. The differences in genome size (163 – 170 Mbp), frond size (0.80 – 1.25 cm^2), frond shape (oval/circular symmetry/asymmetry), stomata (20.57 – 24.00 µm in length and 10.00 – 21.30 µm in width) and root number (4.75 – 6.69 roots) among six representatives S. polyrhiza clones were recorded. The chromosome number of these clones was uniformly 2n=40.

1. INTRODUCTION

Duckweeds are small, free-floating, largely asexual and highly neotenous organisms. They belong to the monocot order Alismatales and display highly reduced organs, the fastest growth rate among flowering plants and are of growing interest both in aquaculture and genome biology with potential become a new generation of sustainable crops. Duckweeds comprise 36 species within 5 genera: 02 *Spirodela*, 01 *Landoltia*, 12 *Lemna*, 10 *Wolffiella*, and 11 *Wolffia* species (Bog et al., 2010, Tippery et al., 2015, Bog et al., 2020). "Frond" was used to describe the leaf-like organism structure of duckweeds which lack a stem. Progressive evolutionary reduced roots and body size from 1.5 cm in diameter with many roots in *Spirodela* (the most ancient genus) to less than 1 mm and no root in *Wolffia* (the most derived genus). All duckweeds reproduce asexually by forming daughter fronds from meristematic pockets (primordia) at the proximal end of a mother frond, although flowers were observed in many species.

Due to noteworthy features of duckweeds such as their worldwide distribution (with the exception of the Arctic and Antarctica), water surface habitat, fast growth rate - yielding up to 100 tons dry mass/hectare/year (Lam et al., 2014; Ziegler et al., 2015) -, the ability to remove contaminants from wastewater (Chaudhuri et al., 2014; Goswami et al., 2014; Teixeira et al., 2014), high quality and quantity of protein (Appenroth et al., 2017; Rusoff et al., 1980) and high starch content in some strains under particular growth conditions (Cui & Cheng, 2015; Fujita et al., 2016; Sree et al., 2015),

duckweeds are seen increasingly attractive as a new crop production platform. They are considered as a potential resource for an increasing world population, useful for wastewater remediation, feedstock for biofuels, animal feed, as well as for human nutrition, without competing with traditional crops for arable land.

Usually, genome sizes and chromosome sets (karyotypes) are rather stable for distinct species and therefore of diagnostic value for taxonomy. Genome size can be a diagnostic feature of individual species and contributes to elucidating whole-genome duplication (WGD) and other events during genome evolution. During the last decades, flow cytometry became the preferred method for genome size measurement in plants. Besides the easiness of sample preparation and high throughput, the capability to estimate genome size, nuclear replication state, ploidy and endopolyploidy levels are advanced features of this method compared to other approaches such as Feulgen densitometry or genome sequencing (Dolezel et al., 2007). The genome size has been reported for different duckweed species with more than a 14-fold variation from 160 Mbp (*S. polyrhiza)* to 2203 Mbp *(Wo. arrhiza)*. However, no significant differences in genome sizes were detected among diploidy clones with 160Mbp in both Spirodela species (*S. polyrhiza* and *S. intermedia)* and 421 Mbp in *La. punctata* while in other genera *Lemna, Wolffiella* and *Wolffia*, genome size variations among different clones of the same species were reported such as in *Le. minor* or *Wo. arrhiza*. Although the two *Spirodela* species have similar genome size (160 Mbp), the chromosome number differ from 2n = 40 in *S. polyrhiza* to 2n = 36 in *S. intermedia.* In addition, a cytogenetic map using chromosome–specific probes of *S. polyrhiza* for subsequence multi-color FISH revealed the homeology and chromosome rearrangements between these *Spirodela* species (Hoang and Schubert, 2017).

