

Phylogenesis of *Halophila ovalis* (R. Br.) Hook. fil. (Hydrocharitaceae) from An Island, Korea

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Halophila ovalis (R. Br.) Hook. fil. was first collected from the Ando, Yeosu, Korea in 2007. *H. ovalis* is widely distributed from sub-tropical to even tropical areas and produces the seeds using bisexual reproduction. Its leaf shape was oblong to ovate. Its leaf blades were rigid in texture, with a strong support to the leaf. Erect shoots arose at irregular intervals along the rhizome. The distance between the intramarginal vein and leaves margin was small. Nucleotides in ITS 1 and ITS 2 regions between the Korean and Japanese *H. ovalis* were found to be 100% similar, whereas Korean *H. ovalis* was found to have four nucleotides in the positions of 202 bp to 206 bp for 5.8S. In the analysis of the phylogenetic relationship using NJ method, Korean *H. ovalis* had a monophyletic genetic tree with Japanese *H. ovalis*, but no phylogenetic relationship with types from the Philippines, Australia, Malaysia, and Vietnam. The first occurrence of *H. ovalis* in Korea was associated with a strong migration of gene flow from Japan and high water temperature caused by the variations in climate.

Key words : Ando, *Halophila ovalis*, ITS, morphology, phylogenetic relationship, unrecorded species

Introduction

It is known that the genus *Halophila* has 13 species [3]. Most have a considerably wide distribution from warm-temperate to even tropical waters [3]. Some researchers have reported their morphological variability based on phenotypic characters and finally taxonomic problems that have recently emerged [3,9]. Advancing in DNA amplification, approaches in *Halophila* are based on the information of genotype properties and nucleic acid sequencings instead of using classical morphology. To resolve taxonomic confusion, some researchers have tested the sequence analysis [5-7,11,14,17].

Halophila ovalis (R. Br.) Hook. fil. belongs to the genus *Halophila* and is widely distributed in the tropical to sub-tropical regions [3]. This species is also reported to experience morphological variations [1]. More recently, Waycott et al. [15] carried out the DNA sequencing inferred from the nuclear internal transcribed spacer (ITS) region and suggested taxonomic revision against geographic distribution of *H. ovalis*. Moreover, it is difficult to differentiate *H. ovalis* from *H. euphlebia* based on morphological features and Uchimura et al. [14] analyzed the DNA sequences between *H. ovalis* and *H. euphlebia*. This study ana-

lyzed the morphological features of Korean *H. ovalis* and sequenced the ITS region, and examined the phylogenetic relationship between Korean *H. ovalis* and Japanese *H. ovalis* including several species of *Halophila*.

Materials and Methods

Specimen

Halophila ovalis was collected from Ando, and along the coastal waters of Yeosu, Korea, in 2007. Leaf shape, leaf length, leaf width, marginal serration, lateral vein, and distance leaf edge per intramarginal described by Kuo and den Hartog were examined [1] Photographs were taken using an Olympus B202 (Olympus, Japan) attached digital camera (Olympus SZ-PT, Japan).

Total RNA extraction

Total RNA was extracted from 0.05 g of blotted wet weight of leaf materials using TRI Reagent kit (Molecular Research Center) after homogenizing the pellets. The RNAs were frozen at -80°C until required.

RT-PCR

Synthesis of complementary DNA (cDNA) from total RNA and PCR amplification using the cDNA as a template were carried out using a random hexamer (Bioneer, Korea) and *AccuPower* RT/PCR premix kit (Bioneer, Korea) ac-

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according to the manufacturer's instructions.

PCR

Amplification of the ITS region was conducted using primers ITS-F (5'-TCCGTAGGTGAACCTGCCG-3') and ITS-R (5'-TCCTCCGCTTATTGATATGC-3') including portion of 18S and 28S ribosomal RNA (Uchimura et al., 2006). PCR amplification was performed with 0.2-0.5 µg of template DNA in a reaction mixture of 25 µl containing 1.25 units of *Taq* DNA polymerase (FastStar *Taq* DNA polymerase, Roche Co.), 0.5 mM of each dNTPs, 10× PCR reaction buffer (Roche Co.) and 20 pmol of each primer. The PCR amplification was conducted over 35 cycles, as follows; 45 sec at 94°C, 45 sec at 50°C, and 60 sec at 72°C. Products from specific PCR amplification were analyzed using a 2% agarose run at 50V for 50 min and visualized after staining in 0.5 µg ml⁻¹ ethidium bromide. The PCR product was purified using PCR Purification kit (NucleoSpin[®] Extract) by the following manufacture's instruction. Purified DNA fragment was stored at -20°C until used. The purified DNAs were ligated with the vector, pUC 18 DNA (Roche Co.), using a One Shot[®] Mach1[™]-T1[®] cloning kit (Invitrogen).

