Coastal Marine Science 35(1): 85–90, 2012

Morphological variations of the seagrass species, *Halophila nipponica* (Hydrocharitaceae, Alismatales)

Satoshi SHIMADA[1*], Maki WATANABE[2], Kensuke ICHIHARA[3] and Masayuki UCHIMURA[4]

[1] *Division of the Natural/Applied Sciences, Graduate School of Humanities and Sciences, Ochanomizu University, 2–1–1 Otuka, Bunkyo-ku, Tokyo, 112–8610, Japan*
**E-mail: shimada.satoshi@ocha.ac.jp*
[2] *Faculty of Science, Ochanomizu University, 2–1–1 Otuka, Bunkyo-ku, Tokyo, 112–8610, Japan*
[3] *Department of Biology, Faculty of Science, Toho University, Chiba, Japan*
[4] *Miura, Kanagawa, 238–0112, Japan*

Received 4 Nov. 2010; accepted 1 April 2011

Abstract—During recent field surveys in the Okinawa Islands, southern Japan, we found a peculiar seagrass growing at sandy ground 10 m in depth at one of the Islands, Ie Island (27 June 2010). The overall gross morphology of this plant was very similar to that of *Halophila* species, while the leaves were much narrower, up to 1 mm in width (up to 3 cm in length). The cross veins were not recognized with naked eyes and under dissecting microscope, but under optical microscope 2–4 cross veins and intramarginal vein were observed in the leaves. In cross section, the leaves were undulated each side of the midrib. To clarify the exact taxonomic status of this plant, the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA was analyzed. As the result, this plant was included in the *H. nipponica* clade and possessed the identical ITS sequence with the plants from Nakagusuku Bay, Okinawa Main Island having broad linear leaves, and from Ooura Bay, Ishigaki Island having elliptical leaves, typical forms in the *H. nipponica* clade. Our present study therefore suggests that *H. nipponica* has a marvelously wide range of morphological plasticity and that the gross morphology of leaves might be of little significance in specific descrimination.

Key words: *Halophila nipponica*, ITS, Japan, molecular phylogeny, morphology

Introduction

The seagrass genus *Halophila* Du Petit-Thouars (1806) is one of the most important marine plants due to its ecological roles as primary producer in marine environments (Larkum et al. 2006). Most of the *Halophila* species are reported to widely occur from warm-temperate to tropical seas around the world (Philips and Menez 1988, Kuo and den Hartog 2001), except *Halophila nipponica* J. Kuo from Aomori Prefecture, northern Japan (Kirihara et al. 2005). There are 21 specific names of *Halophila* in ALGAEBASE (Guiry and Guiry 2010), 16 of which have been flagged as currently accepted. In a recent review of the genus *Halophila* in Japan (Uchimura 2008), four taxa have been recognized as distinct: *H. decipiens* Ostenfeld, *H. major* (Zoll.) Miquel, *H. ovalis* (R.Brown) J.D. Hooker and *H. nipponica* J.Kuo.

During our recent field surveys in the Okinawa Islands (June 2010), we found a peculiar seagrass growing in the sandy ground 10 m deep at one of the Islands, Ie Island. From the overall gross morphology, this plant seemed to belong to the genus *Halophila*, but the leaves of the plant was much narrower than those of the so far described species. To clarify the taxonomic status of the plant, besides morphological observations, we made molecular phylogenetic analyses of the nuclear-encoded internal transcribed spacer region including 5.8S rDNA (Uchimura 2006a, 2006b, 2008). These results will provide a sounder basis for the specific and intraspecific recognition within the genus *Halophila*, marine Angiosperm.

Materials and Methods

The specimens were collected at the Ie Island, Okinawa Prefecture, Japan (Fig. 1) on 27 June 2010 from the sandy ground at 10 m depth (Fig. 2) by SCUBA diving. They were transported alive to the Ochanomizu University, Japan for morphological and molecular phylogenetic studies. Morphological observations were made on fresh specimens or on those preserved in −20° freezer. Sections were made by hand. The voucher specimens are pressed on sheets and housed in the herbarium of Ochanomizu University.