Due to the high potential for research and practical applications of duckweed in general and *S. polyrhiza* in particular (Lam et al., 2014), the genomes of *S. polyrhiza* clones 7498 (Wang et al., 2014) and 9509 (Michael et al., 2017) have been sequenced to produce the reference genetic map for this species. In addition, the cytogenomic study revealed no chromosome rearrangements between the seven *S. polyrhiza* clones, but several misassemblies were uncovered in both previous maps (Hoang et al., 2018)

In Viet Nam, the biodiversity and potential application of duckweeds has not yet been investigated comprehensively. Only a few surveys on the possibility of heavy metal accumulation or transgenic research on duckweeds have been conducted (Bui, 2009; Nguyen, 2016; Tran, 2009). Therefore, this study offeers the first systematic study comprising the collection, identification, and evaluation of duckweed genetic diversity in Viet Nam. Among more than 100 samples collected from different regions along Viet Nam, all 29 *Spirodela* samples were identified *S. polyrhiza* based on DNA sequences of atpF- atpH and psbK – psbI regions, the chromosome set from six representative samples is 2n = 40. Furthermore, the genome size, as well as frond size, frond shape, and root system of the six representative clones, were investigated.

2. MATERIALS AND METHOD

2.1. Plant materials

Twenty-eight *Spirodela* samples collected from different regions in Viet Nam were sterilized and grown in liquid nutrient medium including KH_2PO_4 (60 µM), $Ca(NO_3)_2$ (1 µM), KNO_3 (8 mM), $MgSO_4$ (1 mM), H_3BO_3 (5 µM), $MnCl_2$ (13 µM), Na_2MoO_4 (0.4 µM), FeEDTA (25 µM) (Appenroth et al., 2015) under 16 h white light of 100 µmol $m^{-2}s^{-1}$, at 24°C.

Six representative *S. polyrhiza* samples were used for further investigation collected at Ninhthuan (HNP002), Quangninh (HNP053), Dongthap (HNP079), Lamdong (HNP095), Vungtau (HNP100) and Binhthuan (HNP101).

2.2. DNA isolation, amplification, sequencing, and alignment

For each sample, 0.3 g of fresh and healthy fronds were harvested and cleaned in distilled water, placed into a 2 ml Eppendorf tube with two metal balls, frozen in liquid nitrogen, and ground by a ball mill mixer (Retsch MM400). The genomic DNA of the studied species was isolated using the DNeasy Plant Mini Kit (cat. nos. 69104-Qiagen). DNA was eluted by 200 µl buffer AE and quality checked by electrophoresis.

Among different plastid markers such as *rpl16, rps16, atpF-atpH, psbK-psbI* and amplified fragment length polymorphism (AFLP) were used for barcoding of taxa in duckweed genera (Bog et al., 2010; Bog et al., 2015; Bog et al., 2020; Wang et al., 2010; Borisjuk et al., 2015), *atpF-atpH* and *psbK-psbI* regions are suitable for determination at

the species level. In this study, we used these two plastid markers (*atpF-atpH* and *psbK-psbI*) for barcoding. The investigated regions were amplified using the corresponding primer pairs: for the atpF-atpH region: atpF 5'-ACTCGCACACACTCCCTTTCC-3',

atpH 5'-GCTTTTATGGAAGCTTTAACAAT-3'; and for psbK-psbI region: psbK- 5'-TTAGCCTTTGTTTGGCAAG-3', psbI- 5'-AGAGTTTGAGAGTAAGCAT-3'.

The PCR reactions were carried out in a total volume of 25 µl containing 2.5 µl of a ten-fold concentrated Dream Taq Green buffer with, 2.0 µl dNTPs (2.5 mM), 2.5 $\mu l.l^{-1}$ of each primer (10 mM), 0.5 µl Dream Taq enzyme (5 $U.\mu l^{-1}$) and 2 µl DNA template (20 $ng.\mu l^{-1}$).

Amplification was carried out at 95°C for 5 min, followed by 35 cycles at 95°C for 45 sec, at 55°C (psbK-psbI) or 49°C (atpF-atpH) for 30 sec, at 72°C for 50 sec, and a final extension step at 72°C for 7 min. The amplified DNA fragments were resolved on a 1.2% (w/v) agarose gel, stained with SYBR Green and visualized with a UV light (GelDoc-It®2315 imager UVP-USA).

The PCR amplicons obtained from the successful amplifications were selected for sequencing at Macrogen company (10F, 254 Beotkkot-ro geumcheon-gu, Seoul 08511, Rep. of Korea).

The raw reads were manually edited by ATGC software and generated consensus sequences for each sample. The obtained consensus sequences were compared in NCBI through BLASTN. The MEGA 7.0 (The Molecular Evolution Genetics Analysis) with bootstrap 1000 was used for phylogenetic tree construction.

