# Application of genetic markers for identification of *Halophila* members and genetic variation of *Halophila ovalis* from Western Pacific to Eastern Indian Ocean

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**A:** (3)
…[2]Es ist eine ausführliche Darstellung voranzustellen, die eine kritische Einordnung der Forschungsthemen und wichtigsten Erkenntnisse aus den Publikationen in den Kontext der wissenschaftlichen Literatur zum Thema vornimmt …

**Die voranzustellende ausführliche Darstellung ist in dieser Arbeit aufgeteilt in die Kapitel 1 und 7.**

**B:** (3)
…vornimmt sowie die individuellen eigenen Beiträge und ggf. die Beiträge weiterer Autoren an den jeweiligen Publikationen darlegt.

**Publication (Chapter 2):**

**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.

JP and NXV defined the research topic. NXV carried out the field work and collected the materials. JSB and NXV analyze the leaf morphology. PJ and NXV analyzed the data and wrote the manuscript.

**Publication (Chapter 3):**

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JP and NXV defined the research topic. NXV carried out the field work and collected the materials. LH and NXV carried out the laboratory experiments and generated the data. JP and NXV analyzed the data and wrote the manuscript.

**Publication (Chapter 4):**

**Nguyen** XV, Thangaradjou T, Papenbrock J. 2013: Genetic variation among *Halophila ovalis* (Hydrocharitaceae) and closely related seagrass species from the coast of Tamil Nadu, India – An AFLP fingerprint approach. *Syst Biodivers* 11: 467-476.

JP, NXY and TT defined the research topic. TT carried out the field work, collected the materials and analyzed the morphological trait of leaf. NXV carried out the laboratory experiments and generated the data. JP and NXV analyzed the data and wrote the manuscript.

**Publication (Chapter 5):**

**Nguyen** XV, Matsapume D, Piyalap T, U Soe-Htun, Japar SB, Anchana P, Papenbrock J. 2014: Species identification and differentiation among and within populations of *Halophila* from the Western Pacific to Eastern Indian Ocean by ITS, AFLP and microsatellite analysis. *BMC Evol Biol* 14:92.

The conception and design of the investigation was developed by JP and AP. PT, USH, JSB and NXV carried out field work, collected the materials. MD and NXV carried out the laboratory experiments and generated the data. JP, MD and NXV analyzed the data and wrote the manuscript.

**Publication (Chapter 6):**

**Nguyen** XV, Klein M, Riemenschneider A, Papenbrock J: 2014: Distinctive features and role of sulfur-containing compounds in marine plants, seaweeds, seagrasses and halophytes from an evolutionary point of view. In: Sabkha Ecosystems Vol IV: Cash Crop Halophytes & Biodiversity Conservation, edited by M.A. Khan et al. Springer: 299-312.

JP wrote the review chapter in the review on “Introduction and Conclusion”. MK wrote part of the review chapter in the review on “Selected sulfur-containing metabolites with specific functions in salt-tolerant plants” AR and NXV wrote the review chapter in the review on “Is metal-binding the only function of phytochelatins and metallothioneins?”

**Summary**

The seagrass genus *Halophila* (Hydrocharitaceae) forms a complex group with an unresolved taxonomy due to high plasticity and overlapping morphological characters among currently defined species leading to many misidentifications. Reproductive organs are rarely found to compare among specimens. The Indo-Pacific region, the origin of the Hydrocharitaceae, has the largest number of seagrass species worldwide, especially members of the genus *Halophila*. The species *Halophila ovalis*, distributed from tropical to warm-temperate waters, is the most common *Halophila* species in that region and can grow in variations of temperatures and substratum.

A genetic marker is a gene or DNA sequence that can be used to characterize and identify taxa. Genetic markers provide promising approaches for the classification in both animal and plant taxa. DNA fingerprint approaches also reveal the genetic distance among closely related species as well as genetic differentiation among populations within species. Does molecular analysis confirm morphological identification? Are there genetic differences between *Halophila ovalis* populations growing in different habitats? Is there any genetic differentiation among populations in the Western Pacific and the Eastern Indian Ocean, which are separated by the Thai-Malay peninsula?

Based on seagrass material collected at a broad study site (1 – 22°N; 77 – 119°E) in both the Pacific and the Indian Ocean, the aim of the present study is to determine the genetic markers that can be used to characterize and identify individuals or species to answer the research questions.

With respect to the species identification, the plastid gene encoding the large subunit of ribulose-1,5-bisphosphate-carboxylase-oxygenase (*rbc*L) showed the lowest species resolution, plastid maturase K (*mat*K) showed higher species resolution and the concatenated sequences of the two plastid markers (*rbc*L and *mat*K) resolved almost all members of the *Halophila* genus except *H. ovalis – H. major – H. ovata*. Analysis based on the nuclear ribosomal internal transcribed spacer (ITS1-5.8S-ITS2 or ITS) region resolved *H. major* from the complex. Analysis of ITS and supporting leaf morphological data revealed yet unrecorded populations of *H. major* in Viet Nam, Malaysia and Myanmar. Results from Amplified Fragment Length Polymorphism (AFLP) indicated that *H. ovalis* and *H. ovata* are distinct species. Moreover, genetic differences among populations in the open sea and the lagoon were detected. AFLP and microsatellite (SSRs) analysis demonstrated impressively that the Thai-Malay peninsula forms a geographic barrier to populations in the Western Pacific and the Eastern Indian Ocean. A high correlation between genetic and geographic distances among populations in the Western Pacific and Eastern Indian Ocean was observed. Additionally, the distinctive features and role of sulfur-containing compounds in marine plants, seaweeds, seagrasses and halophytes from an evolutionary point were reviewed.

In summary, the highlight of this study is that the application of molecular markers resolved the genetic relationship among all members of the *Halophila* genus investigated. Moreover, *H. major* was unambiguously described as a new record for Viet Nam, Malaysia and Myanmar, based on both morphological characters and ITS analysis. Geographic and ecological barriers affect the genetic differentiation among *H. ovalis* populations from the Western Pacific to the Eastern Indian Ocean.

**Keywords:** Eastern Indian Ocean, evolution, genetic distance, genetic markers, *Halophila*, *Halophila ovalis*, Western Pacific Ocean.

**Zusammenfassung**

Seegräser der Gattung *Halophila* (Hydrocharitaceae) bilden eine komplexe Gruppe mit einer noch ungelösten Taxonomie. Durch hohe Plastizität und überlappende morphologische Merkmalen bei aktuell definierten Spezies kommt es immer wieder zu falschen Identifizierungen, zumal Blüten- und Fruchtbildung nur selten zu beobachten sind und als Bestimmungsmerkmal kaum genutzt werden können. Die indo-pazifische Region, in der auch der Ursprung der Hydrocharitaceae liegt, zeigt die größte Anzahl von Seegras Arten weltweit, vor allem Mitglieder der Gattung *Halophila*. *Halophila ovalis* ist die häufigste *Halophila*-Art in dieser Region und wächst von tropischen bis zu warm-gemäßigten Gewässern, bei verschiedenen Temperaturen und in verschiedenen Substraten.

Genetische Marker sind DNA-Sequenzen, die zur Charakterisierung und Identifizierung von Taxa genutzt werden können. Genetische Marker bieten vielversprechende Ansätze für die Einordnung von Tier- und Pflanzenarten. Über DNA-fingerprinting kann auch die genetische Distanz zwischen eng verwandten Arten sowie genetische Differenzierung zwischen Populationen innerhalb der Arten bestimmt werden. Können molekulare Marker die Identifizierung von Arten basierend auf morphologischen Merkmalen verifizieren? Gibt es genetische Unterschiede zwischen Populationen von *Halophila ovalis*-Pflanzen, die in verschiedenen Lebensräumen wachsen? Kann eine genetische Differenzierung zwischen den Populationen im westlichen Pazifik und im östlichen Indischen Ozean, die durch die Thai-Malay Halbinsel getrennt sind, nachgewiesen werden?

Mithilfe von Seegras-Material, das in einer breit angelegten Studie sowohl im Pazifik als auch im Indischen Ozean (1-22°N; 77-119°E) gesammelt wurde, ist das Ziel der vorliegenden Studie genetische Marker zu finden, die genutzt werden können, um diese Forschungsfragen zu beantworten.

Im Hinblick auf die Identifizierung der Art zeigte das Plastiden-Gen *rbc*L codierend für Ribulose-1,5-bisphosphat-Carboxylase-Oxygenase die niedrigste Auflösung auf Artebene, die Analyse der plastidären Maturase K (*mat*K) zeigte eine höhere Auflösung auf Artebene und die Kombination beider Sequenzen (*rbc*L und *mat*K) führte zu einer Auflösung fast aller Mitglieder der Gattung *Halophila* außer *H. ovalis* - *H. major* - *H. ovata*. Die Analyse der kernlokalisierten „internal transcribed spacer"-Region (ITS1-5.8S-ITS2 oder ITS) führte zu einer eindeutigen Zuordnung von *H. major* aus dem Komplex in eine Klade und unterstützt Merkmalsunterschiede in der Blattmorphologie. Basierend auf diesen Ergebnissen konnten wir die Erstfunde für *H. major* in Vietnam, Malaysia und Myanmar beschreiben.