Molecular phylogenetic analysis was conducted based on Shimada et al. (2003) and Shimada et al. (2008). Total DNA was extracted from two samples using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) and protocol. The region selected for PCR amplification and automated se-

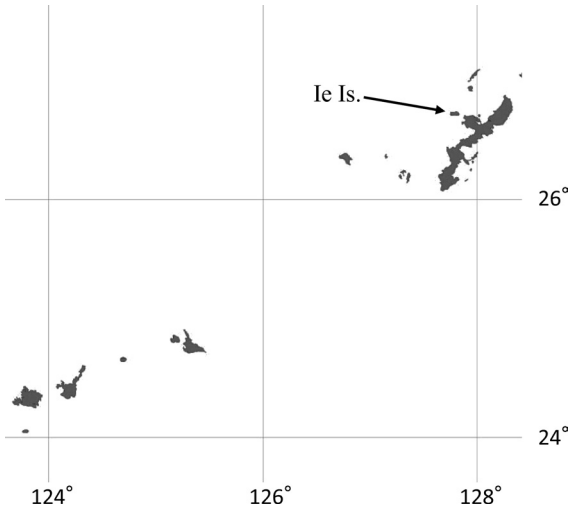


Fig. 1. Map showing locality of *Halophila* samples with the narrow leaves collected in this study, Ie Island, Okinawa Prefecture, Japan.

Fig. 2. Field habitat of the *Halophila* samples with the narrow leaves in this study, Ie Island, Okinawa Prefecture, Japan.

quencing was the nuclear ribosomal internal transcribed spacer (ITS) region including the 5.8S gene. The following pair of primers was used for PCR and cycle-sequencing reactions: ITS1 (5′-TCCGTAGGTGAACCT GCGG-3′) and ITS4 (5′-TCCTCCGCT TATTGATATGC-3′). They were derived from Waycott et al. (2002) published ITS sequences. PCR amplification was run on a PROGRAM TEMP CONTROL SYSTEM (Astec, Fukuoka, Japan) and the profile of the reactions was: initial denaturation 1 min at 94°C followed by 35 cycles of denaturation 45 sec at 94°C, primers annealing 45 sec at 50°C and extension 60 sec 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' protocol. Cycle-sequencing reactions consisted of an initial step of 96°C for 10 sec, followed by 25 cycles (96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min) and a final hold at 4°C. Both forward and reverse strands were sequenced using a DNA autosequencer (ABI PRISM, 3130 Genetic Analyzer, Applied Biosystems, CA, USA). The newly determined sequences were added to the alignment files used in the previous report (Uchimura et al. 2008, Table 1, including 62 GenBank data). Identical sequences within each species were excluded from the alignment. As outgroup species, *Halophila beccarii* Ascherson (GenBank accession no. AF366441) and *H. engelmanni* Ascherson (GenBank accession No. AF366404) were chosen following Waycott et al. (2002). The alignment is available from the first author upon request.

Phylogenetic analysis was performed using the Maximum Likelihood (ML) algorithm available in the computer program PAUP V. 4.0 b10 (Swofford 2002). The program MODELTEST version 3.7 (Posada and Crandall 1998) was used to find the model of sequence evolution that best fit each dataset by a hierarchical likelihood ratio test (a=0.01). When the best sequence evolution model was determined, ML tree searches were performed using the estimated model parameters with the following options: starting tree option=obtained by stepwise addition, and branch swapping algorithm=TBR. Bootstrap values based on 100 re-samplings in ML dataset were calculated (TBR, full heuristic search option) (Felsenstein 1985).

