Vietnam Academy of Science and Technology
Vietnam Journal of Marine Science and Technology
journal homepage: vjs.ac.vn/index.php/jmst

Characterizing of ITS2 secondary structures reveals the geographical differentiation of seagrass *Halophila ovalis* (Hydrocharitaceae) in the world

Nguyen Thi XuanThuy[1], Nguyen Nhat Nhu Thuy[1], Nguyen Trung Hieu[1], Nguyen Manh Linh[2], Cao Van Luong[2], Nguyen Thi My Ngan[1], Dao Viet Ha[1,3], Nguyen Xuan Vy[1,3,*]

[1]*Institution of Oceanography, VAST, Nha Trang, Khanh Hoa, Vietnam*
[2]*Institute of Marine Environment and Resources, VAST, Hai Phong, Vietnam*
[3]*Graduate University of Science and Technology, VAST, Hanoi, Vietnam*

Received: 21 August 2024; Accepted: 20 October 2024

ABSTRACT

The seagrass *Halophila* is one of the genera of Hydrocharitaceae that shows the highest number of species, with around 20 species. Among them, *H. ovalis, H. major, H. minor*, and *H. nipponica* are closely related species. It is the first time ITS2 secondary structures and their phylogenetic utility in this genus were reported worldwide. Phylogenetic analysis based on 205 bp of ITS2 showed four clades corresponding to above species. ITS2 secondary structures showed insight into *Halophila ovalis* from the East coast of Africa. *Halophila ovalis* from the East coast of Africa showed a distinct variant in Helix 1, 2, and 3 compared to the worldwide populations. Therefore, the ITS2 locus should be used as a DNA barcode for identifying *Halophila* species.

Keywords: *Halophila*, ITS2, phylogeny, secondary structure, world-wide.

*Corresponding author at: Department of Marine Botany, Institute of Oceanography, 01 Cau Da, Nha Trang City 65000, Khanh Hoa, Vietnam. *E-mail addresses*: nguyenxuanvi@gmail.com

https://doi.org/10.15625/1859-3097/20455
ISSN 1859-3097; e-ISSN 2815-5904

INTRODUCTION

Seagrasses, a flowing plant group adapted to life within the coastal zone, diverged from other alismatid monocots approximately 105 million years ago. They are known among the most valuable ecosystems globally [1, 2]. The role of seagrasses in coastal ecosystems is crucial, as they can form extensive meadows that promote high biodiversity. Seagrass distribution is estimated at 160,387 km^2 to 266,562 km^2 globally [3]. Seagrasses comprise around 72 species within 12 genera and are found on all continents except Antarctica [4]. Based on species distributions, species distributional ranges, and tropical and temperate influences, Short et al., [4] suggested six global bioregions, in which the tropical Indo-Pacific (South Asia, East Africa, and tropical Australia to the eastern Pacific) shows the largest and highest diversity with 24 species. Unfortunately, seagrass net change in 1880–2016 has been extensive, with 5,602 km^2 (19.1%) worldwide [5].

Halophila (Thouars) is one of the genera of Hydrocharitaceae, and it is highest species with around 20 species [6]. Section 2 - *Halophila* consists of 13 species including *Halophila ovalis* and the closely related species such as *H. major, H. minor, H. nipponica,…* presented over-lapping of morphological characteristics among taxa that lead to misidentification [7]. Annaletchumy et al. [8] reported that leaf dimensions of *Halophila ovalis* were changed under different substratum and light exposures. So far, Waycott et al., [9] have suggested that *H. ovalis* is paraphyletic and may contain cryptic species.