Amplified Fragment Length Polymorphismus- (AFLP) Ergebnisse zeigten, dass *H. ovalis* und *H. ovata* verschiedene Arten sind. Darüber hinaus wurden die genetischen Unterschiede zwischen den Populationen im offenen Meer und der Lagune erkannt. AFLP- und Mikrosatelliten (SSR)-Analyse demonstrierten eindrucksvoll, dass die Thai-Malay Halbinsel eine geografische Barriere für die Populationen im westlichen Pazifik und im östlichen Indischen Ozean bildet. Eine hohe Korrelation der genetischen und geographischen Distanzen zwischen den Populationen im westlichen Pazifik und dem östlichen Indischen Ozean wurde beobachtet. Zusätzlich wurden die Besonderheiten und die Rolle der schwefelhaltigen Verbindungen in marinen Pflanzen, Algen, Seegras und Halophyten aus evolutionärer Sicht betrachtet.

Es lässt sich festhalten, dass die Anwendung von molekularen Markern, die genetische Beziehung zwischen allen Mitgliedern der Gattung *Halophila* klar aufgelöst hat. Darüber hinaus wurde das Vorkommen von *H. major* erstmals für Vietnam, Malaysia und Myanmar beruhend auf morphologischen Merkmalen und der

ITS-Analyse beschrieben. Geographische und ökologische Barrieren beeinflussen die genetische Differenzierung zwischen den Populationen von *H. ovalis* vom westlichen Pazifik bis zum östlichen Indischen Ozean.
**Schlüsselwörter**: Evolution, genetische Distanz, genetische Marker, *Halophila*, *Halophila ovalis*, östlicher und westlicher Indischer Ozean.

## Content

## Abbreviations

| | |
|---|---|
| α | Significance level |
| AFLP | Amplification Fragment Length Polymorphism |
| AMOVA | Analysis of Molecular Variance |
| ANOVA | Analysis of Variance |
| BA | Bayesian Analysis |
| BD | Bodgaya Island |
| bp | base pair |
| BLAST | Basic Local Alignment Search Tool |
| CA | Character Attributes |
| CAOS | Character Analysis Organization System |
| CBOL | Consortium for the Barcode of Life |
| CM | Cu Mong |
| CTAB | Cetyl trimethylammonium Bromide |
| *df* | Degrees of freedom |
| dNTP | Desoxyribonucleic Triphosphate |
| DMSP | Dimethyl-sulfoniopropionate |
| DMS | Dimethyl Sulfide |
| DNA | Deoxyribonucleic Acid |
| DTT | Ditiotreitol |
| EDTA | Ethylenediaminetetraacetic Acid |
| *F* | F-statistics |
| GS | Gusunan Island |
| HK | Hong Kong |
| ITS | Internal Transcribed Spacers |
| JO | Johore |
| KA | Kanyakumari |
| KN | Kanom |

| | |
|---|---|
| MA | Marakanam |
| *mat*K | Chloroplast Maturase K |
| MB | Mabul Island |
| MG | Maiga Island |
| ML | Maximum Likelihood |
| MP | Maximum Parsimony |
| MTs | Metallothioneins |
| MY | Myanmar |
| NOAA | National Oceanic and Atmospheric Administration |
| NCBI | National Center for Biotechnology Information |
| NJ | Neighbor Joining |
| NT | Nha Trang |
| PB | Palk Bay |
| PCs | Phytochelatins |
| PCS | Phytochelatin Synthase |
| PCR | Polymerase Chain Reaction |
| PCoA | Principal Coordinates Analysis |
| RAPD | Random Amplified Polymorphic DNA |
| *r* | Intra-marginal vein distance |
| R | Lamina margin distance |
| *rbc*L | Ribulose-1,5-bisphosphate-carboxylase-oxygenase |
| rDNA | Ribosomal DNA |
| RL | Restriction Ligation |
| SA | Satun |
| SB | Sibangat Island |
| sp. | Species |
| SP | Sulfated Polysaccharides |
| SP6 | SP6 Promoter Region |
| SSRs | Simple Sequence Repeats |

| | |
|---|---|
| subsp. | Subspecies |
| T7 | T7 Promoter region |
| *Taq* | *Thermus aquaticus* polymerase |
| TEA | Tris-Acetate-EDTA |
| TG | Tiga Island |
| TN | Thi Nai |
| TR | Trang |
| Tris | Tris-(hydroxymethyl)-aminomethane |
| TT | Thuy Trieu |
| UPGMA | Unweighted pair group method with arithmetic mean |
| VE | Vellar Estuaty |
| VP | Van Phong |

# CHAPTER 1

# GENERAL INTRODUCTION

## Seagrasses

Seagrasses are marine angiosperms and adapted to aquatic life several times independently in tropical as well as in temperate regions (Den Hartog, 1970). They play important roles in the coastal ecosystem (Short et al., 2007). Seagrasses grow in shallow, sheltered and soft-bottomed coastal habitats such as coastlines, estuaries and lagoons (Den Hartog and Kuo, 2006). Seagrass can reproduce through both sexual and asexual methods (Den Hartog, 1970). Most seagrass species produce flowers of a single sex on each individual, so there are separate male and female plants (Waycott and Les, 1996).

The roles of seagrasses are clearly reflected in biological, physical and chemical aspects. In terms of biological aspects, seagrasses are a primarily food source for many organisms. Many marine species also utilize seagrass habitats as feeding grounds and nursery areas (Beck et al., 2003; Hori et al., 2006). Several other species spend their juvenile stage in seagrass habitat, eventually moving on to mangroves as they mature (Phillips, 1979). Seagrasses are efficient at removing dissolved nutrients from waters that often enter coastal waters as a result of runoff from the land (Phillip and Menez, 1988). Seagrasses also trap fine sediments and particles from both substratum and water. The removal of particles and nutrients from the water results in high water clarity and nutrient-poor waters required for the survival of coral reefs (de Boer 2007). In terms of physical aspects, seagrasses stabilize bottom sediments with their dense roots and rhizomes that form a secure mat (de Boer 2007; Short and Wyllie-Echeverria, 1996). This sediment stabilization and erosion prevention is especially important during storms and hurricanes that often threaten the coastline. In terms of chemical aspects, seagrasses are well documented for the presence of potent diverse secondary metabolites (Puglisi et al., 2007). There are several bioactive compounds such as phenolic acid, tannins, antibacterial activity, antifungal activity, antidiabetic, antioxidant and vasoprotective effects (Buchsbaum et al., 1990; Arnold et al., 2008; Bushmann and Ailstock, 2006; Kannan et al., 2010). Among bioactive compounds, sulfated polysaccharides are exploited as antithrombotic and anticoagulant agents and suggested to be immunostimulants (Assreuy et al., 2008; Baba et al., 1990).

## Distribution of seagrass

Global distribution of seagrasses based on seagrass assemblage of different taxonomic groups was divided into six bio-geographical regions including (i) Temperate North Atlantic (North Carolina, USA to Portugal), (ii) Tropical Atlantic (including the Caribbean Sea, Gulf of Mexico, Bermuda, the Bahamas, and both tropical coasts of the Atlantic), (iii) Mediterranean (including the Mediterranean Sea, the Black, Caspian and Aral Seas and Northwest Africa), (iv) Temperate North Pacific (Korea to Baja, Mexico), (v) Tropical Indo-Pacific (East Africa, South Asia and tropical Australia to the eastern Pacific) and (vi) Temperate Southern Oceans (New Zealand and temperate Australia, South America, and South Africa) (Short et al., 2007).

## Morphology and systematics of seagrass

There are about 66 species of seagrass belonging to 14 genera recorded globally. They belong to one of four plant families including Posidoniaceae, Zosteraceae, Hydrocharitaceae and Cymodoceaceae, in the class of monocotyledonous plants in the order of Alismatales (Den Hartog and Kuo, 2006).

Morphologically, the seagrass size ranges from tiny leaves of two to three mm (*Halophila minor* (Zoll.) den Hartog) to large leaves of more than one meter (*Enhalus acoroides* (L.f.) Royle). Leaves of different seagrass species can be shaped like a flattened ribbon (*E. acoroides, Halodule* spp), look like a fern (*Halophila spinulosa* (R. Brown) Ascherson), round like a clover (*Halophila baillonis* Ascherson ex Dickie), or even spaghetti-shaped (*Syringodium isoetifolium* (Ascherson) Dandy) (Phillips and Menez, 1988). The plant consists of three main parts including roots, stems and leaves. Seagrasses are unique amongst flowering plants that they can live entirely immersed in seawater except *E. acoroides* which must emerge to the surface for reproduction (Den Hartog, 1970). All others can flower and be pollinated under water. Adaptation to a marine environment imposes major constraints on morphology and structure (McKenzie, 2008).

The morphology of species in the *Halophila* genus is unique among seagrasses in having a petiolate leaf lacking a leaf sheath (Den Hartog, 1970). The species are either monoecious or dioecious. Both annual and perennial marine plants are found with creeping, monopodial rhizomes, rooting and with erect lateral shoots at the nodes. Leaves in pairs, arise from an extremely short lateral shoot. The lamina are variable in shape and

size (Kuo et al., 2006). In the genus *Halophila* five sections have been described, based on differences in the gross vegetative morphology of the plants. They are (i): Section *Halophila* contains most of the species, (ii): Section *Spinulosae* – only one species *H. spinulosa* (R. Brown) Ascherson, (iii): Section *Microhalophila* contains only one species *H. beccarii* Ascherson, (iv): Section *Americanae* includes *H. engelmanni* Ascherson and *H. baillonis* Ascherson ex Dickie, and (v): In section *Tricostatae* exists only one species *H. tricostata* Greenway (Den Hartog and Kuo, 2006).