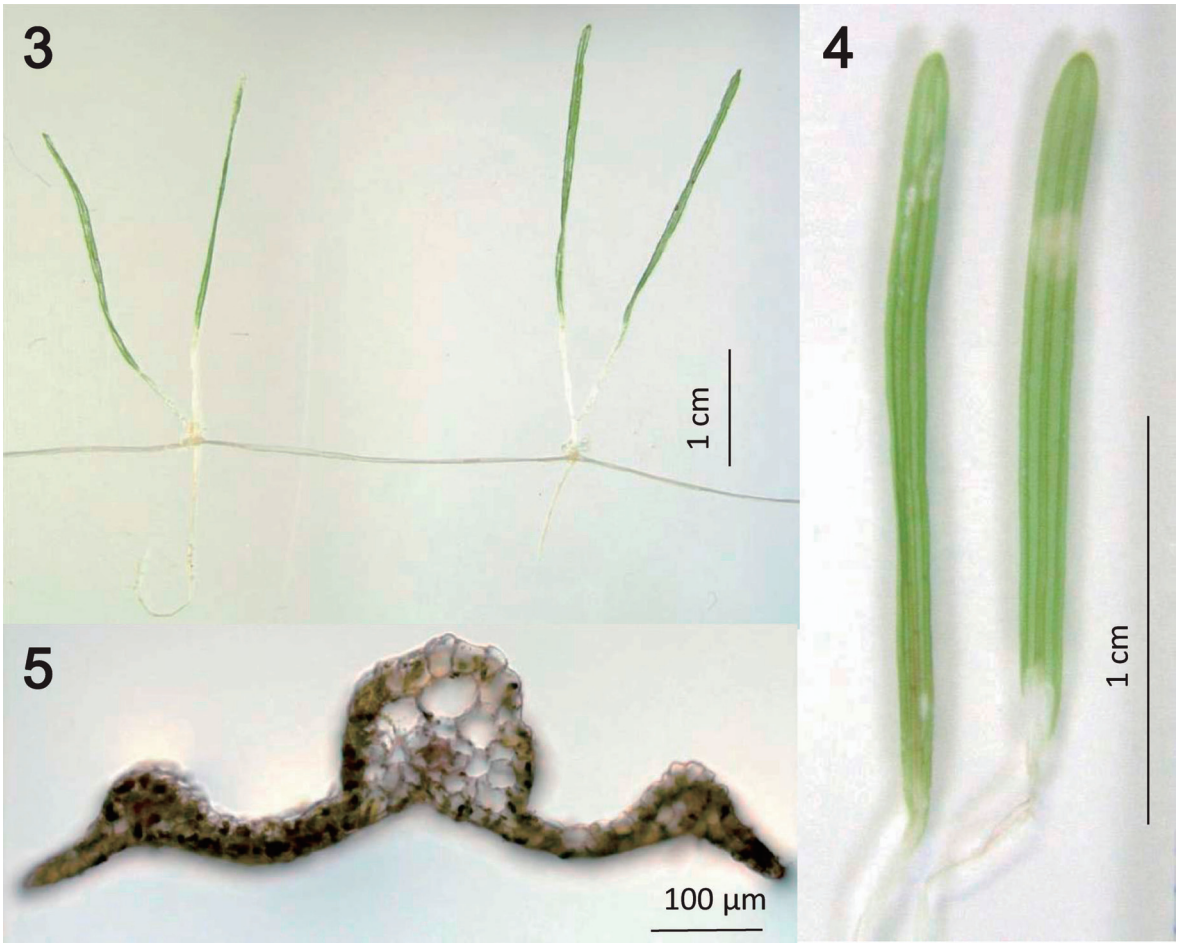
Results

Morphology

Plants are generally robust and consist of irregularly segmented rhizomes bearing at each node a single root downward and an erect shoot upward (Fig. 3). The rhizome is creeping, often buried in the sediments, irregularly branched, 1 mm thick. Internodes are 1.4–2.8 cm long. Erect shoots consist of petiolate leaves growing in pair (Fig. 3). Petioles are 0.5–1.4 cm long, 0.3–0.5 mm in diameter, enveloped at the base with one transparent scale, obovate, up to 3 mm long. Leafy blades are rigid in texture with glabrous surfaces and smooth margins. They are yellow-green to dark-green in color, very narrowly linear or oblanceolate in shape, rounded or obtuse at apex, up to 3 cm long, up to 1 mm wide. The intra-marginal vein and cross veins were not observed with naked eyes and under dissecting microscope (Fig. 4), but an intra-marginal vein and 2–4 cross veins were recognized in the leaves under optical microscope. In cross section, the leaves were undulated each side of the midrib (Fig. 5). The cross veins were not closely arranged to one another, alternate to sub-opposite along a central mid-vein. The distance

Table 1. Samples information used in the molecular phylogenetic analysis in this study.