Sequencing

The purified DNA using an Applied Biosystem model ABI 3730XL automated sequencer and a Big Dye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, UK). For the sequencing reaction, 30 ng of purified PCR products, 2.5 pmol of primer, and 1 µl of Big Dye terminator were mixed and adjusted to a final volume of 7 µl with dH₂O. The reaction was run with 5% DMSO for 30 cycles of 15 sec at 95°C, 5 sec at 50°C, and 4 min at 60°C. Both strands were sequenced for crosscheck.

Phylogenetic analysis

Sequences were aligned using the multiple alignment program Clustal W [13] and were determined by parsimony, distances and maximum likelihood (ML) methods. To understand the possible genetic relationships, PHYLIP (Phylogenetic Inference Package) ver. 3.573c (Felsenstein, 1993) was used in this study. PHYLIP was used for *Halophila* species derived from GenBank data (*H. becarii* AF366441, *H. engelmannii* AF366404, *H. spimulosa* AF366440, *H. tricostata* AF366438, *H. decipiens* AB243980, *H. stipulacea* AF366436, *H. australis* AF366414, *H. euphlebica* AB243965, *H.*

minor AF366405, *H. hawaiiiana* AF366426, *H. ovalis* AF366416, *H. ovalis* AF366429, *H. ovalis* AF366437, *H. ovalis* AF366420, *H. ovalis* TNS-752693 AB243975, *H. ovalis* TNS-752696 AB243970, *H. ovalis* TNS-752694 AB243974, *H. ovalis* TNS-752692 AB243973, *H. ovalis* TNS-752691 AB243972, *H. ovalis* TNS-752695 AB243976, *H. ovalis* TNS-752690 AB243971). This search for parsimony analysis was repeated several times from different random starting points using the stepwise addition option to make certain the most parsimonious tree was found. For distance analysis, the subprogram DNADIST in PHYLIP was used to obtain a matrix of Kimura's two-parameter distance [2]. Distance matrix was analyzed by subprogram NEIGHBOR in PHYLIP with algorithms based on Saitou and Nei's neighbor-joining (NJ) method [12]. All nucleotide substitutions were equally weighted and unordered alignment gaps were treated as missing information. The data set was iterated 100 times using a subprogram SEQBOOT. Reliability of the tree was constructed using the subprogram CONSENSE in PHYLIP after pairwise sequence distances were estimated by Kimura's two-parameter method, which attempts to correct observed dissimilarities for multiple substitutions in sequences evolving with a transition bias.

GenBank accession number

The determined ITS region sequences were deposited at the National Center for Biotechnology Information (NCBI) data library. Their accession numbers are EU477609.

Results and Discussion

Morphology and ecology

This species was generally robust and consisted of segmented rhizomes. It experienced dioecious reproduction. A morphological illustration is shown in Fig. 1. Its leaf shape was oblong to ovate (Fig. 1a). The rhizome was creeping, with irregular slender branches. Erect shoots arose at irregular intervals along the rhizome and consisted of petiolate leaves. Internodes had one unbranched root and a pair of petiolate leaves at each node. Petioles enveloped at the base with one transparent scale. Leaf blades were rigid in texture, rounded at the apex and base. The distance between the intramarginal vein and leaves margin was small, ranging approximately 0.1 mm at the lower parts of the leaves (Fig. 1b).