One of the uses of molecular markers is their application for species identification, which allows the detection of variations or polymorphisms among individuals in the population for specific regions of DNA [10]. For seagrass, a combination of nuclear ribosomal ITS1-5.8S-ITS2 (ITS) sequence analyses and morphological examinations indicated that the restricting the list of *Halophila* representatives in Japan may be following four species, including *H. decipiens*, *H. major*, *H. ovalis*, and *H. nipponica* [11]. Several studies applied single or/and concatenated ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L), maturase K (*mat*K), *trn*H-*psb*A intergenic space, ITS for species identification of *Halophila* members [12–14]. The previous study on the vicariant evolutionary diversification of *H. ovalis* showed that there are four sub-clades following four geographic regions, including East Asia, Southeast Asia, Australia/USA and the Red Sea. Thirteen genotypes of *H. ovalis* were found in the world [15]. Based on the targeted nuclear and chloroplast gene regions and ddRAD, Waycott et al. [16] revealed that *H. johnsonii* in Florida, USA, *H. ovalis* in Antigua, and *H. ovalis* obtained from the east coast of Africa (Kenya and Zanzibar) was resolved a well-supported clade. Several well-supported clades (tropical and eastern Australia; Solomon Islands; Thailand and Singapore; and Western Australia) were shown, but the authors indicated that these groups are poorly resolved. Previously, several studies revealed that barcode ITS2 should be used as a valuable tool for identifying of plants [17], fungi [18] or algae [19].

RNA secondary structure information of ITS2 was revealed very helpful for phylogenetic analyses [20]. ITS2 secondary structure provides additional data for resolving phylogenetic relationships in closely related taxa [21]. Since compensatory base changes (CBCs) are not anticipated to occur inside a single chromosome, their occurrence in ribosomal internal transcribed spacer 2 (ITS2) stems can have significant evolutionary consequences. Thus, even closely related species can be distinguished using a CBC in an ITS2 sequence-structure alignment [22]. ITS2 secondary structure was widely applied to improve discrimination between closely related species of plants, for example, *Akebia quinata* and *A. trifoliata* [23], *Protasparagus* spp [24], *Physalis* spp. (Solanaceae) [25]. Kurtuluş [26] reported that the well-annotated ITS2 sequences and the CBC concept can be used to distinguish most Coluteocarpeae members.

The application of ITS2 secondary structure in discrimination is very limited for seagrass. In the genus *Halophila*, two species, including *Halophila engelmannii* and *H. stipulacea*, the ITS2 structure also indicated that they are two distinct species [27]. Unfortunately, the authors did not include other members such as *Halophila ovalis, H. major*, *H. nipponica,* and *H. minor,* although

they were considered as closely related species [7]. Therefore, this study aims to find the distinctive characteristics and geographical differentiation of *Halophila ovalis* worldwide.

MATERIALS AND METHODS

Data collection

The dataset of ITS2 of *Halophila ovalis* was collected from various seas or oceanic systems (Figure 1). There are 19 sites, including 15 sequences from Fiji (1) [14], 1 sequence from French Polynesia (2) [28], one sequence from Hawai'i, USA (3), one sequence from Floria, USA (4) [9], four sequences from Egypt (5) [15], 27 sequences from Tanzania (6) [29], eight sequences from India (7) [30, 31], 20 sequences from Sri Lanka (8) [32], 28 sequences from Thailand (9) [33], 40 sequences from Singapore (10) [34], five sequences from South Viet Nam (11) [35], one sequence from North Viet Nam (12) [9], five sequences from Malaysia (13) [9, 30], six sequences from China (14) [30, 36], two sequences from the Philippines (15), three sequences from Indonesia (16) [9], 65 sequences from Taiwan (17) [37], eight sequences from Japan (18) [7] and 14 sequences from Australia (19) [9].

