

Is *Halophila major* (Zoll.) Miquel a big *H. ovalis* (R. Brown) J.D. Hooker? An evaluation based on age, morphology, and ITS sequence

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ABSTRACT: The common seagrass, *Halophila ovalis* (R. Brown) J.D. Hooker, is highly variable morphologically. It adapts well to various environmental conditions rendering the various forms unclear taxonomically. *Halophila* species were collected along the coast of southern Thailand. The morphology was quantified according to different parts of the leaf and the ages of leaves. Some samples had significantly different characters from *H. ovalis*: the lengths of their leaves ranged from 11.7–29.4 mm, and the widths from 5.6–14.8 mm; there were 9–18 cross veins. Phylogenetic analyses based on ribosomal internal transcribed spacer sequences divided them into two groups: one agrees with *H. ovalis* and the other with *H. major*. We suggest that leaf size at maturity (age iii–iv) and the ½ ratio between the leaf width and the space between the intra-marginal vein and lamina margin are important characters that distinguish *Halophila* species.

KEYWORDS: seagrass, nrITS, leaf age, Thailand

INTRODUCTION

The genus *Halophila* Thouars (1806), family Hydrocharitaceae (Alismatales, Monocots)¹ has a broad global distribution², and is one of the most important marine plant due to its ecological roles as primary producer in marine environments³. *Halophila ovalis* (R. Brown) J.D. Hooker is the most common species in this genus found in the Indo-Pacific, the temperate North Pacific, the temperate Southern Oceans, and has recently been observed in the Tropical Atlantic Ocean⁴. It is well known for its variable morphology and adaptability to various environmental conditions^{5–8}. Although *H. ovalis* is widely distributed, it has been represented as a single collective species^{1,9}.

The five species of *Halophila* reported from Thailand are *H. ovalis*, *H. beccarii*, *H. minor*, *H. decipiens*, and *H. major*^{10–12}. *H. ovalis* is also common in Thai waters, forming extensive beds along the Andaman coast. It is well documented as food for the dugong, an endangered marine mammal^{11,13,14}. Seagrass studies in Thailand are however scant^{15,16}

and taxonomic studies have not been revised in the last 10 years. For example, only *H. decipiens*, *H. ovalis*, and *H. ovata* have been reported in the Flora of Thailand¹⁷, where *H. ovata* was placed as a synonym of *H. minor*. Later however it was recognized as two distinct species¹⁸. *H. ovata* is now an illegitimate name and is proposed as *H. gaudichaudii*¹⁹; but a recent study¹² suggested that *H. gaudichaudii* was a synonym of *H. nipponica*. These observations reveal that the taxonomic status of the group is still unclear. Besides, molecular studies by Uchimura et al¹² revealed that *H. major* occurred in Thailand, which was the first record of *H. major* in Thailand. A recent report by Nguyen et al²⁰ suggested the occurrence of *H. major* in Thailand; however its morphological features had never been examined.

During our recent surveys, we have found many *Halophila* specimens in several locations with similar characters to *H. ovalis*, but some with greater leaf size and thick leaf. Both forms grow in the subtidal zones also mixed with *Thalassia hemprichii* and *Cymodocea serrulata*. Recent studies using var-

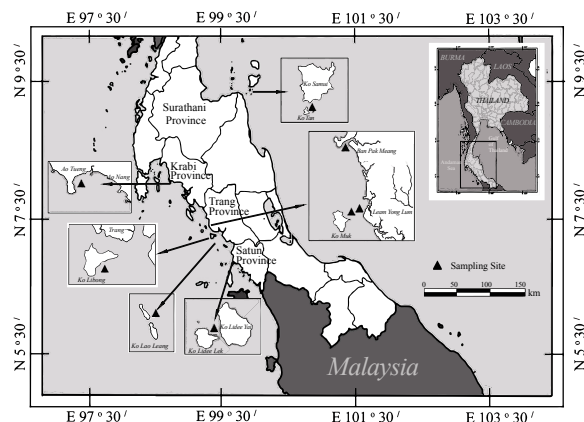


Fig. 1 Field collection sites along the coastal southern Thailand.

ious genetic markers of plastid sequences have clarified the identification of the *Halophila* species^{21,22}. Ribosomal internal transcribed spacer (ITS) sequences could be used to study and identify the genetic relation between *Halophila* closely related species^{12,20,23,24}. It is unknown whether there is any difference in ITS molecular analysis between big and small leaf morphological forms of *H. ovalis*. Thus this study evaluates the taxonomic status of this large *Halophila* sp. by analysing the nuclear ribosomal internal transcribed spacer (nrITS) sequences and measuring different leaf parts at various ages.

MATERIALS AND METHODS

Seagrasses were collected from the intertidal and subtidal zones along the coastal line in southern Thailand (Fig. 1). Samples were collected by walking survey during low tide at intertidal zone area and by using SCUBA diving or snorkelling at the subtidal area. At each sampling point plants containing leaf, root, and rhizome having at least 3–4 leaf pairs were selected, cleaned, and preserved as dried herbarium specimens. New and/or young leaves of *Halophila* with no epiphytes were preserved in silica gel for molecular studies. These vouchers herbarium specimens were deposited at Princess Maha Chakri Sirindhorn Natural History Museum, Prince of Songkla University, Hat Yai.