Num.	Taxon	First Identification	Collection information
1	*Halophila beccarii* Ascherson		Gia Luan, Vietnam
2	*Halophila engelmannii* Ascherson		Florida Bay, USA
3	*Halophila tricostata* Greenway		Whitsunday Island, Australia
4	*Halophila spinulosa* (R. Br.) Ascherson		Pulau Perhentian, Malaysia
5	*Halophila australis* Doty et Stone		Limeburners, Greater Geelong, Victoria, Australia
6			Cowes City, Phillip Island, Victoria, Australia
7			Bay of Shoals, Kangaroo Island, Australia
8	*Halophila decipiens* Ostenfeld		Nakagusuku Bay, Okinawa
9			Nakagusuku Bay, Okinawa
10			Maui Corsair Point, Hawaii, USA
11			Oura Bay, Okinawa
12			Izena-Gyoko, Izena Island, Okinawa
13			Southeastern Costa Rica
14			Indian River, Florida, USA
15			Kuala Setiu, Malaysia
16			Kepple Isles, Middle Island, Capricorni Group, Queensland, Australia
17	*Halophila stipulacea* (Forsskal) Ascherson		Sicily, Italy
18	*Halophila major* (Zoll.) Miquel		Nakagusuku Bay, Okinawa
19			Trang, Thailand
20		"*H. australis*"	Two Peoples Bay, southwestern Australia
21		"*H. mikii*"	Urata, Tanegashima Island, Kagoshima
22			Nakagusuku Bay, Okinawa
23			Shishikui, Tokushima
24			Mugi-Ooshima, Tokushima
25			Urasoko, Ishigaki Island, Okinawa
26			Itona, Ishigaki Island, Okinawa
27			Kambing, Bay of Bima, Sumbawa, Indonesia
28			Sindhu Beach, Bali, Indonesia
29		"*H. ovalis*"	Hilutangan, Philippines
30	*Halophila ovalis* (R. Br.) J. D. Hooker	"*H. hawaiiana*"	Oahu, Waialae Beach, Hawaii, USA
31		"*H. hawaiiana*"	Oahu, Hawaii, USA
32			Dingo Beach, Whitsundays Queensland, Australia
33			Wair Terang, Flores Island, Indonesia
34			Darat Pante, Flores Island, Indonesia
35			Parigata Beach, Sanur, Bali, Indonesia
36		"*H. minor*"	Kajouwulu, Flores Island, Indonesia
37		"*H. johnsonii*"	Florida, USA
38			Nakagusuku Bay, Okinawa
39			Kayou, Okinawa
40			Trang,Thailand
41			Trang, Thailand
42			Kabila Bay, Ishigaki Island, Okinawa
43			Taketomi Island, Okinawa
44	*Halophila nipponica* J. Kuo	"*H. gaudichaudii*"	Agania Bay, Guam
45		"*H. gaudichaudii*"	Tumon Bay, Guam
46		"*H. okinawensis*"	Shirahama, Iriomote Island, Okinawa
47		"*H. okinawensis*"	Saki-Eda, Ishigaki Island, Okinawa
48		"*H. okinawensis*"	Nakagusuku Bay, Okinawa
49		"*H. okinawensis*"	Oura Bay, Okinawa
50		"*H. okinawensis*"	Nakagusuku Bay, Okinawa
51			Odawa Bay, Kanagawa
52			Mukoujima, Naoshima, Kagawa
53			Koshiki Is., Kagoshima
54			Mutsu Bay, Aomori
55			Suou-Oohshima, Yamaguchi
56			Mugi-Ooshima, Tokushima
57			Ezura, Shirahama-cho, Wakayama
58			Urumi, Chibu Island, Shimane
59			Oohama, Takuma, Kagawa
60			Mihokogaura Park , Miyazaki
61			Sasebo, Nagasaki
62			Hasama, Tateyama, Chiba
63			Ie Island, Okinawa



Figs. 3–5. Morphological features of the *Halophila* samples collected in this study. Fig. 3: Fragments of wet living specimens. Fig. 4: Detail of leaves under dissecting microscope. Fig. 5: Cross section of middle region of a leaf.

between intra-marginal vein and the margin is markedly small, ranging from 0.1 mm at the lower parts of the leaves and about 0.2 mm at the apex (Fig. 2). The mid-veins unite with intra-marginal veins at leaf apices without extending further.