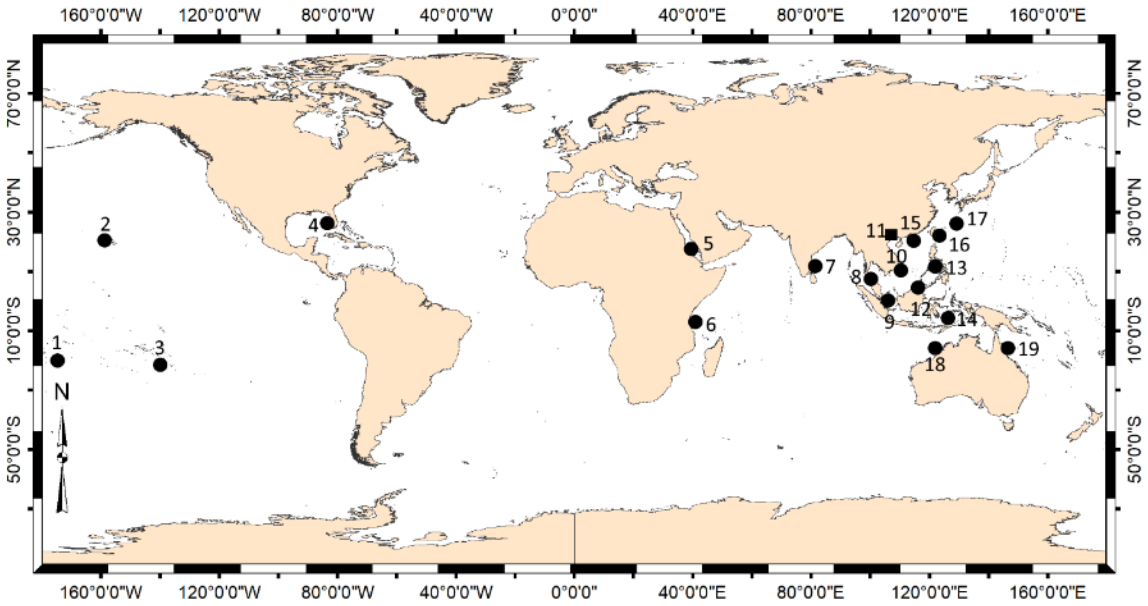

Figure 1. The world map shows different locations where samples were collected. 1. Fiji, 2. Hawai'i (USA), 3. French Polynesia, 4. Florida (USA), 5. Egypt, 6. Tanzania, 7. Sri Lanka and India, 8. Thailand, 9. Singapore, 10. South Viet Nam, 11. North Viet Nam, 12. Malaysia, 13. Philippines, 14. Indonesia, 15. China, 16. Taiwan, 17. Japan, 18 and 19: Australia

Phylogenetic analysis

The dataset of ITS2 (254 sequences x 212 characters) from *Halophila ovalis* and three closely related species including *H. major, H, minor* and *H. nipponica* were aligned by the MAFFT algorithm with the selection of the q-ins-i option [38]. We used the software jModelTest version 2.1.6 [39] with the corrected AIC (Akaike Information Criterion) to find the best model for the analysis. Both Maximum Likelihood (ML) and Bayesian Inference (BI) were used for the phylogenetic analysis. For ML, the phylogenetic analyses were carried using RAxML version 8.1 [40] and the BI analyses were performed in MrBayes v.3.2.2 [41]. The software DendoScope was used to present the consensus tree [42].

Prediction of ITS2 secondary structure

186 ITS2 primary sequences representing *Halophila ovalis* and closely related species (*H. major, H. minor*, and *H. nipponica*) were analyzed. The dataset predicted their secondary structures by comparing with the most modeled structure in the ITS2 Ribosomal RNA database [43, 44]. The sequences, including associate secondary structures, were aligned simultaneously by 4SALE program to generate the consensus secondary structure of *Halophila*'s dataset [45, 46]. Partitioning the ITS2 main sequences into paired and unpaired sections was done. Each base pairing of the secondary structure was coded comparable characters [47]. Then, MAFFT aligned the paired site transformed sequences to analyze variation to determine compensatory base changes (CBCs) and hemi-compensatory base changes (hCBCs). The structural variants of each helix were visualized using VARNA version 3-93 [48] and colored substations by Inkscape version 1.3 (https://inkscape.org).

RESULTS

Phylogenetic analysis

The phylogenetic analysis based on ITS2 revealed four clades, including I - *Halophila major*, II - *Halophila minor*, III - *Halophila nipponica*, and IV - *Halophila ovalis* with high support values (ML = 89, BI = 1.0). Within clade IV, four subclades were formed, but low support values. Six samples formed subclade 1 from two sites in Africa (Egypt and Tanzania), and 19 samples from Singapore were grouped in subclade 2. Subclade 3 was 22 samples from Fiji. The remaining samples were in one group (worldwide group) (Figure 2). Nucleotide differentiations among four subclades were from 1 bp to 8 bp (or 0.5–3.9%). In detail, the highest nucleotide differentiation was between Africa and Singapore (8 bp or 3.9%), and the lowest nucleotide differentiation was between the worldwide group and Singapore (1 bp or 0.5%) (Table 1).