Total DNA was extracted from 38 samples (Table 1) using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) following the protocol of the manufacturer. The nuclear ribosomal internal transcribed spacer (nrITS) region including the 5.8S gene was selected for PCR amplification and auto-

mated sequencing. The following pair of primers was used for PCR and cycle-sequencing reactions: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR amplification was run on a PROGRAM TEMP CONTROL SYSTEM (Astec, Fukuoka, Japan) and the profile of the reactions was an initial denaturation 1 min at 94°C followed by 35 cycles of denaturation 45 s at 94°C, the primers annealing 45 s at 50°C, and extension 60 s at 72°C, terminated by a final hold at 4°C. The presence of the PCR-amplified products was verified by agarose gel electrophoresis, followed by staining with ethidium bromide. Prior to cycle-sequencing, PCR-amplified products were cleaned using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and directly sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, CA, USA) according to the manufacturers. Cycle-sequencing reactions consisted of an initial step of 96°C for 10 s, followed by 25 cycles (96°C for 10 s, 50°C for 5 s, 60°C for 4 min) and a final hold at 4°C. Only the forward strand was sequenced using a DNA autosequencer (ABI PRISM, 3130 Genetic Analyser, Applied Biosystems, CA, USA).

The sequences were aligned using CLUSTAL X²⁵. Identical sequences within each species were excluded from the alignment. Additional 29 ingroup sequences were loaded from GenBank (Table 1). *H. decipiens* Ostenfeld (AF366412) and *H. stipulacea* (Forssk.) Asch. (AF366436) designated as outgroups. Phylogenetic analysis were implemented using maximum likelihood (ML) and Bayesian Inference (BI). Prior to ML and BI analysis, the best-fit model of nucleotide substitution was selected using the jMODELTEST 2.1.1 tool²⁶. The ML tree was constructed using RAXML²⁷ with the HKY+I+G model. Support for branches was obtained from 1000 bootstrap replications. BI analysis was performed using MRBAYES v.3.2.1²⁸, with a random starting tree run for 5 000 000 generations, sampling tree every 1000 generations and a with a burning of 5000 trees.

The herbarium specimens were closely examined under a stereo microscope (Olympus SZX 12) and photographed using an Olympus DP 71. Each leaf was divided into 4 equal sections from the base to the apex (Fig. 2). The leaf morphological characters were quantified as follows: leaf length (LL) and leaf width (LW) in each of the four sections, number of cross veins (CV), counted from the base of all cross vein (secondary vein) which are connected with the mid rib (primary vein), cross

Table 1 Samples collected from southern Thailand and sequence data that use in this study.