2.3 Genome size measurement

Genome size measurements were performed according to (Dolezel et al., 2007) using a CyFlow Space flow cytometer (Sysmex/Partec). For nuclei isolation and staining the DNA staining kit 'CyStain® PI Absolute P' was used. As internal reference standard, *Raphanus sativus* 'Voran' (IPK gene bank accession number RA 34; 2C = 1.11 pg) was used. The absolute DNA contents (pg/2C) were calculated based on the values of the G1 peak means and the corresponding genome sizes (Mbp/1C) according to (Dolezel et al., 2003). In total, for each sample at least 6 independent measurements on two different days were performed.

2.3. Mitotic chromosome preparation

Spreading of mitotic chromosomes was carried out according to Cao et al. (2016) with some slight modifications. In brief, healthy fronds were incubated in 2 mM 8-hydroxylquinoline at 37°C and then fixed in fresh 3:1 absolute ethanol: acetic acid for at least 12 h. The samples were washed twice in 10 mM Na-citrate buffer, pH 4.6, for 10 min each before and after softening in 2 ml pectinase/cellulase enzyme mixture, prior to maceration and squashing in 45 and 75% acetic acid. After freezing in liquid nitrogen, the slides were treated with pepsin, post-fixed in 4% formaldehyde in 2x SSC (300 mM Na-citrate, 30 mM NaCl, pH 7.0) for 10 min, rinsed twice in 2x SSC, 5 min each, dehydrated in an ethanol series (70, 90 and 96%, 2 min each) and air-dried.

2.4. Frond and stomata size measurement

Spirodela fronds were placed with their upper side on the adhesive tape because stomata are located on the upper surface in floating plants (Landolt, 1986, Shtein et al., 2017). A shaped razor blade was used to carefully remove other parts of the fronds until only the transparent layer of epidermis stuck on the tape.

A light microscope was used to obtain images of fronds and stomata, then Image J software to calculate the diameter of fronds and stomata. At least 10 fronds and 20 stomata were used for measurements per sample.

3. RESULTS AND DISCUSSION

3.1. Species identification by plastidic sequences barcoding

The phylogenetically basic genus *Spirodela* comprises two species, *S. polyrhiza* L. (Schleich) and *S. intermedia* W. Koch, which have a similar genome size (~160 Mbp/1C) (Bog et al., 2015). *Spirodela* genus can be easily distinguished morphologically with other genera according to its biggest frond size (~1.5cm), round leaf-shape. Some morphology characteristics were reported that could be used to distinguish the two *Spirodela* species, however these features also differ due to growth condition. Therefore, we would like to use molecular factor for a correct identification of the species.

The results generated from the two plastid markers (psbK-psbI, and atpF-atpH) for species identification showed that all 29 *Spirodela* samples collected along Viet Nam are *S. polyrhiza.*

atpF- atpH region

The sequencing efficiency for *atpF – atpH* was 89.7% (26/29 samples were sequenced) and all 26 obtained sequences showed no SNPs (Single Nucleotide Polymorphisms) among 29 tested samples and with *S. polyrhiza* reference on NCBI. The length of sequences ranged 662-684bp.

psbK - psbI region

The length of *psbK - psbI* sequences ranged 662-684bp. The sequencing efficiency for this region was 96.6% (28/29 samples were sequenced), 4 SNPs at positions 235 (T/G), 257 (A/G), 425 (A/G) and 474 (A/G) among the 28 obtained sequences were found. Depending on the position of SNPs, the 28 samples were divided into 7 groups (Table 1).

Table 1. SNPs in *psbK – psbI* region occurred in 28 *S. polyrhiza* sequences

Clone ID	235	257	425	474	Clone ID	235	257	425	474
HNP131	T	A	A	A	HNP133	T	G	A	A
HNP095	T	A	A	A	HNP111	T	G	A	A
HNP065	T	A	A	G	HNP100	T	G	A	A
HNP105	T	A	A	G	HNP002	T	G	A	A
HNP003	T	G	G	G	HNP060	T	G	A	A
HNP129	T	G	A	G	HNP069	T	G	A	A
HNP091	T	G	A	G	HNP079	T	G	A	A
HNP028	T	G	A	G	HNP101	T	G	A	A
HNP087	T	G	A	G	HNP055	G	G	A	G
HNP093	T	G	A	G	HNP062	G	G	A	G
HNP053	T	G	A	G	HNP063	G	G	A	G
HNP109	T	G	A	G	HNP082	G	G	A	G
HNP081	G	G	A	A	HNP112	G	G	A	G
HNP117	G	G	A	A	HNP118	G	G	A	G