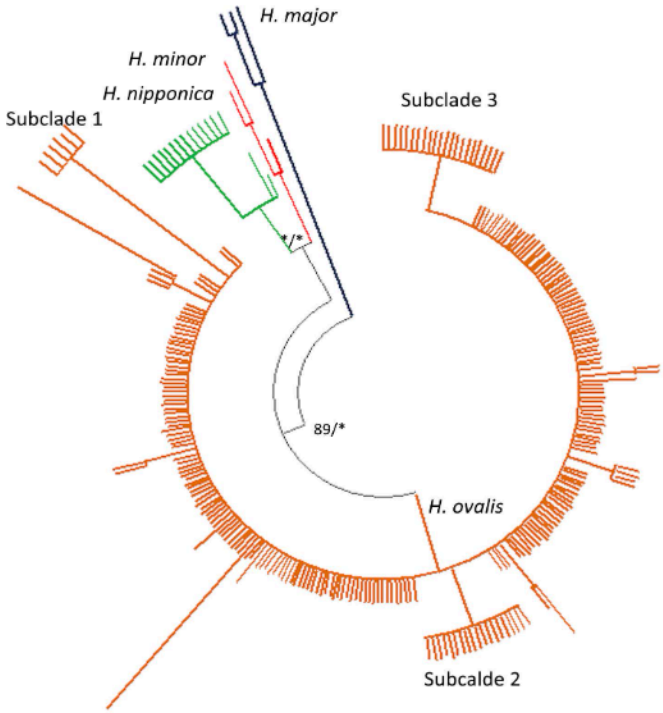

Figure 2. Phylogeny of *Halophila* species based on 205 bp DNA sequences of ITS2. Bootstrap values and posterior probability of each method are shown at each node: (left) ML; (right) BI; * denotes full support (ML=100%, BI= 1.0). - = Bootstrap values lower than 50%. Subclade 1: East Coast Africa, Subclade 2: Singapore, Subclade 3: Fiji

Table 1. Nucleotide differentiation (bp, shadow) and *p*-distance (%) between four subclades

	Africa	Singapore	Fiji	Worldwide
Africa		3.9	3.4	3.4
Singapore	8		1.5	0.5
Fiji	7	3		1.0
Worldwide	7	1	2	

ITS2 secondary structure

The aligned length of ITS2 alignments ranged from 201 bp to 205 bp. The consensus ITS2 secondary structure of *Halophila ovalis* showed four Helices (H1-4) radiating from a central loop; among them Helix 3 was the longest. CBCs were found in Helix 1 (one CBC), Helix 2 (one CBC), and Helix 3 (4 CBCs), whereas Helix 3 showed non-structure base change. In addition, hCBCs were found in Helix 2 (one hCBC) and Helix 3 (one hCBC) (Figure 3). Details of substitution, length of each helix, base change, number of each type in helix, and number of types in helix were present in Table 2.