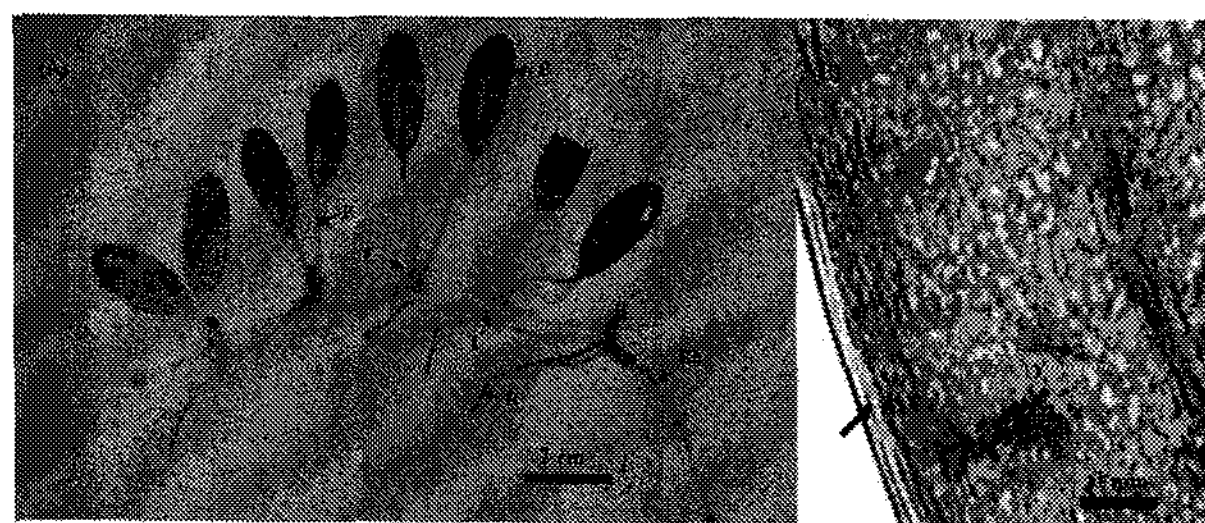


Fig. 1. Photograph showing *Halophila ovalis* collected from Ando, Yeosu, Korea, in 2007. A. General features of *H. ovalis*. P, petiol; F, flower; B, blade; I, internode; Rh, rhizome; R, root. B. Enlarged portion of leaf apex showing the space between intra-marginal vein (arrow) and margin.

Korean waters are influenced by different minor systems (the Kuroshio current; the Tsushima warm current; the East Korea warm current; the North Korea cold current; the Yellow Sea warm current; and the Korean coastal waters) [8], and this may be associated with an abundant nutrients and cause changes in water temperature. In this sense, Ando based on geographical characteristics is highly vulnerable to a strong intrusion of offshore water current (i.e., the Tsushima warm current). Warm water currents are not only associated with a desirable development of morphometric traits, but also with the healthy growth of *H. ovalis* in An Island.

Phylogenetic relationship

The primer pair ITS-F and ITS-R toward to a portion of 18S rRNA and ITS and partial of 28S rRNA was used successfully to obtain a PCR product of predictable size for *H. ovalis* (Fig. 2). Nucleotide sequences for Korean and Japanese *H. ovalis* were aligned (Fig. 3). The position of 101 to 400 in nucleotide showed the ITS region that ITS1 contained the position of 101 to 200 in nucleotide and 5.8S had the position of 201 to 300 in nucleotide and 300 to 400 in nucleotide comprised ITS2. Interestingly, Korean *H. ovalis* showed the insertion of four nucleotides at 202 to 205 while ITS1 and ITS2 between Korean and Japanese *H. ovalis* had 100% similarity. In 28S rRNA, four nucleotides at 506 to 509 were found in Korean *H. ovalis*. The length of the ITS region in *H. ovalis* from Japan, Australia, the Philippines, and Malaysia had a 300 bp, but Korean *H. ovalis* showed a 304 bp. In the analysis of PHYLIP inferred from the distance of the aligned sequencing targeted to ITS using neighbor-joining method, the genetic position of Korean *H. ovalis* was close to Japanese *H. ovalis*, which