Among the five sections in the genus *Halophila*, the *Halophila* section is known as one the most complex taxonomic challenges mainly based on the high morphological plasticity. This is illustrated by the following examples: McDermid et al. (2003) found that the leaves and rhizomes showed great variability among collection sites of *H. hawaiiana* Doty & B.C. Stone in Hawaii Islands, USA. The shape of this species change from spatulate shape, elongated shape, paddle shape, long and narrow to strap-like. The great variations of leaf morphology were also found in *Halophila nipponica* J. Kuo in Japan (Shimada et al., 2012). Procaccini et al. (1999) found that the recently established population of *Halophila stipulacea* (Forss.) Ascherson on Sicily, Italy, exhibited significant morphological variations in different depth. Recently, Kuo et al. (2006) suggested the classification of several new species including *Halophila major* J. Kuo, *H. mikii* J. Kuo, *H. nipponica, H. okinawensis* J. Kuo and *H. gaudichaudii* J. Kuo based on leaf morphology of the Japanese *Halophila* complex. However, Short et al. (2011) argued that morphology and species boundaries between these new species and *H. ovalis* are not clear. Moreover, the identification keys including number of cross veins, lamina size, lamina shape, angles of cross veins for differentiating among *Haplophila ovalis* (R. Br.) Hook, *H. johnsonii* Eiseman, *H. minor* (Zollinger) den Hartog, *H. major* and *H. ovata* Gaudichaud are overlapping each other, leading to difficulties in species identification based on morphological classification (Kuo et al., 2006).

## Genetic marker

A genetic marker is a gene or DNA sequence that can be used to characterize and identify individuals or species. Genetic markers provide promising approaches for classification in both animal and plant taxa (Pierce 2010). DNA barcoding, using a short gene sequence from a standardized region of the genome, is a species identification tool which would not only aid species discovery but would also have applications ranging from large-scale

biodiversity surveys through to identification of a single fragment of material in forensic contexts (Cowan and Fay, 2012). For animals, the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene has been employed as a possible DNA marker for species and a number of studies in a variety of taxa have accordingly been carried out to examine its efficacy (Waugh, 2007). However, DNA barcoding of plants presents a number of challenges compared to DNA barcoding in many animal clades, also due to the fact that the CO1 animal DNA barcode is not effective for plants (Fazeka et al., 2012). Nowadays, single nucleotide polymorphism (SNP) of marker genes, including plastid and nuclear, are used to identify the boundaries among the species. However, plastid and nuclear sequences fail to resolve genetic relation among closely related species in some cases (Drespres et al., 2003). Genetic differentiation is not only found among different species but also among different individuals of the same species based on DNA fingerprinting approaches.

Several DNA fingerprinting have been applied to investigate the genetic relations among individuals within population or among populations of the same species. Some DNA fingerprinting types commonly used are: Restriction Fragment Length Polymorphism (RFLP); Amplified Fragment Length Polymorphism (AFLP); Random Amplification of Polymorphic DNA (RAPD); Variable Number Tandem Repeat (VNTR); Microsatellite Polymorphism; Single Nucleotide Polymorphism (SNP); STR Short Tandem Repeat (STR); SFP Single Feature Polymorphism (SFP) and Diversity Arrays Technology (DArT). These techniques are well established and their advantages as well as limitations have been realized. Advanced marker techniques tend to amalgamate advantageous features of several basic techniques (Mueller and Wolfenbarger, 1999). Among DNA fingerprinting mentioned above SNP, RADP, AFLP and SSRs are commonly used to investigate the genetic distance among individuals and among populations (Edwards et al., 1991; Selkoe and Toonen, 2006; Pourcel et al., 2009; Vos et al., 1995; Welsh and McClelland, 1990).

## The applications of genetic markers in seagrass

Today, genetic markers are widely applied for the species identification as well as genetic diversity, population structure of the species. For plastid sequences, the plastid gene encoding the large subunit of ribulose-1,5-bisphosphate-carboxylase-oxygenase (*rbc*L) was in the focus of numerous plant studies concerning phylogeny and molecular evolution

(Les et al., 1997). Chloroplast maturase K (*mat*K) is highly conserved in plants (Wanke et al., 2007) and it has been shown to evolve at approximately three times the rate of the *rbc*L gene (Johnson and Soltis, 1995). Based on *rbc*L sequences, Les et al. (1997) clearly showed the genetic relation among families of marine Angiosperms (or seagrass). Combination of *rbc*L and *mat*K revealed that the three genera *Enhalus, Thalassia* and *Halophila* are monophyletic (Tanaka et al., 1997). Recently, Lucas et al. (2012) suggested that the concatenated sequences of the two plastid markers (*rbc*L and *mat*K) could be used as DNA barcoding sequences for seagrasses because of high species resolution. However, the position of some members of the *Halodule* and *Halophila* genera were not completely resolved. Recently, Ito and Tanaka (2011) showed the very close genetic distance of two species *H. uninervis* (Forssk.) Asch. and *H. pinifolia* (Miki) Den Hartog based on analysis of the the concatenated sequences plastid markers (*rbc*L) and nuclear encode *phy*B sequences.

For the nuclear genome, the nuclear ribosomal internal transcribed spacer (ITS1-5.8S-ITS2) region was applied to resolve the genetic relation among the members of *Halophila*. Based on analysis of ITS sequences, Uchimura et al. (2006; 2008) suggested that *H. gaudichaudii, H. okinawensis* and *H. nipponica* may be conspecific and *H. ovalis* and *H. major* are two distinct species. Recently, result based on ITS analysis was shown that *H. johnsonii* and *H. ovalis* are synonyms (Short et al., 2010). In the case of *H. hawaiana* Doty & B. C. Stone, the results from genetic marker analysis confirm that genetic variation among population in Hawaii, USA is very low although high variation of leaf morphology was observed (McDermid et al., 2003). ITS analysis is also helpful tool to reveal new records for species of *H. decipiens* Ostenfeld for Hawaii, USA and Kenya (McDermid et al., 2002; McMahon and Waycott, 2009). Studies on leaf morphology of *H. nipponica* also stated that there is no nucleotide difference in the ITS sequence between elliptical-type and linear-type leaves (Shimada et al., 2012). The studies of Waycott et al. (2002) based on ITS sequences showed that some specimens identified as *H. ovalis* belonged to different clades, and this clearly points to the need for critical taxonomic revision of the members of the *Halophila* complex from the entire geographic distribution of this genus.

RAPD has been successfully used to assess genetic diversity of seagrasses. Data sampled from Warnbro Sound, Western Australia, showed the intra-population variability in

*Posidonia australis* (Linnaeus) Delile (Waycott, 1998). Alberto et al. (2001) revealed that high genetic homogeneity is characteristic for *Cymodocea nodosa* (Ucria) Ascherson sampled in the Northern Atlantic. In the Mediterranean Sea, Jover et al. (2002) presented that genotypic diversity of *Posidonia oceanica* (Linnaeus) Delile strongly depend on the spatial structure, age, and maturity of the meadows. Other studies indicated a low degree of gene flow between populations of *Zostera muelleri* Irmisch ex Ascherson (Jones et al., 2008) and *Posidonia oceanica* from Santa Marinella meadow (Rotini et al., 2011) among others. Furthermore, RAPD markers revealed a decreased genetic diversity in *Posidinia oceanica* along the anthropogenic disturbance gradient, both at small scale within a meadow and at large scale in the Mediterranean Sea (Micheli et al., 2005). This gives a clue that these techniques can also be used to assess the health of the seagrass beds at any given time.

AFLP may be helpful to solve the genetic relation among closely related species (Després et al., 2003). AFLP is a DNA fingerprinting technique that is based on selective PCR amplification of restriction fragments from a total digest of genomic DNA and considered as a useful approach to resolve closely related species and/or genetic diversity of populations (Vos et al., 1995). The variation in genetic diversity based on AFLP among populations has been noted for seagrass species such as *Halodule wrightii* Ascherson (Travis and Sheridan, 2006), *Thalassia testudinum* Banks ex König (Waycott and Barnes, 2001), *Zostera marina* Linnaeus (Reusch, 2002; Olsen et al., 2004), and *Posidonia oceanica* (Procaccini et al., 1996). Almost all previous studies based on this method indicated that genetic distance much depend on geographic distance or habitat of species. Such an AFLP-based approach to test genetic diversity of *Halophila ovalis* has not been applied so far. The major advantage of the AFLP technique is the large number of polymorphisms that the method generates compared with other markers.

Microsatellites or simple sequence repeats (SSRs) which contains the repeated units generally di-, tri- tetra- or pentanucleotides and tend to be highly polymorphic are commonly applied to investigate the genetic differentiation among seagrass populations (Queller et al., 1993; Reusch, 2002). Several studies on genetic variation and genetic structure of seagrass populations have been published during the last years. Reusch et al. (2002) indicated that *Zostera marina* populations in the Baltic Sea were genetically less diverse compared to those in the Wadden Sea and correlation between genetic and

geographic distance were weak in both areas. *Zostera noltii* (Linnaeus) populations along the Iberian coast also revealed a split between northern and southern populations (Diekmann et al., 2005). For the species *Cymodocea nodosa*, Alberto et al. (2008) found that the extremely low genotypic richness at the Atlantic northern edge contrasts with the high values (low clonality) at the Atlantic southern edge and in most of the Mediterranean Sea. Another study on *Thalassia testudinum* collected at the western tropical Atlantic, Gulf of Mexico, and Florida indicated that the populations exhibited high levels of genetic diversity suggesting strong recruitment of sexually derived propagules (Bricker et al., 2011). On the species of *Posidonia oceanica*, the analysis of SSRs showed the significantly genetic different among populations in the basin from Spain to Turkey (Procaccini et al., 2002).