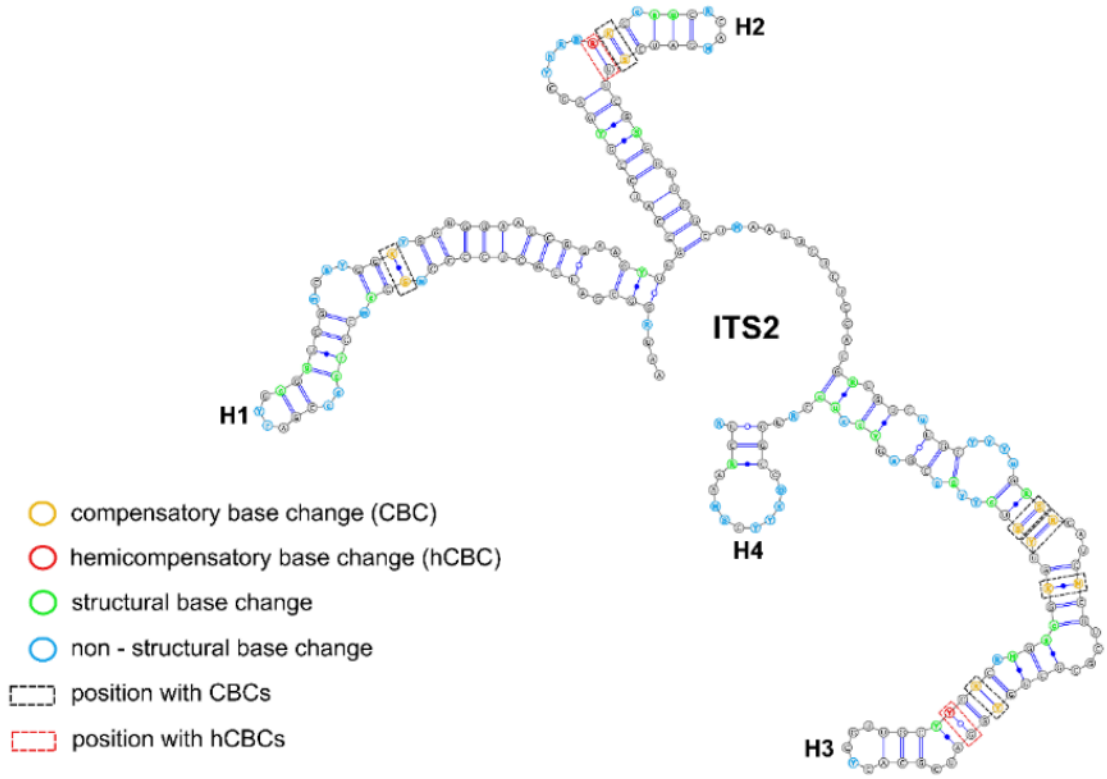

Figure 3. Secondary structures of ITS2 sequences of *Halophila ovalis* and closely related species showing four helices (H1-4)

Structure of Helix 1:

The structure of Helix 1 showed one CBC between *Halophila ovalis* and *H. major* SL type from Vietnam. Within *Halophila ovalis*, there were three variants based on non-structural based changes. Variant 1 (Figure 4A) was specific to samples collected in Africa. In contrast, variant 2 (Figure 4B) was widely found in the Southeast Asian Countries, tropical Australia, India, Hawaii and French Polynesia, and variant 3 (Figure 4C) was specific to a material collected from the Philippines (AF366417).

Table 2. Type and distribution of hCBCs and CBCs in four helices of the consensus ITS2 secondary structure

Substitution (number)	Helix (length)	Base change (type)	Number of each type in Helix	Total number of types in helix
hCBCs (2)	II (16)	AU --> GU (H1)	1/1	1
	III (26)	GU --> GC (H2)	1/1	1
CBCs (6)	I (21)	CG --> GU (C3)	1/1	1
	II (16)	GC --> UG (C5)	1/1	1
	III (26)	CG --> GC (C2)	1/1	4
		GC --> AU (C4)	1/1	
		AU --> CG (C1)	1/1	
		UA --> CG (C6)	1/1	

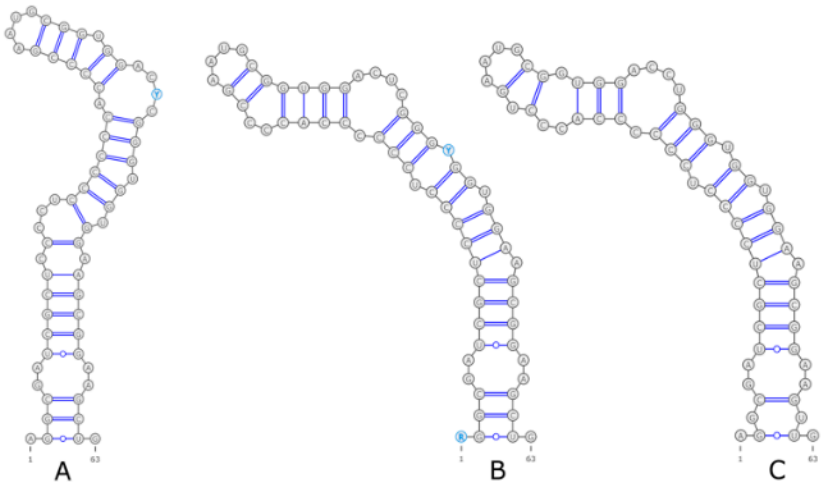

Figure 4. Three variants in Helix 1. A: Africa, B: world-wide, C: Philippines

Structure of Helix 2:

In the structure of Helix 2, three variants were also found. *Halophila* subsp. *ramamurthiana* was specific by one hCBC at position 84 (AU → GU). There was no structure among *H. ovalis* in the world. However, the non-structural base changes can be identified into three variants. Again, samples collected from the East Coast of African countries (Egypt and Tanzania) revealed a distinct variant (Figure 5A); the second variant (Figure 5B) can be widely found in the world, including India, Sri Lanka, Southeast Asian countries (Singapore, Viet Nam, Thailand, Indonesia, Malaysia, Philippines), East Asian countries (China, Taiwan, Japan, Korea), Fiji, French Polynesia, USA. Finally, the third variant (Figure 5C) was found in tropical Australia. In particular, *H. major* can be seen by one CBC at position 85 (GC → UG) in Helix 2 (Figure 5D).

Structure of Helix 3:

Helix 3 showed the longest and highest diversity of specific variants. The different variants can be found in CBCs, hCBCs, and structural and non-structural base changes. Therefore, ten variants can be found in the structure of Helix 3. Variant 1 (Figure 6A), variant 2 (Figure 6B), and variant 3 (Figure 6C) were specific in Africa, Taiwan, and India, respectively. *Halophila* subsp. *ramamurthiana* also showed a distinct variant 4 (Figure 6D). In the Southeast Asian countries, there were three more variants: Indonesia (variant 5, Figure 6E), Philippines (variant 6, Figure 6F) and Singapore (variant 7, Figure 6G). Variant 8 (Figure 6H) was specific to Fiji. Finally, variants 9 (Figure 6I) and 10 (Figure 6 J) can be found in Sri Lanka and tropical Australia.

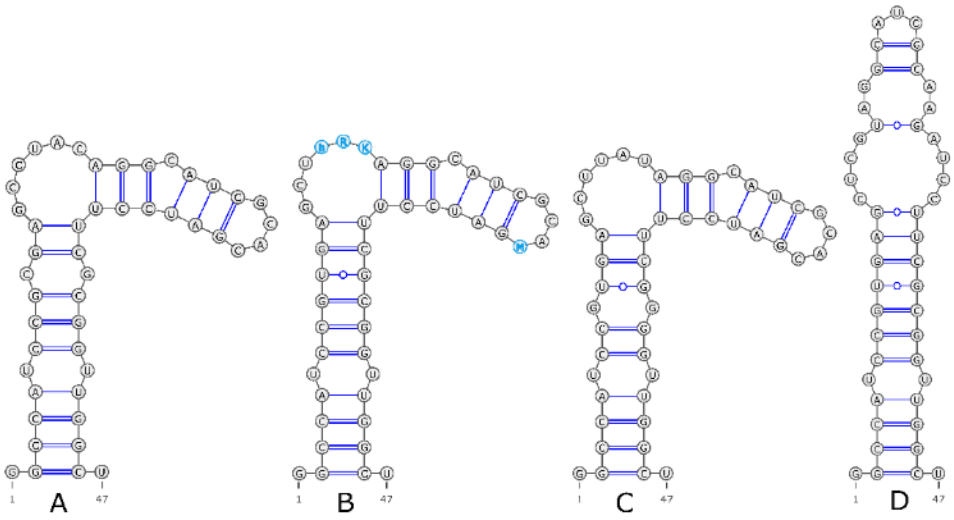

Figure 5. Comparison of three variants of *Halophila ovalis* and *H. major* in Helix 2. A: Africa, B: worldwide, C: Australia, D: *H. Major*