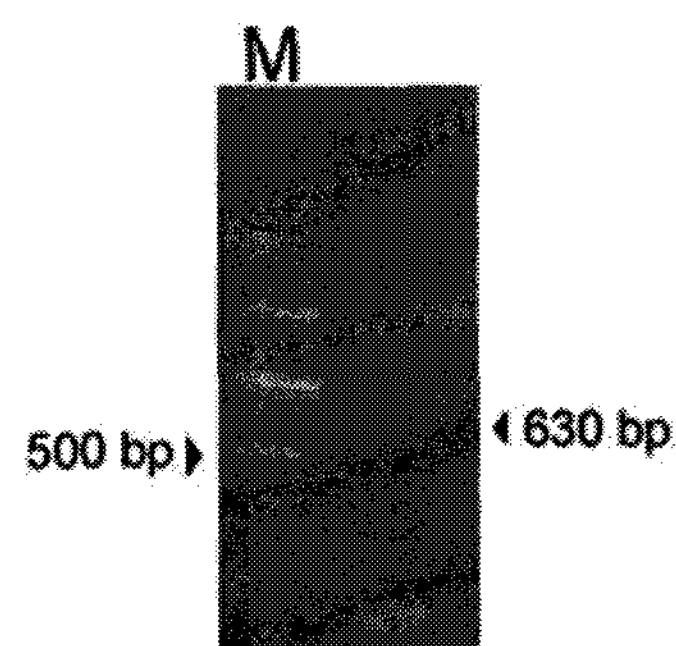


Fig. 2. PCR product of *Halophila ovalis* inferred from 18S rRNA and ITS region and 28SrRNA using species-specific primers ITS-F and ITS-R. M. molecular mark (100 bp).

supported a strong bootstrap value of 100%, but did not join *H. ovalis* from the Philippines, Australia, Vietnam, and Malaysia (Fig. 4). Phylogenetic trees based on parsimony and ML methods had the same topology.

Waycott et al. [15] suggested that ITS sequence analysis might be useful for investigations of sub-generic relationships within other seagrass genera. Likewise, the use of ITS sequencing data in this study also provides a high resolution of differentiating the individual of *H. ovalis* in Korea from those in the Philippines, Australia, Vietnam, and Malaysia. On the basis of genetic tree, the genetic placement of the Korean *H. ovalis* had a monophyletic relationship with Japanese *H. ovalis*, while it did not show a close phylogenetic relationship and strong bootstrap between Korean and those from the Philippines, Australia, Vietnam, and Malaysia, although they were morphologically similar. den Hartog [1] has already pointed out that the *H. ovalis* 'complex' was an unusual range of morphological variability and required for greater study to understand the relationship between environment and genetic variation. In effect, the environmental parameters in Korea are closer to Japan than to the Philippines, Australia, Vietnam, and Malaysia. On the basis of genetics, similar environmental conditions may possibly play a major role in the formation of monophyletic relationships and same functional genotype. Furthermore, *H. ovalis* is dioecious and the movement of pollen allows for a high level of genetic diversity. Larkum [4] suggested that seeds dispersed to distant sites by a floating rather than by a sinking mechanism. However, seagrass is generally limited to out-crossing, but the out-crossing rate is positively correlated with clonal diversity [10]. The occurrence of *H. ovalis* in Korea may be caused by a strong water movement of seeds from Japan. On the other hand, ballast water, used