Advantages of microsatellites as genetic markers include locus-specificity, a high degree of polymorphism and therefore it is possible to work also with partially degraded DNA. Another advantage of SSRs is co-dominance (heterozygotes can be distinguished from homozygotes) that is not found in other DNA fingerprint techniques such as AFLP and RAPD (Kimberly and Toonen, 2006).

## Tropical Asia – A hotspot and center of seagrass biodiversity

The Indo-Pacific region has the largest number of seagrass species worldwide, with huge meadows of mixed species stands and this region was considered as the origin of the Hydrocharitaceae family (Chen et al., 2012; Short et al., 2011). The highest concentration of seagrass species is found in this region (Duarte, 2001; Spalding et al., 2003). There are 24 species found in this region, higher than any regions in the world (Short et al., 2011). There are about 24 species recorded in this region, however, exactly member of *Halophila* species have not resolved due to overlapping of leaf morphology among the species. (Short et al., 2007, Kuo et al., 2006). Actually, there is new record of *Halophila sulawesii* former identified as *H. ovalis* in Indonesia (Kuo, 2007). Beside the species diversity was found in this region, morphological diversity within species was also documented in several studies. *Halophila hawaiiana* showed very great variation of leaf morphology in different depth (McDermid et al., 2003). Diversity of leaf morphology was also found in *H. nipponica* (Shimada et al., 2012). Japar et al. (2010) indicated that *H. ovalis* in Malaysia had the different variation of leaf morphology with different substratum the plant grows.

The distribution of halophyte species/clones can be understood not only by geographical but also by latitudinal temperature ranges. Geographically, the South China Sea and Gulf of Thailand are isolated from Andaman Sea and Bay of Bengal by the Thai-Malay peninsula. Recently, several studies have been published on mangroves (Liao et al., 2009; Su et al., 2006) and animals (Khamnamtong et al., 2009; Zhang et al., 2006) to reveal the genetic variation caused by the Thai-Malay peninsula barrier. *H. ovalis* is commonly found from South China Sea via Gulf of Thailand to Andaman Sea and Bay of Bengal in India. Perhaps, diversity of habitats (lagoon, estuary, open sea, creek, littoral area etc.), long geographic distance (0°- 22°N; 77° – 122° E) and geographic barriers (Thai-Malay peninsula) in tropical Asia causes the high variation of leaf morphology and maybe the genome. Clearly, this led to several hypotheses: Does a molecular analysis of these leaf samples confirm the morphological identification? Are there any samples misidentified as *H. ovalis*? Are there genetic differences among population in lagoons (low salinity) and the open sea (high salinity)? Is the Thai-Malay peninsula a geographic barrier for *H. ovalis* populations? Are there any correlations between genetic and geographic distances?

## Sulfur-containing compounds and heavy metal accumulation of seagrass

Coastal areas are considered as places receiving high amounts of pollutants including high concentrations of heavy metals (Govindasamy et al., 2011). Halophytes in general and seagrass in particular occur in this zone where several stresses such as high nutrient loads, flood, daily changing of salinity, and heavy metal accumulation act on the plants. Researches on heavy metal concentration in the sediment of coastal areas reveal that they are much higher than WHO standard recommendations and are a very serious health hazard. Hence, seagrasses have developed strategies to overcome these stresses. Sulfur-containing compounds and proteins seem to play a pivotal in the adaptation to these environmental conditions. Phytochelatins (PCs) and metallothioneins (MTs) are Cys-rich metal chelators that represent the two principle groups of metal-binding molecules found across most taxonomic groups (Grennan, 2011). PCs, glutathione-derived metal binding peptides, usually with the structure of $(1'\text{-Glu-Cys})_n$ - Gly (n= 2-11) are enzymatically synthesized peptides considered to be involved in heavy metal detoxification, mainly Cd and As, which has been demonstrated in plants, algae and some transformed yeast strains grown at high heavy metal concentrations (Clements and Persoh, 2009). MTs are a group

of proteins with low molecular mass and high Cys content that bind heavy metals and are thought to play a role in their metabolism and detoxification (Cobbett and Goldsbrough, 2002).

## Aims of this thesis

- To analyze species boundaries of members of the *Halophila* genus based on plastid genes (single *rbc*L, *mat*K and the concatenated sequences).
- To identify *Halophila* spp. collected in Viet Nam based on nuclear sequence (ITS)
- To determine the genetic relation of the closely related species *H. ovalis, H. ovalis* subsp. *ramamurthiana* and *H. ovata* by application of DNA fingerprinting (AFLP) when plastid and nuclear gene fail to resolve.
- To define genetic diversity of *Halophila ovalis* population from the South China Sea via the Gulf of Thailand, Andaman Sea and Bay of Bengal and role of geographic barrier based on ITS, AFLP and microsatellite analyses.
- To show the important role of sulfur-containing compounds in marine plants, seaweeds, seagrasses and halophytes

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# CHAPTER 2

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# Variability of leaf morphology and marker genes of members of the halophila complex collected in Viet Nam

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ABSTRACT

Seagrasses are marine angiosperms and adapted to aquatic life several times independently. In tropical as well as in temperate regions they play an important role in the coastal ecosystem. Classification according to morphology remains difficult due to very similar adaptations to the aquatic lifestyle and small simple flowers which are often not even formed. Especially the *Halophila* genus (Hydrocharitaceae) forms a complex group with an unresolved taxonomy due to overlapping morphological characters and high plasticity leading to many misidentifications. Hence, this led to the hypothesis that phylogenetic analysis on the molecular level may resolve taxonomic classification. The aim of this study is to identify and unambiguously characterize *Halophila* species collected in Viet Nam using tree- and character-based analysis of *rbc*L and *mat*K sequences. Results obtained from molecular data and leaf morphology indicate that there are at least three species found in Viet Nam. Topologies based on single locus or combined datasets were similar but not equal. Analysis of *rbc*L sequences showed lowest species resolution when only *Halophila beccarii* Aschers was resolved at the *Halophila* complex. *mat*K shows better resolution with respect to *H. beccarii* and *H. decipiens* Ostenfeld. Combined *rbc*L and *mat*K consensus trees showed the highest species resolution when all species form three distinct clades representative for three difference species including *H. beccarii, H. decipiens*, and *H. ovalis* (R. Br.) Hook. *f.*



## 1. Introduction

Seagrasses, which are important contributors to primary production, part of marine ecosystems and providing valuable ecosystem services, have received relatively little attention in both scientific and popular media (Phillips, 1980; Beck et al., 2003; Hori, 2006). There are about 66 species of seagrass belonging to 14 genera recorded globally. They belong to the class of monocotyledonous plants in the order of Alismatales (den Hartog and Kuo, 2006). Comparison of morphological traits is the key factors in describing and naming species within the field of taxonomy (Radulovici et al., 2010). The long-standing approach can be very tedious and a matter of subjectivity since it is up to the taxonomist to choose those morphological characters believed to delineate species (Coyne and Orr, 2004). Molecular markers provide promising approaches for classification (Alberte et al., 1994; Procaccini et al., 1996; Reusch et al., 1999). For seagrasses, genetic markers have been used by several scientists since the 1980s (Les, 1988; McMillan, 1991). Studies of other authors showed the importance of allozyme markers in terms of polymorphisms (Laushman, 1993; Ruckelshaus, 1995; Waycott, 1995; Williams and Davis, 1996; Waycott et al., 1997). The plastidic gene encoding the large subunit of ribulose-1,5-bisphosphate-carboxylase-oxygenase (*rbc*L) was in the focus of numerous plant studies concerning phylogeny and molecular evolution (Les et al., 1997; Barrett and Freudenstein, 2008). Chloroplast maturase K (*mat*K) is highly conserved in plants (Wanke et al., 2007; Dhivya et al., 2008). It has been shown to evolve at approximately three times the rate of the *rbc*L gene (Johnson and Soltis, 1995) and seems to be suitable for phylogenetic analysis of plants at both the genus and species level.

The *Halophila* section is known as one the most complex taxonomic challenges (McMillan and Williams, 1980; McMillan, 1986; Kuo, 2000; Kuo and den Hartog, 2001; Uchimura et al., 2006, 2008; Yip and Lai, 2006). The *Halophila ovalis* species complex has little genetic variation but wide morphological plasticity (Short et al., 2010). Genetic markers including Internal Transcribed Spacers (ITS) (McDermid et al., 2002; Waycott et al., 2002; Ruggiero and Procaccini, 2004; Uchimura et al., 2006, 2008; McMahon

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and Waycott, 2009; Short et al., 2010; Shimada et al., 2012) and *rbc*L/*mat*K (Tanaka et al., 1997; Chen et al., 2012; Lucas et al., 2012) were used to identify *Halophila* species as well as for analyzing genetic relationships by several authors. Those studies indicated that genetic markers were useful at different major groups within the *Halophila* genus and were informative for investigating relationships within other seagrass genera.