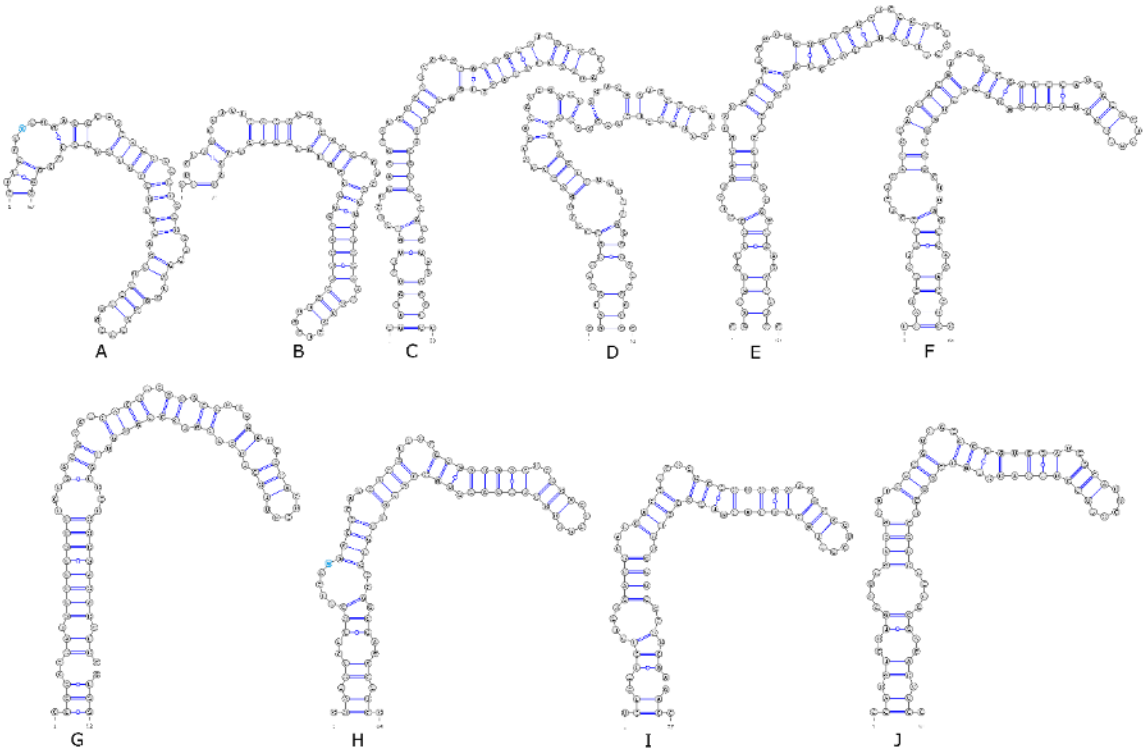

Figure 6. 10 variants on Helix 3 of *Halophila ovalis.* A: East coast of Africa, B: Taiwan, C: India, D: *Halophila* subsp. *ramamurthiana*, E: Indonesia, F: Philippines, G: Singapore, H: Fiji, I: Sri Lanka, and J: Tropical Australia

Structure of Helix 4:

Among four Helices, Helix 4 was the shortest. The results showed that there is no CBC or hCBC among *Halophila ovalis*. The structural base change and non-structural base change revealed three variants. Variant 1 was

worldwide and can be found in Southeast Asia, India, tropical Australia, Japan, China, Korea and Hawaii (Figure 7 A), variant 2 (Figure 7 B) was found from the Philippines, whereas, variant 3 (Figure 7 C) was found in Fiji and French Polynesia. There was no difference in structure on Helix 4 for the two species *Halophila minor* and *H. nipponica* (Figure 7 D).

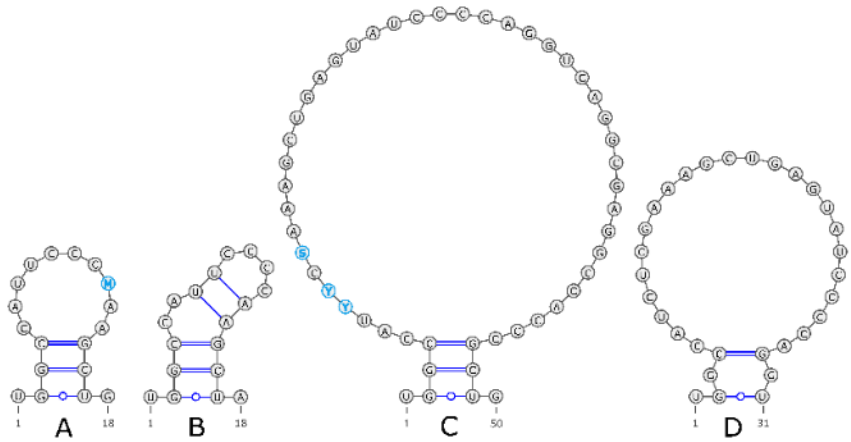

Figure 7. Comparison structure in Helix 4 among variants of *H. ovalis* and other two sister species. A: World-wide, B: Philippines, C: Fiji, French Polynesia, and *H. minor/H. Nipponica*

DISCUSSION

Within seagrass, *Halophila* showed the highest diversity of species. Three closely related species including *H. ovalis*, *H. minor,* and *H. major*, had overlapping morphological characteristics that led to misidentify in some case. This present study revealed the insight of *Halophila ovalis* in the world based on phylogenetic analysis and ITS2 secondary structure.