No.	Taxon	Location	Voucher No.	GenBank No.	Source
1	<i>H. ovalis</i>	Okinawa, Japan		AB243973	23
2	<i>H. ovalis</i>	Okinawa, Japan		AB243975	23
3	<i>H. ovalis</i>	Trang, Thailand		AB436938	12
4	<i>H. ovalis</i>	Trang, Thailand		AB436939	12
5	<i>H. ovalis</i>	Queensland, Australia		AF366431	29
6	<i>H. ovalis</i>	Marakanam, India		KF620355	20
7	<i>H. ovalis</i>	Kanyakumari, India		KF620353	20
8	<i>H. ovalis</i>	Trang, Thailand		KF620350	20
9	<i>H. ovalis</i>	Nakhon Si Thammarat, Thailand		KF620345	20
10	<i>H. ovalis</i>	Satun, Thailand		KF620347	20
11	<i>H. ovalis</i>	Lantau Island, Hong Kong		KF620337	20
12	<i>H. ovalis</i>	Cu Mong Lagoon, Vietnam		KC175909	20
13	<i>H. ovalis</i>	Sarawak, Malaysia		KF620338	20
14	<i>H. ovalis</i>	Tiga Island, Malaysia		KF620339	20
15	<i>H. ovalis</i>	Johore, Malaysia		KF620346	20
16	<i>H. ovalis</i>	Flores Island, Indonesia		AB436930	12
17	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	PT 45.1.1*	KP408228	† (Group Ho.1)
18	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	PT 45.2.1	KP408229	† (Group Ho.1)
19	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	PT 49.1.1*	KP408230	† (Group Ho.1)
20	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	PT 49.2.2	KP408231	† (Group Ho.1)
21	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	PT 49.3.4	KP408232	† (Group Ho.1)
22	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	PT 52.1.2	KP408233	† (Group Ho.1)
23	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	CK 13-1	KP408234	† (Group Ho.1)
24	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	CK 14-2	KP408235	† (Group Ho.1)
25	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	CK 14-6	KP408236	† (Group Ho.1)
26	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	CK 14-7	KP408237	† (Group Ho.1)
27	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	CK 15-5	KP408238	† (Group Ho.1)
28	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	CK 15-7	KP408239	† (Group Ho.1)
29	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	CK 19-5	KP408240	† (Group Ho.1)
30	<i>H. ovalis</i>	Koh Tan, Suratthani, Thailand	CK 24-1	KP408241	† (Group Ho.1)
31	<i>H. ovalis</i>	Koh Tan, Suratthani, Thailand	CK 24-2	KP408242	† (Group Ho.1)
32	<i>H. ovalis</i>	Koh Tan, Suratthani, Thailand	CK 24-3	KP408243	† (Group Ho.1)
33	<i>H. ovalis</i>	Koh Tan, Suratthani, Thailand	CK 24-3.4	KP408244	† (Group Ho.1)
34	<i>H. ovalis</i>	Koh Tan, Suratthani, Thailand	CK 24-4	KP408245	† (Group Ho.1)
35	<i>H. ovalis</i>	Koh Tan, Suratthani, Thailand	CK 24-5	KP408246	† (Group Ho.1)
36	<i>H. ovalis</i>	Ban Pak Meang, Trang, Thailand	PT 46.1.2	KP408247	† (Group Ho.1)
37	<i>H. ovalis</i>	Ban Pak Meang, Trang, Thailand	PT 46.1.3	KP408248	† (Group Ho.1)
38	<i>H. ovalis</i>	Ban Pak Meang, Trang, Thailand	PT 46.2.4	KP408249	† (Group Ho.1)
39	<i>H. ovalis</i>	Ban Pak Meang, Trang, Thailand	PT 46.3.1	KP408250	† (Group Ho.1)
40	<i>H. ovalis</i>	Ban Pak Meang, Trang, Thailand	PT 47.2.2	KP408251	† (Group Ho.1)
41	<i>H. ovalis</i>	Ban Pak Meang, Trang, Thailand	PT 47.1.3	KP408252	† (Group Ho.1)
42	<i>H. ovalis</i>	Ban Pak Meang, Trang, Thailand	PT 62.1	KP408253	† (Group Ho.1)
43	<i>H. ovalis</i>	Koh Lidee, Satun, Thailand	PT 57.1.1	KP408254	† (Group Ho.1)
44	<i>H. ovalis</i>	Leam Yong Lum, Trang, Thailand	PT 49.1.5*	KP408255	† (Group Ho.2)
45	<i>H. major</i>	Trang, Thailand		AB436927	12
46	<i>H. major</i>	Okinawa, Japan		AB243967	23
47	<i>H. major</i>	Sumbawa, Indonesia		AB436926	12
48	<i>H. major</i>	Bali, Indonesia		AB436928	12
49	<i>H. major</i>	Nha Trang, Vietnam		KC175910	20
50	<i>H. major</i>	Gyeiktan, Myanmar		KF620352	20
51	<i>H. major</i>	Mabul Island, Malaysia		KF620340	20
52	<i>H. major</i>	Leam Yong Lum, Trang, Thailand	PT 48.1.1*	KP408256	† (Group Hm.2)
53	<i>H. major</i>	Leam Yong Lum, Trang, Thailand	PT 48.1.2*	KP408257	† (Group Hm.3)
54	<i>H. major</i>	Leam Yong Lum, Trang, Thailand	PT 48.2.3	KP408258	† (Group Hm.1)
55	<i>H. major</i>	Leam Yong Lum, Trang, Thailand	PT 48.3.1*	KP408259	† (Group Hm.1)
56	<i>H. major</i>	Leam Yong Lum, Trang, Thailand	PT 51.1.2*	KP408260	† (Group Hm.1)
57	<i>H. major</i>	Koh Libong, Trang, Thailand	PT 133.10	KP408261	† (Group Hm.1)
58	<i>H. major</i>	Koh Muk, Trang, Thailand	CK 16-3	KP408262	† (Group Hm.1)
59	<i>H. major</i>	Ko Lao Liang, Trang, Thailand	SP 348	KP408263	† (Group Hm.1)
60	<i>H. major</i>	Ao Nang, Krabi, Thailand	AD 178 a	KP408264	† (Group Hm.1)
61	<i>H. major</i>	Ao Nang, Krabi, Thailand	AD 178 b	KP408265	† (Group Hm.1)
62	<i>H. australis</i>	South-Western Australia		AF366414	29
63	<i>H. hawaiiiana</i>	Hawaii, USA		AF366426	29
64	<i>H. johnsonii</i>	Florida, USA		AF366425	29
65	<i>H. mikii</i>	Kagoshima Pref., Japan		AB436929	12
66	<i>H. minor</i>	Guam		AF366405	29
67	<i>H. minor</i>	Philippines		AF366406	29
68	<i>H. stipulacea</i>	Sicily, Italy		AF366436	29
69	<i>H. decipiens</i>	Malaysia		AF366412	29

* measurement samples in this study

† this study

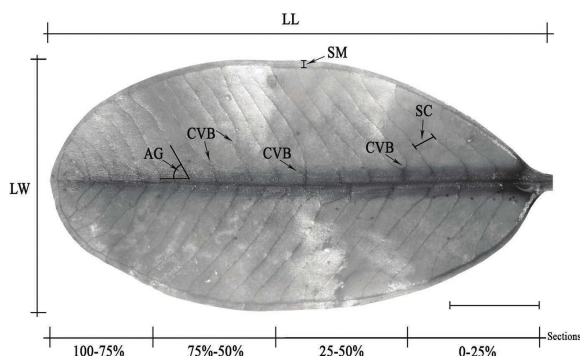


Fig. 2 Leaf characters of *Halophila major* that were measured in each section. LL: leaf length; LW: leaf width; AG: angle between cross veins and mid veins; CVB: cross veins branching; SC: space between cross veins; SM: distance between intramarginal veins and lamina margin. Scale bar is 5 mm.