Molecular phylogeny

The phylogenetic tree obtained with the ML method is presented in Fig. 6. Likelihood settings from the best-fit model (GTR+G) were selected by a hierarchical likelihood ratio test in the program MODELTEST version 3.7: assumed nucleotide frequencies A=0.19020, C=0.30500, G=0.29200, and T=0.21280; substitution-rate matrix with AC=1, AG=2.789900, AT=1, CG=0.5352400, CT=1, and GT=1; proportion of invariable sites=0.3781; gamma distribution with shape parameter=0.4012. Based on these settings, a heuristic search was performed with the TBR branch swapping option ($-\ln L=2231.92689$) after 15851 rearrangements.

The plant in question with very narrow, linear-type leaves from the Ie Island (Fig. 7c) was included in the *H. nipponica* clade and possessed the identical ITS sequence with the plants collected from the Nakagusuku Bay of the Okinawa Island (Fig. 7b) and that from the Ooura Bay of the Ishigaki Island (Fig. 7a). The *H. nipponica* clade showed wide range of morphological variation from elliptical leaves to linear-type leaves (Fig. 7).

Discussion

Kuo et al. (2006) reported that there are eight morphological species of *Halophila* in Japan. However, in the molecular reassessment of the *Halophila* species focusing on Japanese representatives, Uchimura et al. (2008) concluded that four phylogenetic species are recognizable in Japan: *H.decipiens, H. major, H. ovalis* and *H. nipponica*. Among the latter three species with glabrous leaves and smooth margins, *H. nipponica* widely distributes from subtropical (Okinawa Prefecture) to warm and temperate region (Aomori Prefecture) and is known to have three distinctive morphotypes in leaves (wide-leaf type, narrow-leaf type and intermediate-leaf type). In this species, the narrowest leaves are reported to be over 1.5 mm in width (Uchimura et al. 2008). As our