Viet Nam locates in South East Asia and is considered within the area of seagrasses' origin (Chen et al., 2012). Among 14 species of seagrasses in Viet Nam (Nguyen et al., 2002), four species of *Halophila* including *H. ovalis, H. minor, H. beccarii* and *H. decipiens* were recorded. They are commonly found at different ecological environments including offshore islands, littoral areas and lagoons with different morphological variations of leaves. Additionally, specimens collected at Cu Mong were stored as *H.* cf *johnsonii.* Difficulties on morphological classification occur during species identification due to morphological overlapping among species of *Halophila.*

In Viet Nam the number of species in the *Halophila* complex may be lower than the four species determined in earlier studies. Therefore leaves of different members of the genus *Halophila* were collected along the coast of Central Viet Nam and classified by several morphological parameters. Does a molecular analysis of these leaf samples confirm our morphological identification? Our approach to evaluate the *Halophila* status in Viet Nam by molecular tools is based on the seagrass barcoding system developed by Lucas et al. (2012).

## 2. Experimental

### 2.1. Sampling and species identification

Sampling of *Halophila* was carried out along the coast of Central Viet Nam (Thuy Trieu Lagoon, Nha Trang Bay, Van Phong Bay, Cu Mong Lagoon and Thi Nai Lagoon) (Fig. 1) at the same time of the year in April 2011 to exclude seasonal variations. We used scuba diving to collect *H. ovalis* (depth of 4–6 m) and *H. decipiens* (9–10 m) plant material at Nha Trang Bay. Plant material at the remaining sampling sites was collected at low tide when the meadows were exposed. At each sampling point plants containing root, rhizome and leaf were selected, washed with seawater in the field to remove the epiphytes and debris that were attached to the plants. Each plant sample was placed in a single plastic bag and kept on ice. Plant material was transferred to the laboratory in the same day. In the laboratory, materials were re-washed with de-ionized water to remove seawater. One plant was divided into two parts, one part was pressed as herbarium voucher specimen and the remaining part was desiccated in silica gel (Chase and Hills, 1991) for later DNA extraction. Parts with a size of 10–12 cm long in a developmentally comparable state from 10 to 15 different plants were haphazardly collected across the beds with a distance of 10–15 m among individuals, stored in high-salt cetyl trimethylammonium bromide (CTAB) buffer (Štorchová et al., 2000) to measure morphological parameters of the leaves. Herbarium voucher specimens are currently deposited at Institute of Oceanography, Viet Nam. Materials desiccated in silica gel and stored in CTAB buffer were brought to the Institute of Botany, Leibniz University Hannover, Germany, for further analysis. Three most important and differentiating parameters of leaf morphology including number of paired cross veins, the ratio of the distance between intra-marginal vein (*r*) and lamina margin (*R*) and the angle of cross veins were measured under the microscope Olympus SZ (Olympus, Tokyo, Japan). Photographs were taken using a U-TV1X-2 digital camera (Olympus) connected to a computer. The test for equal variances of each data set of leaf morphology among groups was checked by Levene's test for homoscedasticity. Levene's test, one-way analysis of variance (ANOVA), Tukey test and whisker plots were carried out by Minitab software (State College, PA, USA). Specimens were identified using the keys of den Hartog (1970), Kuo (2000), Kuo and den Hartog (2001), and Kuo et al. (2006). All *rbc*L (15) and *mat*K (12) sequences of *Halophila* and outgroup sequence from other geographic distributions including India (Lucas et al., 2012), Australia (Les et al., 1997), Japan (Tanaka et al., 1997) and the Natural History Museum of Denmark (Petersen et al., 2006) were obtained from National Centre for Biotechnology Information (NCBI) for comparison (Table 1).

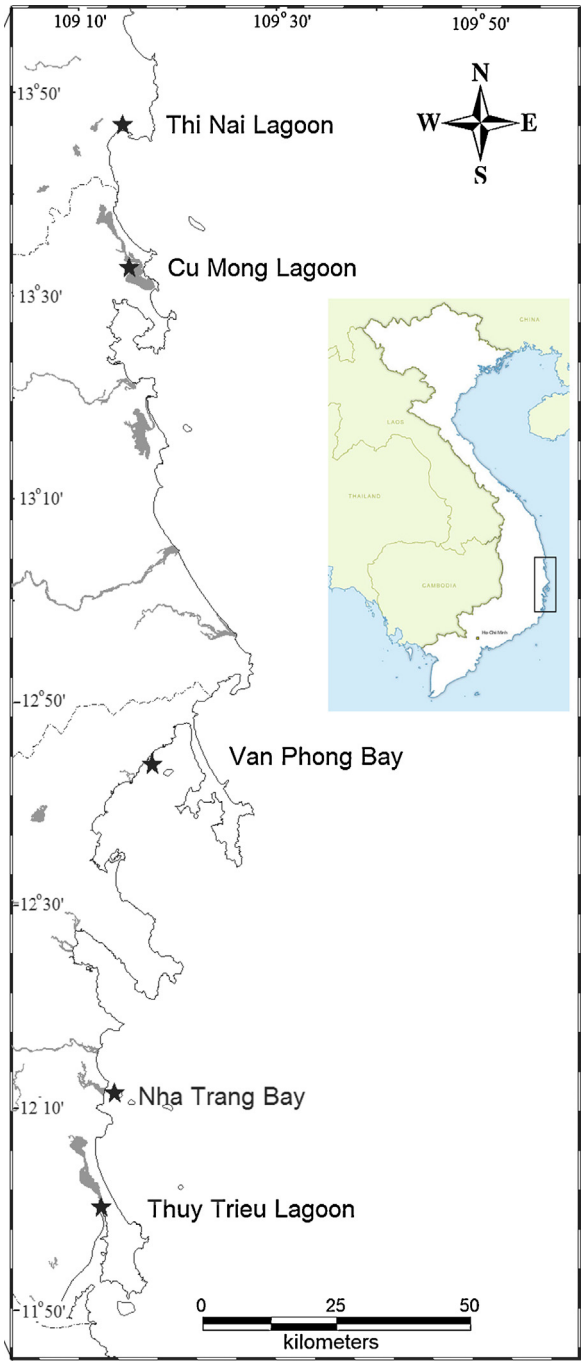


**Fig. 1.** Sample collection sites (⋆) in Viet Nam.
*Source*: Digital map, Department of Survey and Mapping, Ministry of Natural Resources and Environment, Viet Nam.

**Table 1**
List of *Halophila* taxa included in the molecular analysis in this study.

| Samples | Geographic source (GPS data) | Herbarium voucher number or citation | Genbank accession number (NCBI) | |
|---|---|---|---|---|
| | | | *rbc*L | *mat*K |
| *Halophila* sp. | Natural History Museum of Denmark | Petersen et al. (2006) | DQ859168 | |
| *Halophila decipiens* | Australia | Les et al. (1997) | U80698 | |
| *Halophila engelmannii* | Australia | Les et al. (1997) | U80699 | |
| *Halophila ovalis* | Japan | Tanaka et al. (1997) | AB004890 | AB002570 |
| *Halophila ovalis* | Chilika Lagoon, India | Lucas et al. (2012) | JN225349 | JN225366 |
| *Halophila ovalis* | Palk Bay, India | Lucas et al. (2012) | JN225348 | JN225365 |
| *Halophila ovalis* subsp. *ramamurthiana* | Palk Bay, India | Lucas et al. (2012) | JN225355 | JN225380 |
| *Halophila ovata* | Chilika Lagoon, India | Lucas et al. (2012) | JN225347 | JN225367 |
| *Halophila beccarii* | Chilika Lagoon, India | Lucas et al. (2012) | JN225339 | JN225363 |
| *Halophila* sp. A | Chilika Lagoon, India | Lucas et al. (2012) | JN225337 | JN225361 |
| *Halophila stipulacea* | Chilika Lagoon, India | Lucas et al. (2012) | JN225356 | JN225381 |
| *Halophila decipiens* | Palk Bay, India | Lucas et al. (2012) | JN225340 | JN225364 |
| *Halophila* sp. B | Chilika Lagoon, India | Lucas et al. (2012) | JN225340 | JN225362 |
| *Halophila ovalis* | Thuy Trieu Lagoon, Viet Nam (109°11′50″E/12°02′34″N) | HO2011010[a] | JX457593 | JX457600 |
| *Halophila ovalis* | Nha Trang Bay, Viet Nam (109°13′50″E/12°10′08″N) | HO2011011[a] | JX457595 | JX457602 |
| *Halophila ovalis* | Van Phong Bay, Viet Nam (109°17′22″E/12°29′05″N) | HO2011012[a] | JX457597 | JX457604 |
| *Halophila ovalis* | Cu Mong Lagoon, Viet Nam (109°13′44″E/13°35′57″N) | HO2011013[a] | JX457594 | JX457601 |
| *Halophila ovalis* | Thi Nai Lagoon, Viet Nam (109°13′39″E/13°48′36″N) | HO2011014[a] | JX457596 | JX457603 |
| *Halophila decipiens* | Nha Trang Bay, Viet Nam (109°17′04″E/12°10′08″N) | HO2011015[a] | JX457598 | JX457605 |
| *Halophila beccarii* | Thuy Trieu Lagoon, Viet Nam (109°11′50″E/12°02′34″N) | HO2011016[a] | JX457599 | JX457606 |

[a] Herbarium of Institute of Oceanography, Nha Trang City, Viet Nam. The samples were collected in April 2011.