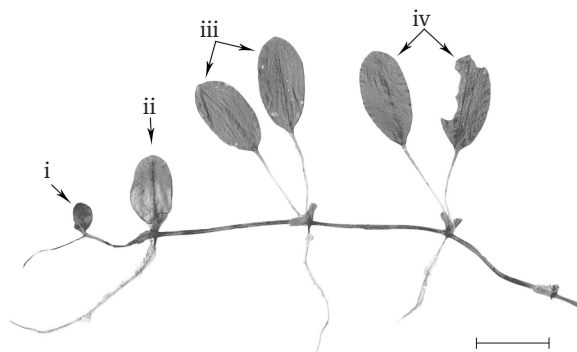


Fig. 3 Leaf age classes. (i) Young or new leaf without the petiole; (ii) young leaf nearly mature and short petiole length (2nd of leaf pairs); (iii) fully young mature (3rd leaf pairs); (iv) old leaf (4th–5th leaf pairs). Scale bar is 3 cm.

vein branching counted from the base of cross vein which branched in the end of cross vein. The space between cross veins (SC) and the space between the intra-marginal vein and the lamina margin (SM) measured as the distance between each characters. The angle of cross veins ascending measured at the angle between the mid vein and the cross vein in each section. All these character parameters were examined using an image analysis program (imageJ software version 1.46r). Three replicates of each character parameters were made. The leaves were also divided into 4 age classes (Fig. 3): Age (i) is the young or new apical leaf with petiole development, age (ii) is young leaf nearly mature and with a short petiole (2nd leaf pairs), age (iii) is fully young mature (3rd leaf pairs), and age (iv) is an old leaf (4th leaf pairs). Leaves at each age were divided

into the 4 sections and examined as above. The homoscedasticity of data was tested using Levene's test; and Two-ways ANOVA was employed to compare the difference in those characters with respect to species at each age; and Welch ANOVA was employed if data are heteroscedasticity.

RESULTS

Molecular phylogeny and ecological aspects

The phylogenetic tree obtained with the ML method is presented in Fig. 4. Both maximum likelihood (ML) and Bayesian Inference trees have the same topology. Thirty eight ITS sequences were divided into two clades consisting of clade I and clade II with 99 bootstrap percentages and 1 of Bayesian Inference posterior probabilities. In clade I, both haplotype *Ho.1* and *Ho.2* were grouped with known sequences, *H. ovalis* from GenBank. The 27 samples (*Ho.1*) were identical but different by 2 bp from *H. ovalis* AB243973 (JP). The haplotype *Ho.2* was identical with *H. ovalis* sequences KF620345(TH), KF620347(TH), AB436938(TH), KF620337(HK), KF620338(ML2) and KF620339(ML3) but different by 2 bp from haplotype *Ho.1*. In clade II, haplotype *Hm.1*, *Hm.2* and *Hm.3* were clustered with known sequences, *H. major* from GenBank. Haplotype *Hm.1* (8 samples) were identical with *H. major* AB436927(TH). Haplotype *Hm.2* and *Hm.3* were grouped with AB436928 and AB436926 from Indonesia, respectively. *Hm.1* differed by 5 bp from *Hm.2* and 6 bp from *Hm.3*, while *Hm.2* and *Hm.3* differed only 1 bp. The results showed the nucleotide differences among individuals of *H. ovalis* clade and *H. major* clade were 0–15 nucleotides and 0–14 nucleotides, respectively. Intra-species variations were 0–0.014% (*H. major*), 0–0.015% (*H. ovalis*) and 0–0.018% (*H. nipponica*). Inter-species variations between *H. major*/*H. ovalis*, *H. major*/*H. nipponica* or *H. ovalis*/*H. nipponica* were 0.04–0.057%, 0.05–0.068% or 0.031–0.047%, respectively.

H. ovalis has occurred in both Andaman Sea and Gulf of Thailand while *H. major* was found only in the Andaman Sea. *H. ovalis* and *H. major* from In Leam Yong Lum, Trang province showed great genetic variations, covered both haplotypes of *H. ovalis* (*Ho.1* and *Ho.2*) and all of *H. major* (*Hm.1*, *Hm.2*, and *Hm.3*).

Morphological observations

The molecular analysis revealed that the large-leaved *Halophila* species is *H. major* (Zoll.) Miq.