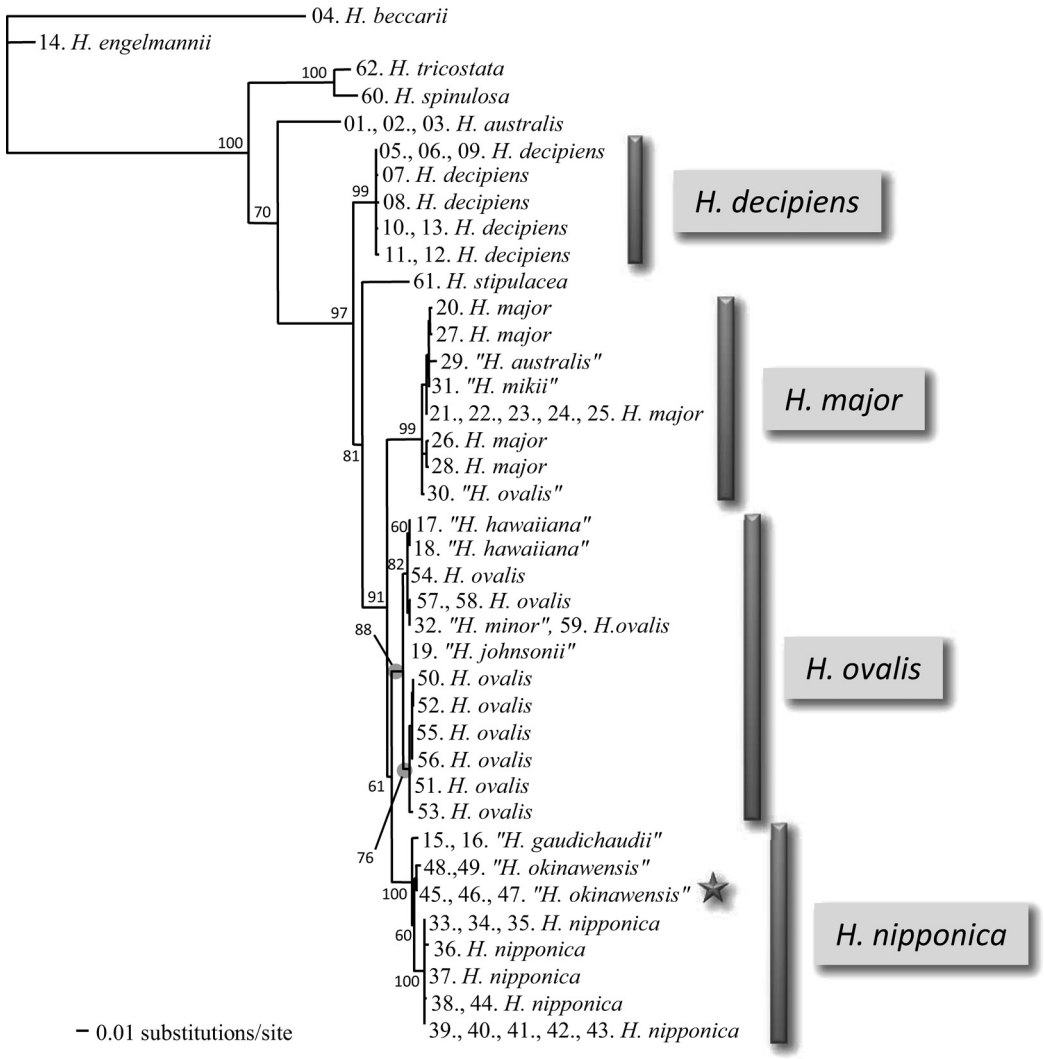


Fig. 6. Phylogenetic tree of the maximum likelihood (ML) analysis inferred from the nuclear encoded ITS regions including 5.8S rDNA of *Halophila*. Numerals at internal nodes are bootstrap values above 50% for 100 replicates in ML analysis. Star indicates the phylogenetic position of our plant.

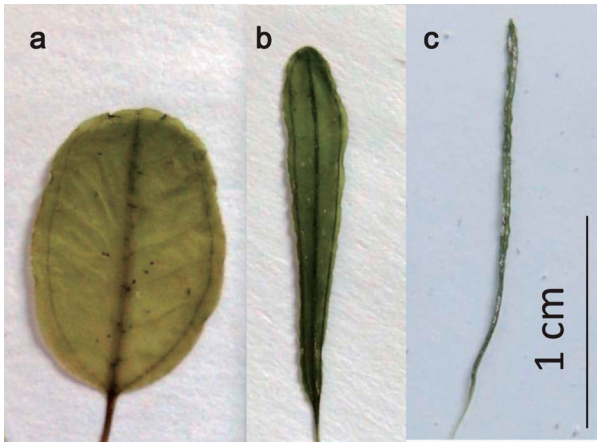


Fig. 7. Intra-species variation in *H. nipponica*. a: elliptical leaves, typical form of this species, collected from the Ooura Bay of the Ishigaki Island, Okinawa Pref. b: linear-type leaves, narrow-leaf type of this species, collected from the Nakagusuku Bay of the Okinawa Island, Okinawa Pref. c: our plant.

plant has narrower leaves (up to 1 mm wide) than those of *H. nipponica*, we hesitate to place it in the species immediately. However, our molecular analysis has shown that it is included in the H. nipponica clade (Fig. 6).

Recently, Frederic et al. (2010) reported that both morphological and genetic analyses of the *Halophila johnsonii* Eiseman collected in Salt Pond, Antigua, determined it to be *H. ovalis*, and concluded that the morphology of *H. johnsonii* falls within the bounds of what has been reported for *H. ovalis*. *Halophila ovalis* is widely distributed in temperate North Pacific, tropical Indo-Pacific and temperate Southern Oceans (Frederic et al. 2010). These results suggest that *Halophila* species with a wide distribution might possess several intra-specific entities with wide genetic diversity, and have wide range of morphological variations. Detailed morphological observation of voucher herbarium specimens using molecular phylogenetic analyses, and culture experiments will be needed to exactly understand these inter-

and/or intra-specific variations.