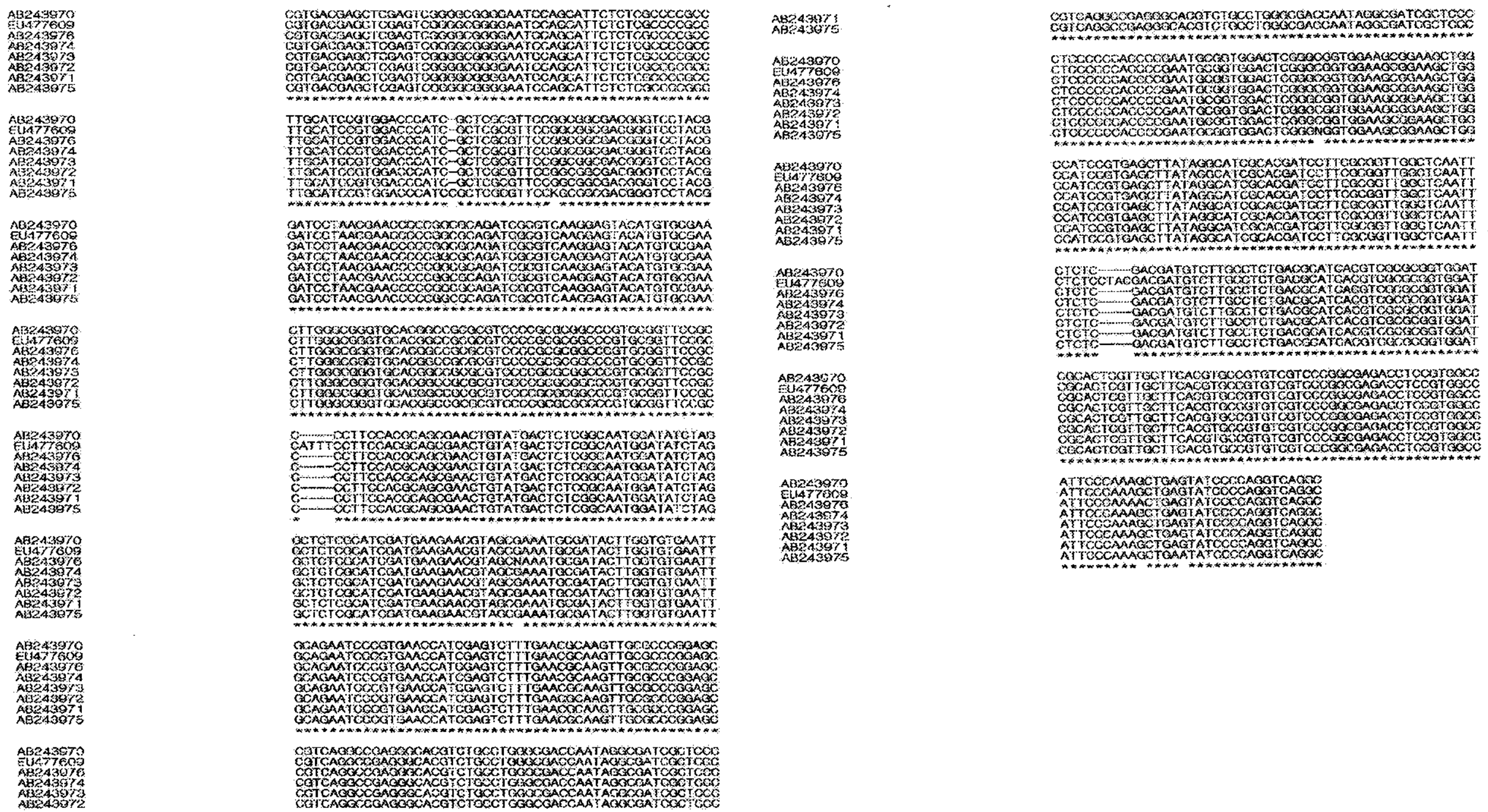


Fig. 3. Sequence alignment of 18S rRNA and ITS region and 28S rRNA. The alignment was generated by the multiple alignment program CLUSTAL W using a gap weight of 3.0 and a gap length weight of 0.1. 18S rRNA spans from 1 to 100 bp; ITS1 was from 101 to 200 bp; 5.8S was from 201 to 300 bp; ITS2 was from 301 to 400; 28S rRNA was 401 to 630 bp. A hyphen represented a gap and a period showed a base identical to that of the top sequence. An asterisk indicated an identical sequence on vertical lines. The source of each sequence was as follows: From the number of AB243970 to AB243975 was Japanese *H. ovalis*, EU477609 was Korean *H. ovalis*.