3.2 Genome size measurement

Six representative *S. polyrhiza* samples were used for genome size measurement. The obtained results showed that the genome size of Viet Nam *S. polyrhiza* samples is a bit larger (163 – 170 Mbp) (Table 2) compared to reported data (158 – 160 Mbp) for other *S. polyrhiza* clones. This might be caused by the use of different internal reference standards or different equipment, but most likely by assuming a different genome size of *Arabidopsis thaliana* as a basis for calculation (157 Mbp in (Hoang et al., 2019), *versus* 147 Mbp in (Wang et al., 2011).

The R-gate charts of six *S. polyrhiza* and the reference standard (*R. sativus)* genome size measurement showed that all tested samples produced a nice peak with more than 100 counts/measurement. The small third peak (on the right side) indicated for 4C cells of *R. sativus* (Fig. 1).

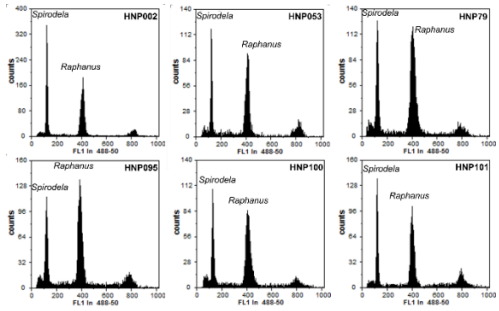

Figure 1. Genome size measurement of *S. polyrhiza* samples with *R. sativus* as reference

3.2. Chromosome number

Preparing nice mitotic chromosome spread of *S. polyrhiza* was a challenge due to the small genome size and the high number of chromosome (~160 Mbp and 2n = 40). Minor modifications were performed according to published protocol (Cao et al., 2016) yielded nice spread of all six tested clones. The images (Fig 2) were captured by SIM (Super Iluminated Microscope) for a high-quality image and precise counting. All tested *S. polyrhiza* clones comprised 40 chromosomes.

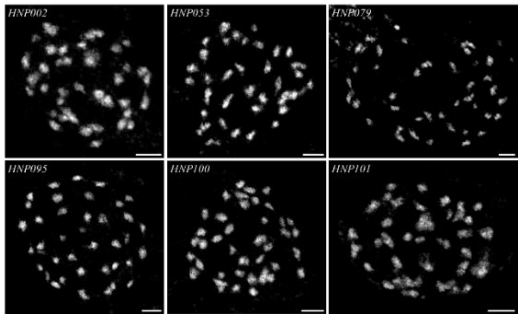

Figure 2 Chromosome spreads of *S. polyrhiza* samples (2n = 40) collected in Viet Nam

Scale bar: 0.5 µm

Combined data from the DNA barcode, genome size and chromosome counting revealed that *S. polyrhiza* samples collected in Viet Nam share similar values with other reported *S. polyrhiza* clones (Hoang et al., 2019, Bog et al., 2015) with genome size (160 – 170 Mbp).

3.3. Morphology variation

Variation between frond shape, frond size, root system and stomata among six *S. polyrhiza* clones were investigated. The frond shape changes from circular to oval, symmetry to asymmetry, and surface area (cm^2) of the largest frond cluster also vary from 0.75 (HNP053) to 1.25 cm^2 (HNP101) (Table 2 and Fig 4). The number of fronds per

cluster mostly is usually 3 (one mother frond and two daughter fronds). A "bridge" was formed before a mature daughter frond separates itself from its mother frond to produce the next generation cluster (Fig 3).

The variation in genome size; frond, root and stomata morphology among six representative *S. polyrhiza* samples observed, showed that, *S. polyrhiza* operates changes in its morphology according to its environment (Tab 2 and Fig 4). The most notable difference could be seen in the shape of the stomata of *S. polyrhiza* HNP095 (collected at Condao Island – Vungtau Province), which is narrow oval (22.75 x 12.25µm), while stomata of other samples are round oval (with 17 – 21µm in width).