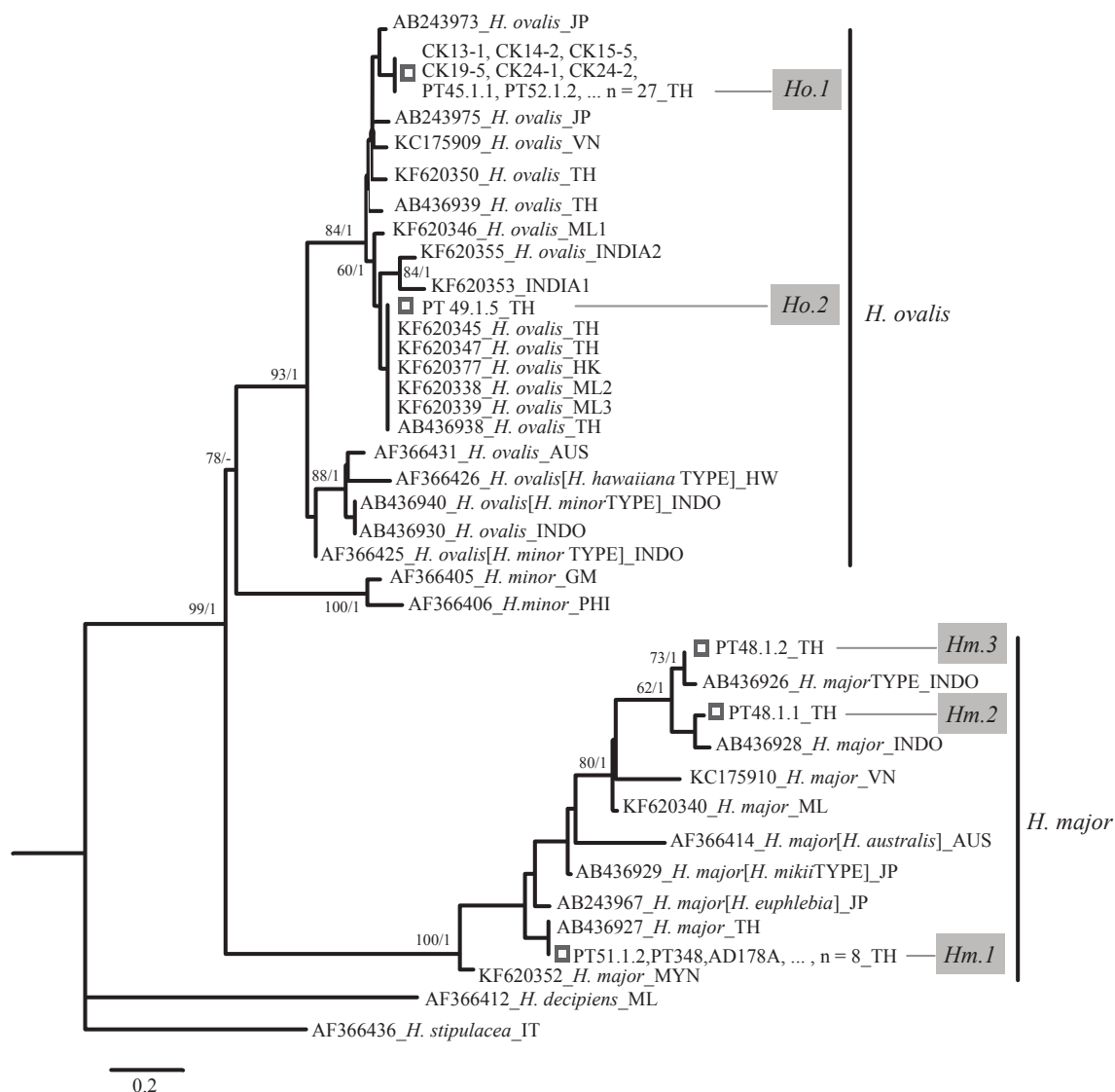


Fig. 4 ML tree of *Halophila* species (nrITS: 635 bp). ML bootstrap values (> 50) and BI posterior probabilities (> 0.80) are indicated at nodes.

A total of 20 morphological leaf characters in 4 age classes were examined and compared between *H. major* and *H. ovalis*. The leaf characters showed the variation between the species among age classes (Table 2). Out of the 20 examined characters, there were 9 morphological leaf characters that featured significant differences between species in each age; which were leaf length, leaf width in each section, number of cross veins, space between cross veins (SC) in each section (except at 25% leaf area), space between the intra-marginal vein and lamina margin (SM) at the leaf tip and ratio between $\frac{1}{2}$ leaf width and the space between the intra-marginal vein and lamina margin (Fig. 5a-i). The differences in leaf

character between *H. major* and *H. ovalis* were closely observed as also summarized in Table 2. Leaf length and width ranged 23.9–29.4 mm and 10.8–12.6 mm in *H. major* and 10–17 mm and 4.3–836 mm in *H. ovalis*, respectively, (Fig. 5a–d). The numbers of cross veins were 14–18 veins in *H. major* and were 9–16 veins in *H. ovalis* (Fig. 5e). Space between cross veins at 50%, 75% and 100% leaf area were slightly increased in space with aged. However, *H. major* had wider space between cross veins than *H. ovalis* (Fig. 5f–h). Interestingly, the ratio between $\frac{1}{2}$ leaf width and the space between the intra-marginal vein and lamina margin was clearly different. A much greater ratio was found

Table 2 Summary of morphological characters between *H. major* and *H. ovalis* populations.