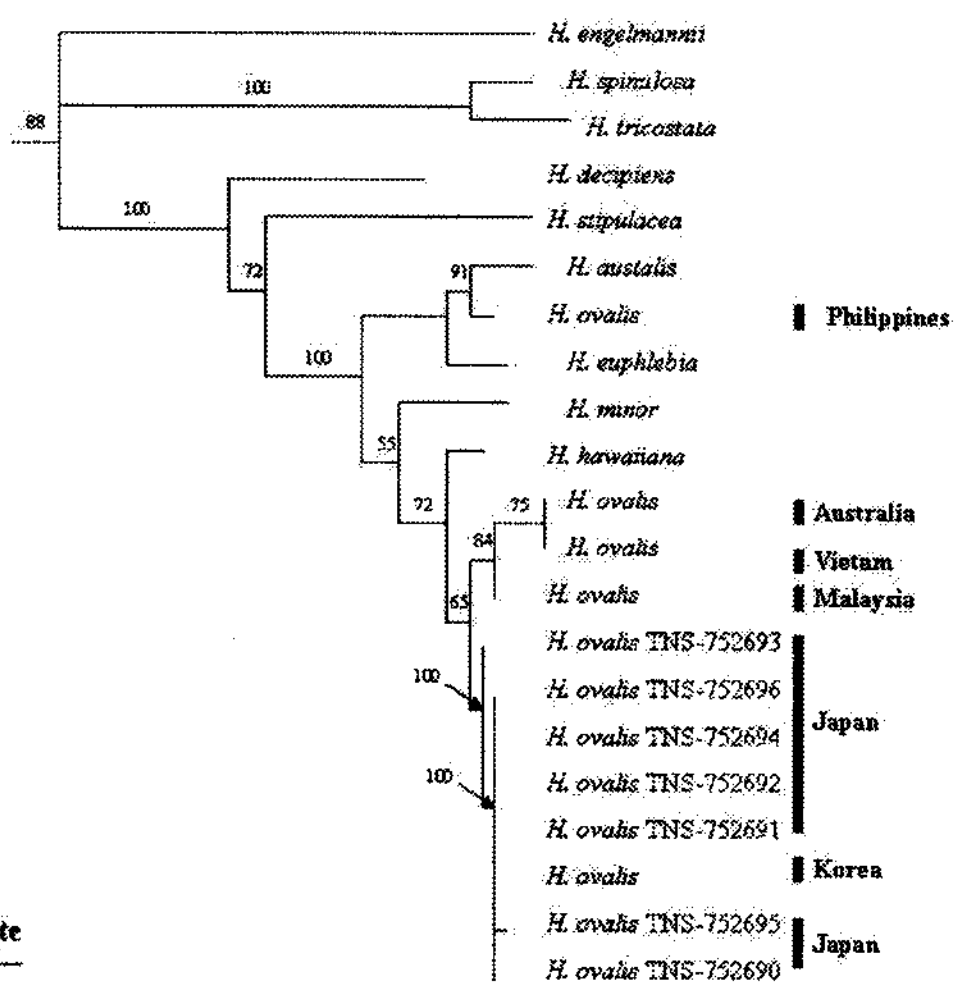


Fig. 4. PHYLIP analysis of 10 species obtained from GenBank database including Korean *Halophila ovalis*. Phylogenetic tree was constructed by inferring from nucleotide sequences of ITS region. The tree was generated using subprogram NEIBOR in PHYLIP with the option of Kimura's two-parameter method. Bootstrap values (100 replications) are given above the internal nodes using the subprogram CONSENSE. Bootstrap of above 50% shows a hyphen on node. This is a rooted tree. *H. beccari* is an outgroup.

to balance a ship's cargo, has been responsible for the introduction of seeds in Japan. Seeds in Japan can survive for several years in ballast tanks and once released into new environments are able to grow in Korea. With increasing population, human activity may be somewhat associated with the introduction of seeds.

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초록 : 전남 여수시 안도섬에서 발견된 해오말의 유전학적 관계 연구

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2007년 전라남도 여수시 남면 안도리에서 발견된 국내 미기록종 해오말은 지리적으로 열대부터 아열대까지 넓게 분포하는 식물로서 열매를 만든다. 잎의 모양은 계란형에 가깝고, 입쪽지는 견고하게 잎을 지지하고 있다. 뿌리는 불규칙적으로 뻗어있고, 뿌리 사이로 꽃이 형성되어 있다. 잎맥은 잎의 가장자리와 공간을 유지하고 있다. ITS1과 ITS2 부위는 한국산과 일본산 해오말은 100% 동일한 염기서열을 나타내고 있으나, 5.8S에서 한국산 해오말은 202 bp에서 206 bp까지 4개의 염기가 삽입된 것이 보였다. ITS 부위에 대한 한국산 해오말은 일본산과 동일한 유전적 clade을 나타내었으나, 필리핀, 호주, 베트남, 말레이시아산 해오말과는 유전적 분리를 보였다. 따라서 한국산 해오말은 일본에서 gene flow로 된 것으로 추정되며, 아열대성인 해오말이 우리나라 연안에 나타난 것은 기후변동에 의한 수온상승과도 밀접한 관계가 있는 것으로 보인다.