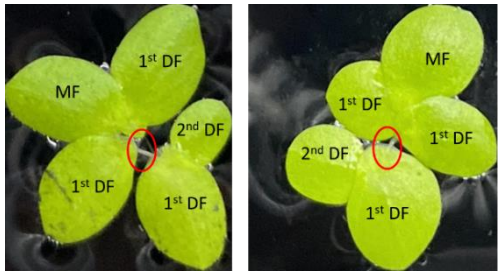

Figure 3. Frond clusters of HNP002 (left) and HNP053 (right)

MF: mother frond; 1st DF: the first generation of daughter frond (formed from MF; 2nd DF: the second generation of daughter frond (formed from 1st DF); Red circle: The "bridge" connects mother and daughter cluster

Table 2. Variation of genome size, frond shape and size, number of frond and root, stomata diameter among six *S. polyrhiza* samples collected in Viet Nam

Sample	Genome size (Mbp)	Frond shape	No. of frond	No. of root	Frond size (cm^2)	Stomata length (µm)	Stomata width (µm)
HNP002	164	oval symmetry	3.5 ± 0.67	6.41 ± 2.55	0.80 ± 0.06	22.17 ± 1.34	18.33 ± 2.50
HNP053	165	circular symmetry	2.9 ± 0.70	5.55 ± 2.01	0.75 ± 0.03	22.67 ± 0.47	17.00 ± 1.58
HNP079	164	circular asymmetry	2.5 ± 0.67	5.92 ± 2.02	0.98 ± 0.05	22.25 ± 0.97	17.25 ± 0.97
HNP095	165	circular asymmetry	2.4 ± 0.72	5.63 ± 2.63	0.97 ± 0.06	22.75 ± 1.09	12.25 ± 1.09
HNP100	170	circular symmetry	2.2 ± 0.75	6.69 ± 2.64	1.07 ± 0.07	24.00 ± 1.55	21.3 ± 2.05
HNP101	163	oval symmetry	2.3 ± 0.46	4.75 ± 2.51	1.25 ± 0.04	20.57 ± 1.68	19.00 ± 2.56

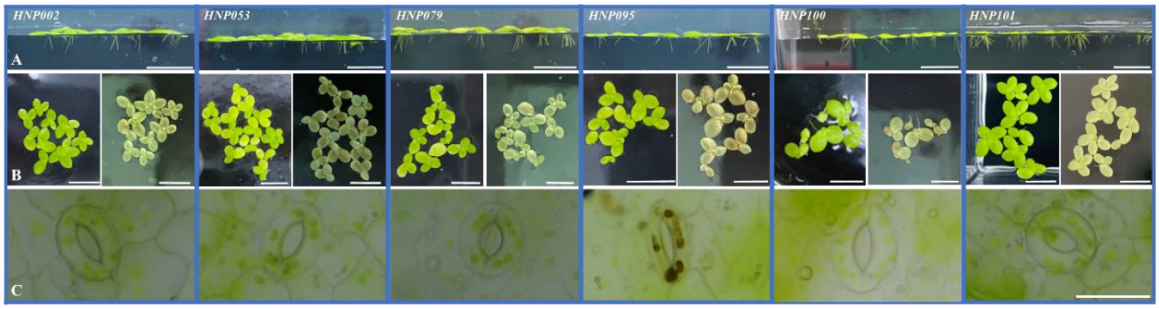

Figure 4. Morphology of frond, root system and stomata of six *S. polyrhiza* samples collected in Viet Nam

(A) the side view of the fronds with the root system. Scale bar: 1 cm; (B) the front view (left) and ventral view (right) of fronds. Scale bar: 1 cm; (C) stomata shape and size. Scale bar: 20 µm

4. CONCLUSION

Our study combining morphology, DNA barcode and cytogenetic approaches showed that all *Spirodela* samples collected along Viet Nam are *S. polyrhiza*. This species has potential applications in wastewater treatment or as biofuel biomass (due to the high concentration of starch) (Lam et al., 2014, Appenroth et al., 2015, Cui et al., 2015). Therefore, our results provide the first systematic survey of the biodiversity of duckweed in general, and *S. polyrhiza* in particular, and provides the background for further research and application based studies.

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