### 2.2. DNA extraction, PCR amplification, cloning and sequencing

DNA extraction was carried out using the Plant Nucleospin II Kit (Macherey & Nagel, Düren, Germany) following manufacture's instruction with slight modifications according to Lucas et al. (2012). To amplify *rbc*L and *mat*K sequences by PCR, modified conditions based on the protocols published by The Consortium for the Barcode of Life (CBOL) plant working group were applied (Hollingsworth et al., 2009). The following primer pairs *rbc*L-F (5′-GTAAAATCAAGTCCACCRCG-3′) and *rbc*L-R (5′-ATGTCACCACAAACAGAGACTAAAGC-3′) (Kress and Erickson, 2007) were used to obtain a *rbc*L fragment of 599 bp, combined primer pairs (5′-ACCCAGTCCATCTGGAAATCTTGGTTC-3′) (Hollingsworth et al., 2009) and (5′-GTTCTAGCACAAGAAAGTCG-3′) (Ford et al., 2009) to obtain *mat*K fragments of 812 bp. PCR amplification was done on a thermocycler (BiozymDiagnostik GmbH, Hess. Oldendorf, Germany) and the profile of the reactions for *rbc*L was: initial denaturation for 4 min at 95 °C followed by 30 cycles of denaturation for 30 s at 95 °C, primer annealing for 35 s at 56 °C and extension for 40 s at 72 °C, terminated by a final hold at 10 °C. For the *mat*K, initial denaturation for 4 min at 95 °C followed by 30 cycles of denaturation for 30 s at 95 °C, primer annealing for 30 s

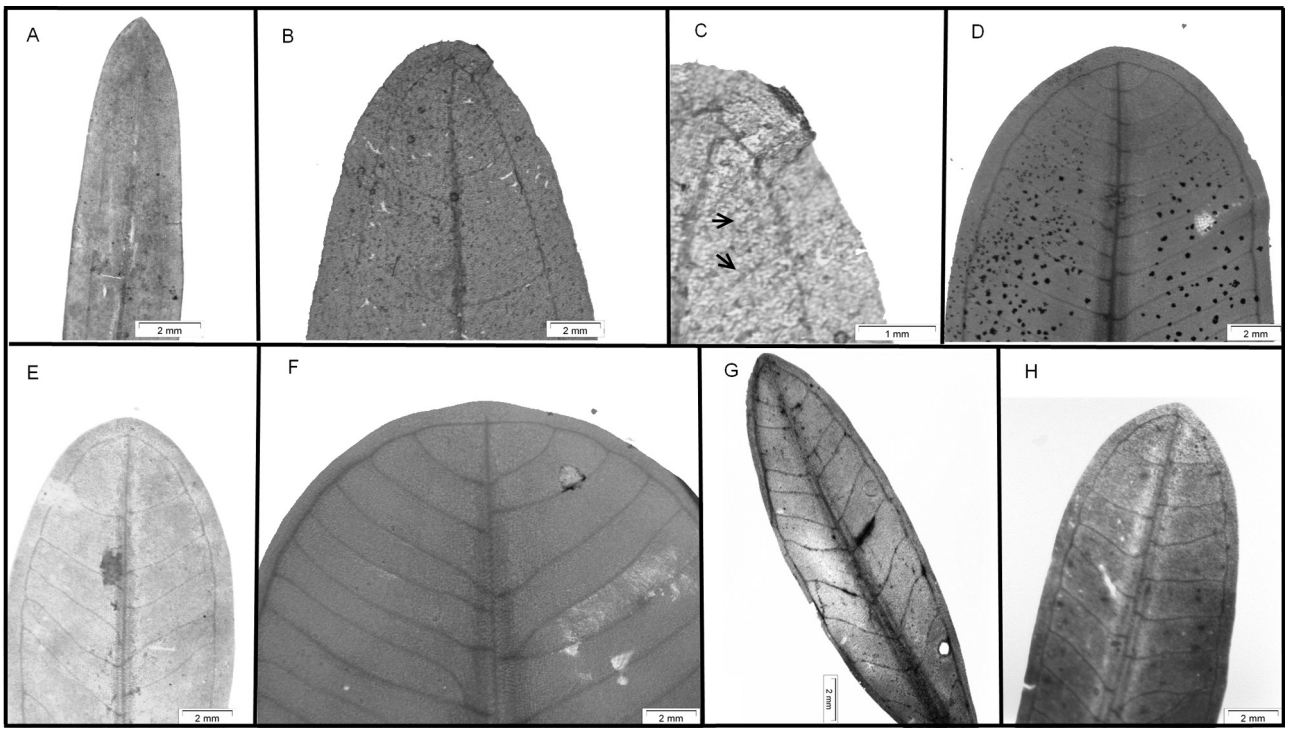


**Fig. 2.** Various leaf morphology of *Halophila* species in Viet Nam. (a) *H. beccarii*; (b) *H. decipiens*; (c) *H. decipiens* show hairs on the surface indicated by arrows; (d) *H. ovalis*, Van Phong Bay; (e) *H. ovalis*, Thuy Trieu Lagoon; (f) *H. ovalis*, Nha Trang Bay; (g) *H. ovalis*, Cu Mong Lagoon; (h) *H. ovalis*, Thi Nai Lagoon.

at 49 °C and extension for 40 s at 72 °C, terminated by a final hold at 10 °C (Lucas et al., 2012). The pGEM®T Cloning Kit (Promega, Mannheim, Germany) was used for cloning the PCR fragment following the producer's protocol. The total ligation volume was used for transformation in competent *Escherichia coli* XL1-blue cells. Plasmid preparation was performed relying on the principle of alkaline lysis (Birnboim and Doly, 1979). The separation of DNA fragments obtained by PCR or restriction analysis was performed using 1% TAE-agarose gels. All PCRs were repeated two to four times independently to reduce errors in the final consensus sequence to a minimum. Sequencing was done by GATC Biotech (Konstanz, Germany) from both directions. For sequencing reaction standard primers T7 and SP6 were used, as proposed for the pGEM®T vector. The obtained raw sequence data was analyzed using Clone Manager 9 (Sci-Ed, Cary, NC, USA). The sequence assembly was manually edited by eye to obtain a consensus sequence. The consensus sequence was subsequently analyzed using BLAST to verify the gene fragment and/or taxon. After verification, the sequence was examined for the appropriate forward and reverse primer sequences. Primer and contaminating vector sequence were cut off. The gained consensus sequences were preceded for phylogenetic analysis. Data from this result (seven *rbc*L and seven *mat*K sequences) were used for phylogenetic analyses. Obtained sequences from Clone Manager 9 were exported to MEGA5 (Tamura et al., 2011) for further analyses.

### 2.3. Phylogenetic analyses

These sequences were aligned by CLUSTAL X (Thompson et al., 1997) and the alignment was further modified by eye. The program jModelTest 0.1.1 (Posada, 2008) was used to find the model of sequence evolution that fitted best with each data set. Phylogenetic analyses were performed using Maximum Likelihood (ML), Neighbor Joining (NJ) (Saitou and Nei, 1987) with the model Tamura 3-parameter, Maximum Parsimony (Felsenstein, 1992) in MEGA5 (Tamura et al., 2011), and Bayesian analysis (BA) (Metropolis-coupled Markov chain Monte Carlo method) performed in MrBayes v.3.2 (Ronquist et al., 2012). In the analyses, trees were tested by the bootstrapping method with 1000 bootstrap replications. The phylogenetic analyses were conducted on each locus, *rbc*L, *mat*K and the concatenated sequences of the two plastid markers by using the Tamura 3-parameter because it was the best-fitting base substitution model. *rbc*L sequences of *Thalassia hemprichii* (JN225341) and *Enhalus acoroides* (JN225336) as well as *mat*K sequences of *T. hemprichii* (JN225373) and *E. acoroides* (JN22536) were used as out-group to construct trees based on *rbc*L, *mat*K and the concatenated sequences of the two plastid markers, respectively. The Tamura 3-parameter model (Nei and Kumar, 2002) was used to estimate pair wise sequence divergence for *rbc*L and *mat*K sequences separately. The program for these tree-making and evolutionary divergence methods was MEGA5. All phylogenetic trees achieved from single gene or combined dataset were analyzed and exactly constructed by "tree of trees" approach (Nye, 2008). For the character-based analysis, first the phylogenetic tree of each gene was constructed by MrBayes, version 3.2 (Ronquist et al., 2012). The following parameters were used: mcmc ngen = 1000,000, nruns = 2, nchains = 4, temp = 0.100, printfreq = 1000, samplefreq = 100, and diagnfreq = 100, stopping the analysis when standard deviation of split frequencies was below 0.01. Trees achieved from MrBayes were converted to nexus format by FigTree v1.3.1 (Rambaut and Drummond, 2009). Tree and aligned sequence nexus file were prepared by Mesquite (Maddison and Maddison, 2011) before loading to the online tool of Character Analysis Organization System (CAOS) (Bergmann et al., 2009). The output files from CAOS–Analyzer were compared with the tree-based approach.