Characters [†]	LL (mm)	LW (mm)	CV (no.)	CVB [*] (no.)	SC (mm)	SM (mm)	SMA (mm)	AG (°)	½ LW/SM
<i>H. ovalis</i> (n)									
age [‡] (i) (8)	6.8–10.1	4.6–5.6	7–8	(2–3)	n/d [#]	0.1–0.4	0.37–0.39	59.3–77.3	1:8–9.2
age [‡] (ii) (7)	12.7–18.2	6.1–8.2	11–14	(1–4)	0.4–1.3	0.2–1.2	0.3–0.5	36.3–89.4	1:3.6–12.7
age [‡] (iii) (11)	11.2–17.0	4.3–8.3	9–16	(0–3)	0.4–1.4	0.2–1.1	0.3–0.5	30.4–88.5	1:4.1–9.7
age [‡] (iv) (12)	10.0–15.0	4.6–7.6	9–14	(0–3)	0.3–1.1	0.2–0.6	0.3–0.5	43.4–79.4	1:6.8–12.1
Japan ¹⁹	12–18	4–8	12–16	n/d	0.8–1.1	0.25–0.4	n/d	n/d	1:10–16
Vietnam ²⁴	9–12	3.7–7.0	8–16	n/d	n/d	0.3	n/d	45–80	1:9–17
<i>H. major</i> (n)									
age [‡] (i) (9)	11.7–19.5	7.1–12.6	9–16	(0–5)	0.5–1.5	0.1–0.5	0.2–0.5	36.5–87.4	1:16.6–22.2
age [‡] (ii) (5)	13.0–25.7	5.6–14.8	12–18	(1–6)	0.5–1.5	0.1–1.5	0.2–0.5	30.1–77.7	1:18.5–19.9
age [‡] (iii) (15)	25.9–29.4	10.8–12.6	14–18	(1–6)	0.6–2.0	0.1–0.6	0.4–0.6	27.7–75.4	1:16.6–27.1
age [‡] (iv) (8)	23.9–27.4	10.8–12.3	15–18	(1–6)	0.6–1.5	0.2–0.6	0.4–0.6	36.9–67.3	1:17.2–27.7
Japan ¹⁹	10–25	9–11	18–22	n/d	0.7–1.25	0.16–0.5	n/d	n/d	1:20–25
Japan ²³	10–30	5–15	12–19	n/d	0.7–1.25	0.1–0.2	n/d	n/d	1:20–25
Vietnam ²⁴	10–18	9–12	16–22	n/d	n/d	0.2–0.25	n/d	60–80	1:24–25

[†] LL: leaf length; LW: Leaf width; CV: cross veins; CVB: cross veins branching; SC: space between cross veins; SM: space between intra-marginal veins and leaf margin; SMA: space between intra-marginal veins and leaf margin at apex of leaf; AG: cross veins angle; ½ LW/SM: Half of leaf width per space between intra-marginal veins and leaf margin.

[‡] Populations in this study from Andaman Sea, Thailand.

^{*} Cross veins branching is common for all populations except for *H. ovalis* from Vietnam²⁴.

[#] n/d = no data.

in *H. major* and ranged between 1:16.58–27.74 and 1:3.59–12.65 in *H. ovalis* without any overlapping values (Fig. 5i). This, in fact, could be a dependable character for the identification of *H. major*.

DISCUSSION

H. major is reported to be distributed in Western Pacific region including Vietnam, Indonesia, Malaysia and the Indian Ocean including Thailand and Myanmar either through the morphological or molecular information^{12,19,20}. This study was the first report, which combined both morphological features and molecular information to identify *H. major* and suggested some key characters to identify this closely similar species. Unlike the study of Nguyen et al²⁰, where high genetic diversity of *H. ovalis* was reported across the Indo-Pacific Ocean, our results showed the identical sequences of *H. major* or between Trang and Krabi provinces (haplotype *Hm.1*, Fig. 4), however a much smaller scale. The distance between those sites are less than 100 km, where both influenced by the same water current thus low genetic diversity was expected. It would be interesting to further understand, dispersal, recruitment and sexual reproduction of this species.

Although *H. major* and *H. ovalis* have similar

shape and shows some overlap in size among leaf age groups (Table 2), they can be clearly distinguished by (1) a significant larger leaf size in all leaf ages, especially in the age (iii) and (iv); and (2) The ½ ratio between leaf width and the space between the intra-marginal vein and lamina margin is significantly higher in *H. major* than in *H. ovalis*. Thus we suggest that leaf ages as well as the ½ ratio are important for distinguishing these 2 closely similar species; and as well as other *Halophila* spp.