In our molecular phylogenetic analysis, *Hlophila ovalis* clade included "*H. hawaiiana* Doty et Stone", "*H. minor* (Zollinger) den Hartog" and "*H. johnsonii*" (Fig. 6). According to Uchimura et al. (2008), *H. minor* and *H. ovalis* are conspecific. These situations strongly suggest that the current taxonomy of *Halophila* is still confusing. To make a better understanding of the genus, more global collection, detailed morphological and molecular phylogenetic analyses would be quite necessary. These studies clear the relationship between phylogenetic species and morphological species.

Acknowledgements

We are deeply indebted to Dr. T. Abe of Hokkaido University, R. Terada of Kagoshima University and Dr. S. Kawaguchi of Kyushu University, for helpful discussions. We are deeply indebted to Dr. T. Abe of Hokkaido University, R. Terada of Kagoshima University and Dr. S. Kawaguchi of Kyushu University, for helpful discussions. We are also thankful to Multilateral Core University Program (Coastal Marine Science) of the Japan Society for the Promotion of Science, for research support.

References

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Frederic, T. S., Gregg E. M. and Kimberly, A. P. 2010. *Halophila ovalis* in the tropical Atlantic Ocean. Aquatic Botany 93: 141–146.

Guiry, M. D. and Guiry, G. M. 2010 AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. 2010; available at: http://www.algaebase.org (searched on 7 Sep. 2010).

Kirihara, S., Fujita, D. and Notoya, M. 2005. Records of seagrass *Halophila ovalis* in Mutsu Bay. J. Jap. Phycol. 53: 237–239.

Kuo, J., Kanamoto, Z., Iizumi, H. and Mukai, H. 2006 Seagrasses of the genus *Halophila* Thouars (Hydrocharitaceae) from Japan. Acta Phytotax. Geobot. 57: 129–154.

Kuo, J and den Hartog, C. 2001. Seagrass taxonomy and identification key. *In* Global seagrass research methods. Short, F. T. and Coles, R. G. (eds.), pp. 31–58, Elsevier Science, Amsterdam.

Larkum, A. W. D., Orth, R. J. and Duarte, C. D. 2006. Seagrasses: biology, ecology, and conservation. Springer, Dordrecht.

Phillips, R. C. and Menez, E. G. 1988. Seagrasses. Smithson. Contrib. Mar. Sci. 34: 1–104.

Posada, D. and Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

Shimada, S., Hiraoka, M., Nabata, S., Iima, M. and Masuda, M. 2003. Molecular phylogenetic analyses of the Japanese *Ulva* and *Enteromorpha* (Ulvales, Ulvophyceae), with special reference to the free-floating *Ulva*. Phycol. Res. 51: 99–108.

Shimada, S., Yokoyama, N., Arai, S. and Masuda, M. 2008. Phylogeography of the genus *Ulva* (Ulvophyceae, Chlorophyta), with special reference to the Japanese freshwater and brackish taxa. J. Appl. Phycol. 20: 979–989.

Swofford, D. L. 2002. PAUP*: Phylogenetic analysis using parsimony (*and other methods), Version 4. Sinauer Associates, Inc., Sunderland, Massachusetts.

Uchimura, M., Faye, E. J., Shimada, S., Arai, S., Inoue, T. and Nakamura, Y. 2006a. A re-evaluation of the taxonomic status of *Halophila euphlebia* Makino (Hydrocharitaceae) based on morphological features and ITS sequence data. Bot. Mar. 49: 111–121.

Uchimura, M., Faye, E. J., Shimada, S., Inoue, T. and Nakamura, Y. 2008. A reassessment of *Halophila* species (Hydrocharitaceae) diversity with special reference to Japanese representatives. Bot. Mar. 51: 258–268.

Uchimura, M., Faye, E. J., Shimada, S., Ogura, G., Inoue, T. and Nakamura, Y. 2006b. A taxonomic study of the seagrass genus *Halophila* (Hydrocharitaceae) from Japan: description of a new species *Halophila japonica* sp. nov. and characterization of *H. ovalis* using morphological and molecular data. Bull. Nat. Sci. Mus. 32: 129–150.

Waycott, M., Freshwater, D. W., York, R. A., Calladine, A. and Kenworthy, W. J. 2002. Evolutionary trends in the seagrass genus *Halophila* (Thouars): Insights from molecular phylogeny. Bull. Mar. Sci. 71: 1299–1308.