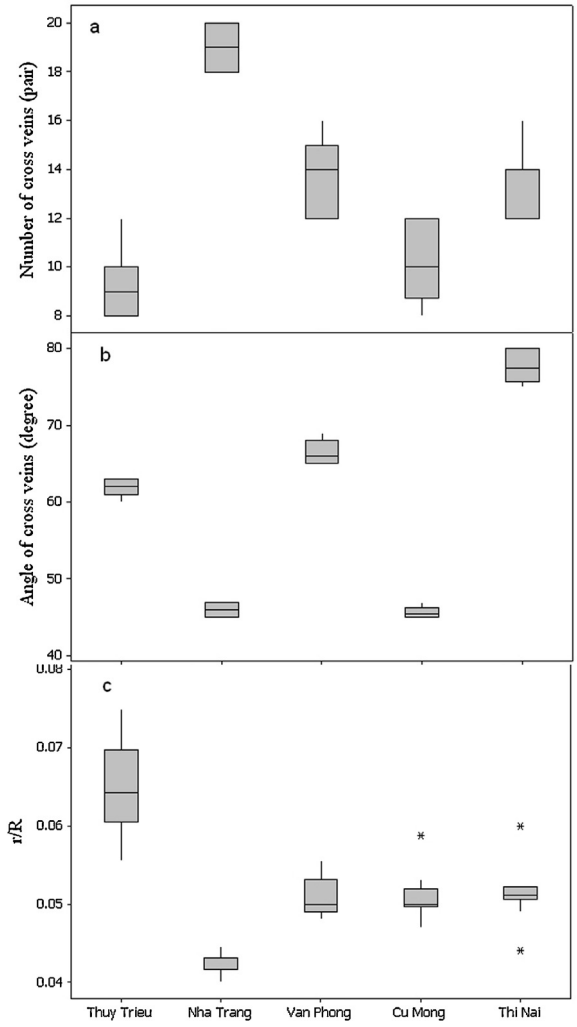


**Fig. 3.** Quantitative analysis of morphological parameters of *Halophila ovalis* leaves. (a) Variation in the number of paired cross veins, (b) angle of cross veins, and (c) the ratio of the distance between intra-marginal vein (*r*) and lamina margin (*R*) at five different sampling sites. Whisker plots were processed by Minitab software, version 15. TT: Thuy Trieu; CM: Cu Mong; TN: Thi Nai; NT: Nha Trang; VP: Van Phong.

## 3. Results

### 3.1. Variability of leaf morphology

Leaf shapes show variations among collection sites. Leaf shapes of the *H. ovalis* population at Van Phong and Nha Trang Bay are oblong (Fig. 2d and f) while the leaf shape of *H. ovalis* population at Thuy Trieu and Thi Nai are between oblong and elliptic (Fig. 2e and h). The leaf shape of *H. ovalis* population at Cu Mong Lagoon is remarkably elliptic (Fig. 2g). Data of mean pairs of cross-veins also indicate that *H. ovalis* collected at Nha Trang shows more pairs of cross veins (18–20) while mean pairs of cross-veins of other collection sites are from 8 to 16 (Fig. 3a). Cross vein angles also show variations among populations and range from 45° to 80° in which mean cross-vein angle of *H. ovalis* collected at Nha Trang and Cu Mong are around 47° while mean cross vein angle of *H. ovalis* collected at other locations is more than 60° (Fig. 3b). At the half-length point of the leaves collected at Nha Trang Bay the ratio of the distance between intra-marginal vein (*r*) and lamina margin (*R*) ranges from 0.040 to 0.046. This ratio is low in comparison to leaves collected in other locations (Fig. 3c). The *p*-values obtained from Levene's test of cross vein number, angles of cross vein and ratio between *r* and *R* (*r*/*R*) were 0.020, 0.026, and 0.051

**Table 2**
Results of ANOVA (single factor) performed on the distance between intra-marginal vein (*r*) and lamina margin (*R*) of *Halophila ovalis* from five collection sites.

| Source of variation | Sum of square | df | Mean | *F* |
|---|---|---|---|---|
| Between | 4.0404E−03 | 4 | 1.0101E−03 | 77.82 |
| Error | 7.7874E−04 | 60 | 1.2979E−05 | |
| Total | 4.8191E−03 | 64 | | |

respectively. Hence, only the data set of the ratio between *r* and *R* shows homoscedasticity ($p$-value > 0.05) and the remaining data sets show heteroscedasticity ($p$-value < 0.05). Single factor ANOVA shows that for the ratio between *r* and *R* significant differences can be observed among the five collection sites ($F = 77.82 > F_{crit.}$, $p < 0.001$) (Table 2). Details resulting from multiple comparisons of each trait obtained by the Tukey test show that there are significant differences of the ratio between *r* and *R* between populations at Thuy Trieu and at Cu Mong and Thi Nai, between populations at Van Phong and at Cu Mong and Thi Nai, and between populations at Cu Mong and at Thi Nai. However, there are no significant differences between populations at Thuy Trieu and at Nha Trang and Van Phong. Additionally, no significant differences are found between populations at Nha Trang and at Van Phong, Cu Mong, and Thi Nai.

### 3.2. Tree-based approach on single locus analysis of rbcL and matK

Since chloroplast genes have a relatively slow rate of nucleotide substitutions (Wolfe et al., 1987), obtaining an unambiguous alignment does not create difficulties. Tree-based methods were used to analyze the genetic relationships among a dataset of 22 *rbc*L sequences. A final alignment of 553 bp was generated for *rbc*L, of which 11 (1.83%) are parsimony informative characters, eight (1.4%) are variable sites, 540 (97.6%) are conserved sites, and five (0.9%) are singleton sites (data not shown). Members of the *Halophila* complex collected in Viet Nam are divided into two major groups (Fig. 4a). *H. beccarii* is resolved within two lineages of species with complex phyllotaxy that are basal to the clade containing *H. decipiens, H. stipulacea, H. ovalis, H. ovalis* subsp. *ramamurthiana*, and *H. ovata*. However, the bootstrap support values of ML, NJ, MP and BA are not high, 53, 56, 60, 69%, respectively. In the remaining *Halophila* complex three sub-groups are formed including (i) *H. decipiens*, (ii) *H. stipulacea*, and (iii) *H. ovalis, H. ovalis* subsp. *ramamurthiana*, and *H. ovata*, but the bootstrap values are less than 50% (data not shown) (Fig. 4a). Our analysis shows that there are eight nucleotide differences (six transitions and two transversions) between *H. beccarii* and *H. decipiens.* Eight nucleotide differences (six transitions and two transversions) are also found between *H. beccarii* and *H. ovalis.* Comparing nucleotide differences between *H. decipiens* and *H. ovalis*, two transitions are found. There are no nucleotide differences between *H. beccarii* from Viet Nam and India; between *H. ovalis* from Viet Nam and *H. ovalis, H. ovata*, and *H. ovalis* subsp. *ramamurthiana* in India (data not shown). Additionally, the number of base substitutions per site between sequences shows that evolutionary divergence between *H. beccarii* collected in Viet Nam and India is 0. Evolutionary divergence between *H. decipiens* and *H. ovalis* is low (0.004). However, 0.015 is the evolutionary divergence between *H. beccarii* and *H. ovalis* and 0.015 is the evolutionary divergence between *H. beccarii* and *H. decipiens* (Table 3).

Tree-based analyses were conducted on a dataset of 19 *mat*K sequences of the *Halophila* complex. A final alignment of 812 bp was generated for *mat*K, of which 18 (2.0%) are parsimony informative characters, 35 (4.3%) are variable sites, 777 (95.7%) are conserved sites, and 17 (2.1%) are singleton sites. The topology of ML, NJ, MP and BA (Tamura 3-parameters model) shows that the *Halophila* complex collected in Viet Nam divides into three major groups including (1) *H. beccarii* that group with *H. beccarii* collected in India, (2) *H. decipiens* stands as single group and (3) the *H. ovalis* group (Fig. 4b). Results indicate that *H. beccarii* is well resolved within the *Halophila* complex and is basal to the clade containing *H. decipiens, H. stipulacea, H. ovalis, H. ovalis* subsp. *ramamurthiana*, and *H. ovata* with a very high bootstrap value (100% for three out of for methods). Similarly, *H. decipiens* is also resolved from *H. stipulacea* and the *H. ovalis* complex and stands as single clade with bootstrap support value of 100, 48, 47 and 83%, respectively. *H. ovalis* collected in different location groups together (Fig. 4b). Results on nucleotide differences and evolutionary divergence between *H. beccarii* and the *H. ovalis* complex indicate that there are 18–19 differences (2.2–2.3%). Fifteen to 16 nucleotide differences are found between *H. decipiens* and the *H. ovalis* complex, evolutionary divergence between *H. decipiens* and *H. ovalis* complex is 1.9–2%. Comparison between *H. beccarii* and *H. decipiens* shows that there are 29 nucleotide differences, evolutionary divergence is 2.3%. Nucleotide differences (0–2 bp) are also found between the members of the *H. ovalis* complex collected at difference locations, so evolutionary divergences among them are 0.0–0.2% (Table 4).

### 3.3. Tree-based analyses on combined dataset

Tree-based analyses were conducted the concatenated sequences of the two plastid markers. A final alignment of 1365 bp was generated for combined *mat*K (812 bp) and *rbc*L (553 bp) sequences, of which 54 bp (4%) are parsimony informative characters, 103 bp (7.5%) are variable sites, 1263 bp (92.5%) are conserved sites, and 49 bp (3.6%) are singleton sites. Results of the ML, NJ, MP and BA method (Tamura 3-parameter model) show that *Halophila* complex collected in Viet Nam depicts three major clades including *H. beccarii, H. decipiens*, and *H. ovalis* (Fig. 5). Topology of the phylogenetic tree based on the concatenated sequences of the two plastid markers is similar to the phylogenetic tree based on single locus *rbc*L (Fig. 4a) or *mat*K (Fig. 4b). *H. beccarii* collected in Viet Nam is well resolved as single clade and group with *H. beccarii* collected in India (99, 99, 99 and 100% of bootstrap value support, respectively). By morphological traits, *H. beccarii* is easy to recognize due to the lacking of veins (Fig. 2a).