Although there are significantly more cross veins in *H. major* in most mature leaves, the number of cross veins in this study ranged only 14–18, which is much lower than those reported from Japanese populations (18–22 cross veins)¹⁹. This suggested that there is high variability in this character in *Halophila* spp. between various populations (Table 2). Overlap of characters is common and conspecific within this group, e.g., *H. minor* and *H. ovalis*, *H. major* and *H. miki*, *H. nipponica*, *H. okinawensis* and *H. gaudichaudii*²³. The nomenclature of the *Halophila* group is still confounding which limits the risk assessment of the dangers to the world's seagrass species²⁹. In addition to the distinguishing morphological and ITS characters, sexual reproductive features, flowers and fruits, would

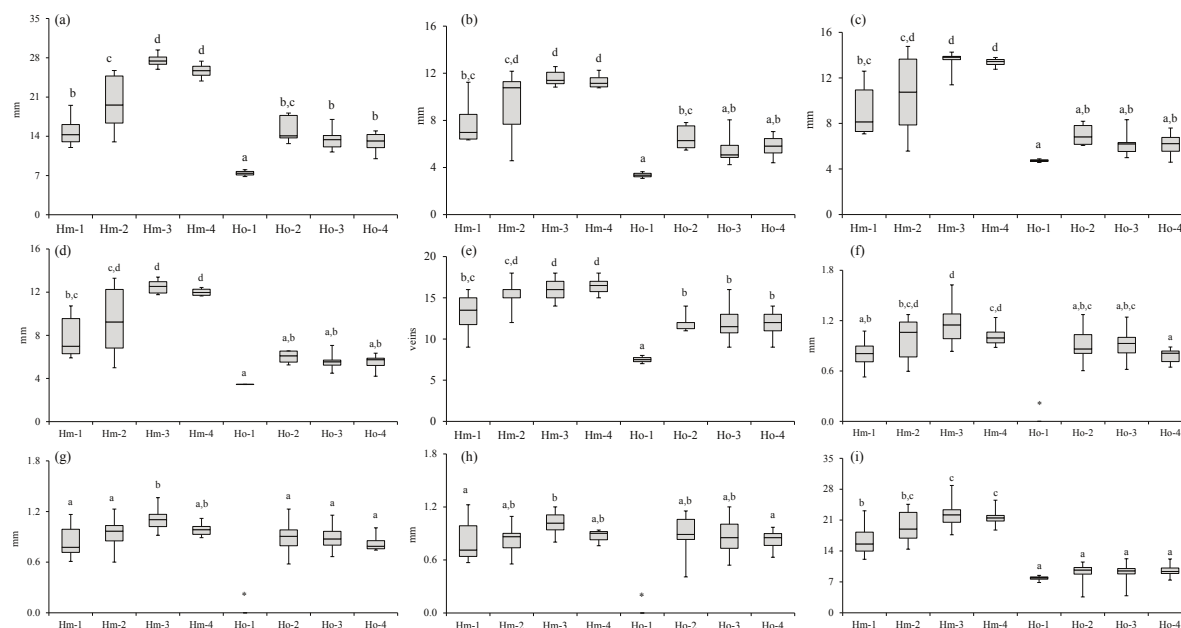


Fig. 5 Summary of morphological characters between *H. major* (Hm-) and *H. ovalis* (Ho-) populations among 4 age classes. Medians are highlighted in bold; bars represent the 25% and 75% quartiles; whiskers represent the lowest and highest data points. (a) Leaf length (Welch = 145.99, p -value = 0.000); (b) leaf width at 25% leaf area (Welch = 90.46, p -value = 0.000); (c) leaf width at the 50% leaf area (Welch = 183.19, p -value = 0.000); (d) leaf width at 75% leaf area (Welch = 597.11, p -value = 0.000); (e) number of cross veins (Welch = 44.69, p -value = 0.000); (f) space between cross veins at 50% leaf area (Welch = 11.09, p -value = 0.000) and * = no data; (g) space between cross at 75% leaf area (Welch = 12.34; p -value = 0.000); (h) space between cross at 100% leaf area (Welch = 5.52, p -value = 0.000) and * = no data; (i) ratio between 0.5 leaf width and space between intra marginal vein and leaf edge (Welch = 77.52, p -value = 0.000).

complete the description of the species. Establishing those characters was outside the realm of this study.

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REFERENCES

- den Hartog C (1970) *Seagrasses of the World*, North Holland Publishing Company, Amsterdam, The Netherlands.
- Short F, Carruthers T, Dennison W, Waycott M (2007) Global seagrass distribution and diversity: A bioregional model. *J Exp Mar Biol Ecol* **350**, 3–20.
- Larkum AWD, Orth RJ, Duarte CM (2006) *Seagrasses: Biology, Ecology, and Conservation*, Springer, Dordrecht, The Netherlands.
- Short FT, Moore GE, Peyton KA (2010) *Halophila ovalis* in the Tropical Atlantic Ocean. *Aquat Bot* **93**, 141–6.
- Ralph PJ, Burchett MD (1995) Photosynthetic responses of the seagrass *Halophila ovalis* (R. Br.) Hook. f. to high irradiance stress, using chlorophyll *a* fluorescence. *Aquat Bot* **51**, 55–66.
- Ralph PJ (1998) Photosynthetic response of laboratory-cultured *Halophila ovalis* to thermal stress. *Mar Ecol Progr* **171**, 123–30.
- Shafer DJ, Sherman TD, Wyllie-Echeverria S (2007) Do desiccation tolerances control the vertical distribution of intertidal seagrasses? *Aquat Bot* **87**, 161–6.
- Tanaka Y, Nakaoka M (2004) Emergence stress and morphological constraints affect the species distribution and growth of subtropical intertidal seagrasses. *Mar Ecol Progr* **284**, 117–31.
- Kuo J, den Hartog C (2001) Seagrass taxonomy and identification key. In: Short FT, Coles RG (eds) *Global Seagrass Research Methods*, Elsevier Science B.V., Amsterdam, pp 31–58.
- Chansang H, Poovachiranon S (1994) The distribution and species composition of seagrass beds along the Andaman Sea coast of Thailand. *Phuket Mar Biol*