The phylogenetic analysis based on 212 bp of ITS2 revealed four clades, and *H. major, H. minor, H. nipponica,* and *H. ovalis* are sister species. The result from this study is very similar to that of the phylogenetic using full ITS in *Halophila ovalis*. Samples collected in the Red Sea and some collected in Tanzania (Africa) formed a subclade. Based on ITS, Nguyen et al. [15] reported that genotype 13 was only found in the Red Sea, and there are14 mutations between populations in the Red Sea population and worldwide. Our result based on ITS2 showed that the nucleotide differentiation and *p*-distance between Africa (Subclade 1, Figure 2) and world-wide are seven bp and 3.4%. Recently, based on seven locus hybridization capture generated for nuclear gene regions, Waycott et al. [16] indicated that populations of *Halophila ovalis* on the East Coast of Africa, *H. ovalis* from Antigua (Caribbean Sea) and putative '*H. johnsonii*' in Florida, USA, is grouped into one subclade (clade 1a), *Halophila ovalis* populations in Asia and tropical Australia formed another subclade. Within populations of *Halophila ovalis* from Singapore, 19 samples showed 2-3 nucleotide differentiations from the remaining samples, therefore, it seemed to be formed a subclade (Subclade 2). However, the supporting values were low. In Fiji Island, Skelton and South [49] reported the occurrence of the subspecies *Halophila ovalis* subsp. *bullosa* is characterized by bullations (blisters or pucker-like structures) present on the leaf blades. Later studies recommended revision and merger of *Halophila ovalis* subsp. *bullosa* and *Halophila ovalis* [14]. Twelve samples of *Halophila ovalis* subsp. *bullosa*/*Halophila ovalis* from Fiji showed 2 nucleotide differentiations from the remaining samples and formed another subclade (Subclade 3) of *Halophila ovalis*.

Insight the ITS2 secondary structure showed that some samples in subclade 1 (East coast of Africa) showed a unique structure in three Helices (1–3). In contrast, subclades 2 (some samples from Singapore) and 3 (some

from Fiji) showed distinct structures in Helix 3 and Helix 4, respectively. The result of the ITS2 secondary structure was similar to that of phylogenetic analysis, for examples. *Halophila minor* and *H. nipponica* differed from *H. ovalis* by two hCBCs, whereas, four CBCs were found between *H. major* and *H. ovalis*. In the phylogenetic tree, *H. major* stands in a distinct clade, whereas *H. ovalis*, *Halophila minor* and *H. nipponica* contributed into three groups in a main clade. Previously, ITS2 secondary structure improved discrimination between closely related species of flowing plants such as *Akebia quinata* and *A. trifoliata* [23], *Chrysanthemum indicum* and its closely related species [50], or in fungi [51, 52]. However, Caisová et al. [53] reported that the phylogeny estimation CBCs in the ITS2 were not diagnosed at the species level in five orders of marine green algae. The single sample *Halophila ovalis* (AF366419) collected in Lucerno, Philippines [9] was a specific case due to two CBCs within *Halophila*. Therefore, more samples from this site should be collected for further analysis. In Viet Nam, *Halophila ovalis* occurs in various habitats. Hence, combining microsatellite and ITS2 secondary structure may be a suitable approach to knowing the genetic diversity and evolution of *Halophila ovalis*.

CONCLUSION

This study shows that phylogenetic analysis based on 205 bp of ITS2 revealed four subclades of *Halophila ovalis* worldwide. The ITS2 secondary structure provides more detail of variants in different geographic distribution of *Halophila ovalis* worldwide. Helix 3 showed the longest and highest diversity of specific variants.

Acknowledgments: We would like to thank the VAST Key Lab on Food and Environmental Safety (Central Viet Nam) for the ability to use their equipment. This work was supported by the Viet Nam Academy of Science and Technology [grant numbers VAST04.09/23–24].

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