- Cent Res Bull* **59**, 43–52.
11. Supanwanid C, Lewmanomont K (2003) The seagrasses of Thailand. In: Green EP, Short FT (eds) *World Atlas of Seagrasses*, UNEP World Conservation Monitoring Centre, Univ of California Press, Berkeley, USA, pp 144–51.
 12. Uchimura M, Faye EJ, Shimada S, Aral S, Inoue T, Nakamura Y (2008) A reassessment of *Halophila* species (Hydrocharitaceae) diversity with special reference to Japanese representatives. *Bot Mar* **51**, 258–68.
 13. Marsh H, Penrose H, Eros C, Hugues J (2002) Dugong: Status reports and action plans for countries and territories. UNEP Early Warning and Assessment Report Series UNEP/DEWA/RS. 02-1.
 14. Hines EM, Adulyanukosol K, Duffus DA (2005) Dugong (*Dugong dugon*) abundance along the Andaman coast of Thailand. *Mar Mamm Sci* **21**, 536–49.
 15. Prathep A, Rattanachot E, Tuntiprapas P (2010) Seasonal variations in seagrass percentage cover and biomass at Koh Tha Rai, Nakhon Si Thammarat Province, Gulf of Thailand. *Songklanakarin J Sci Tech* **32**, 497–504.
 16. Rattanachot E, Prathep A (2011) Temporal variation in growth and reproduction of *Enhalus acoroides* (L.f.) Royle in a monospecific meadow in Haad Chao Mai National Park, Trang Province, Thailand. *Bot Mar* **54**, 201–7.
 17. Haynes RA (2001) Hydrocharitaceae. In: Santisuk T, Larsen K (eds) *Flora of Thailand*, Vol. 7, Part 3, The Forest Herbarium, Royal Forest Department, Bangkok, pp 365–82.
 18. Kuo J (2000) Taxonomic notes on *Halophila ovata* and *Halophila minor*. In: Pergemp G, Pergemp-Marpini C, Buia M, Gambia M (eds) *Proceedings 4th International Seagrass Biology Workshop*, *Biol Mar Mediterr* **7**, 79–82.
 19. Kuo J, Kanamoto Z, Iizumi H, Mukai H (2006) Seagrasses of the genus *Halophila* Thouars (Hydrocharitaceae) from Japan. *Acta Phytotax Geobot* **57**, 129–54.
 20. Nguyen XV, Detcharoen M, Tuntiprapas P, Soe-Htun U, Sidik JB, Harah MZ, Prathep A, Papenbrock J (2014) Genetic species identification and population structure of *Halophila* (Hydrocharitaceae) from the Western Pacific to the Eastern Indian Ocean. *BMC Evol Biol* **14**, 92.
 21. Lucas C, Thangaradjou T, Papenbrock J (2012) Development of a DNA barcoding system for seagrasses: successful but not simple. *PLoS ONE* **7**, e29987.
 22. Nguyen XV, Japar SB, Papenbrock J (2013) Variability of leaf morphology and marker genes of members of the *Halophila* complex collected in Viet Nam. *Aquat Bot* **110**, 6–15.
 23. Uchimura M, Faye EJ, Shimada S, Aral S, Inoue T, Nakamura Y (2006) A re-evaluation of the taxonomic status of *Halophila euphlebia* Makino (Hydrocharitaceae) based on morphological features and ITS sequence data. *Bot Mar* **49**, 111–21.
 24. Nguyen XV, Holzmeyer L, Papenbrock J (2013) New record of the seagrass species *Halophila major* (Zoll.) Miquel in Vietnam: Evidence from leaf morphology and ITS analysis. *Bot Mar* **56**, 313–21.
 25. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–82.
 26. Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–8.
 27. Stamatakis A (2006) Phylogenetic models of rate heterogeneity: a high performance computing perspective. In: *Proceedings of the IPDPS2006 HiCOMB Workshop*, Rhodes Island, Greece.
 28. Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–4.
 29. Short FT, Polidoro B, Livingstone SR, Carpenter KE, Bandeira S, Bujang JS, Calumpong HP, Carruthers TJB, et al (2011) Extinction risk assessment of the world's seagrass species. *Biol Conservat* **144**, 1961–71.