**Table 3**
Estimation of the evolutionary divergence between *rbc*L sequences. The numbers of base substitutions per site between sequences are shown. Analyses were conducted using the Tamura 3-parameter model. The analysis involved 20 nucleotide sequences. There were a total of 553 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. (1) *H. ovalis* Van Phong, (2) *H. ovalis* Cu Mong; (3) *H. ovalis* Nha Trang; (4) *H. ovalis* Thi Nai; (5) *H. ovalis* Thuy Trieu; (6) *H. ovalis* (JN225348); (7) *H. ovalis* (JN225349); (8) *H. decipiens* (JN225340); (9) *H. ovalis* subsp. *ramamurthiana* (JN225355); (10) *H. ovata* (JN225347); (11) *H. ovalis* (AB004890); (12) *Halophila* sp. B (JN225338); (13) *H.* sp. (DQ859168); (14) *H. stipulacea* (JN225356); (15) *H. decipiens* (U80698); (16) *H. decipiens* Nha Trang; (17) *H. beccarii* TT; (18) *H. beccarii* (JN225339); (19) *Halophila* sp. A (JN225337); (20) *H. engelmannii* (U80699).

| Species | 1–13 | 14 | 15 | 16 | 17–19 | 20 |
|---|---|---|---|---|---|---|
| 1–13 | 0.000 | | | | | |
| 14 | 0.005 | | | | | |
| 15 | 0.004 | 0.009 | | | | |
| 16 | 0.004 | 0.005 | 0.007 | | | |
| 17–19 | 0.015 | 0.015 | 0.015 | 0.015 | | |
| 20 | 0.009 | 0.011 | 0.009 | 0.009 | 0.013 | 0.000 |


**Fig. 4.** (a) Phylogenetic tree of *Halophila* inferred from Maximum Likelihood (ML), Neighbor Joining (NJ), Maximum Parsimony (MP) and Bayesian analysis (BA) method (Tamura 3-parameter model) based on 553 bp of 22 *rbc*L sequences. *E. acoroides* (NJ225336) and *T. hemprichii* (JN225341) are used as out-group. (b) Phylogenetic tree of *Halophila* inferred from ML, NJ, MP and BA method (Tamura 3-parameter model) based on 812 bp of 19 *mat*K sequences. *E. acoroides* (NJ225360) and *T. hemprichii* (JN225373) are used as out-group. Bootstrap values (more than 50%) of each method are shown in each node. Above the nodes, left: ML, right: NJ. Below the nodes, left: MP, right: BA. Specimens collected in Viet Nam are marked by symbol (▲). TT: Thuy Trieu; CM: Cu Mong; TN: Thi Nai; NT: Nha Trang; VP: Van Phong.

Similarly, *H. decipiens* forms a defined clade (54, 65, 62 and 100% of bootstrap value support, respectively). It is resolved basal to the clade containing *H. stipulacea, H. ovalis, H. ovata, H. ovalis* subsp. *ramamurthiana*, and *H. decipiens* collected in India. *H. decipiens* is recognized from other *Halophila* species by numerous hairs on the surface (Fig. 2b and c). Sequences of *H. ovalis* individuals collected in different locations including lagoons and open water form a defined clade (Fig. 5).

**Table 4**
Estimation of the evolutionary divergence between *mat*K sequences. The numbers of base differences per site between sequences are shown. Analyses were conducted using the Tamura 3-parameter model. The analysis involved 17 nucleotide sequences. There were a total of 812 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. (1) *H. ovata* (JN225367), (2) *H. ovalis* (AB002570); (3) *Halophila* sp. B (JN225362); (4) *H. ovalis* Thi Nai; (5) *H. ovalis* Thuy Trieu; (6) *H. ovalis* (JN225365); (7) *H. ovalis* (JN225366); (8) *H. decipiens* (JN225364); (9) *H. ovalis* subsp. *ramamurthiana* (JN225380); (10) *H. ovalis* Thi Nai; (11) *H. ovalis* Cu Mong; (12) *H. ovalis* Nha Trang; (13) *H. stipulacea* (JN225381); (14) *H. decipiens* Nha Trang; (15) *H. beccarii* (JN225363); (16) *H. beccarii* TT; (17): *Halophila* sp. A (JN225361).

| Species | 1–9 | 10 | 11 | 12 | 13 | 14 | 15–17 |
|---|---|---|---|---|---|---|---|
| 1–9 | 0.000 | | | | | | |
| 10 | 0.001 | | | | | | |
| 11 | 0.001 | 0.002 | | | | | |
| 12 | 0.001 | 0.002 | 0.002 | | | | |
| 13 | 0.002 | 0.004 | 0.004 | 0.004 | | | |
| 14 | 0.019 | 0.020 | 0.020 | 0.020 | 0.019 | | |
| 15–17 | 0.023 | 0.024 | 0.024 | 0.024 | 0.023 | 0.037 | 0.000 |

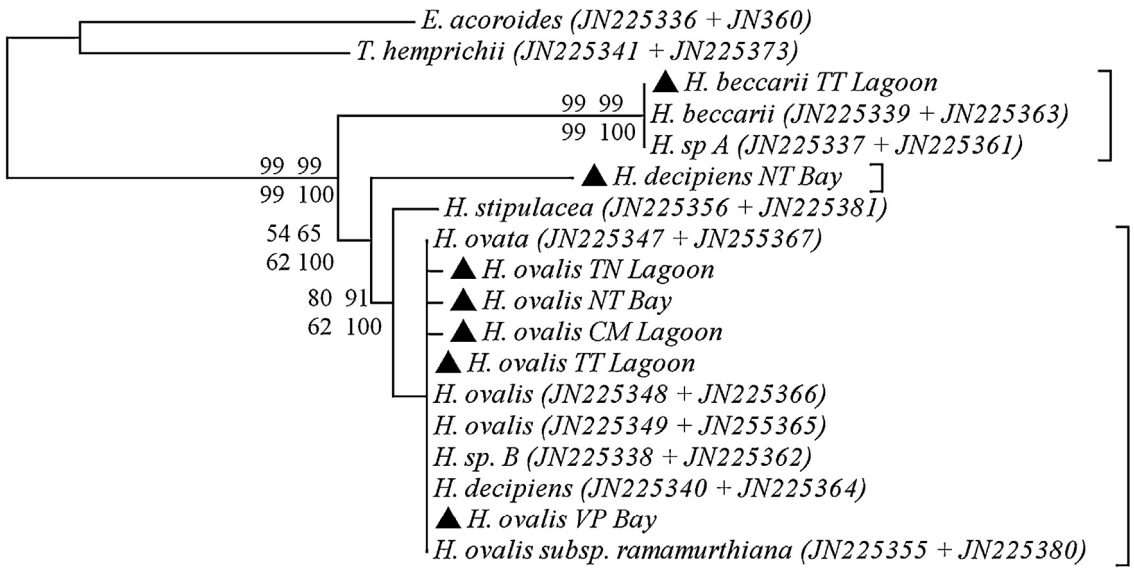


**Fig. 5.** Phylogenetic tree of *Halophila* inferred from ML, NJ, MP and BA method (Tamura 3-parameter model) of 1365 bp in the combined dataset of 18 *mat*K and 18 *rbc*L sequences. *E. acoroides* and *T. hemprichii* are used as out-group. For more details see legend to Fig. 4.

### 3.4. Character-based approach on single locus analysis of rbcL and matK

Character Attributes (CAs) of *rbc*L and *mat*K were analyzed and summarized in Table 5. For the *rbc*L, CAs rank from 1 to 16/553 CAs. Among the *Halophila* complex, *H. engelmannii* shows the highest number of CAs (16). The next species are *H. beccarii* (3), *H. stipulacea* (2), and *H. decipiens* (1), respectively. In the remaining *Halophila* complex, no CAs are found. Hence, three species including *H. engelmannii, H. stipulacea*, and *H. decipiens* can be recognized from the *Halophila* complex. For *mat*K, CAs rank from 1 to 16/812 CAs. *H. beccarii* (16), *H. decipiens* (12), and *H. stipulacea* (2) are found from the *Halophila* complex. Moreover, one CA is found at three *H. ovalis* populations including Thuy Trieu, Nha Trang and Thi Nai. In the remaining species of the *Halophila* complex no CAs are found.

## 4. Discussion

Previous studies on the variation of *H. ovalis* leaf morphology showed that environmental conditions affected leaf morphology and it was also changed in different ecological conditions (McMillan, 1983; Annaletchumy et al., 2005). Significant variations of leaf width of *H. ovalis* were found at different seasons and locations (Hedge et al., 2009). Other ecological conditions including disturbance (Peralta et al., 2005), salinity (Benjamin et al., 1999), intertidal conditions (Cabaço et al., 2009), and depth (Procaccini et al., 1999) also caused variations of leaf morphology of seagrasses. The largest group of specimens sampled in this study is in the *H. ovalis* clade. *H. ovalis* specimens were collected at two main different ecological conditions including offshore island (Nha Trang Bay) and coastal lagoons (Thuy Trieu, Van Phong, Cu Mong and Thi Nai). The *H. ovalis* population in Nha Trang Bay occurred in the depth of 4–6 m at and was less affected by environmental factors. In contrast, *H. ovalis* in the remaining populations was daily affected by salinity fluctuation (high salinity in high tide and low salinity in low tide), exposed to the air at low tide and high turbidity. Detailed analyses of leaf morphology of *H. ovalis* individuals in populations showed significant differences among populations. However, they are in the range of *H. ovalis* morphological parameters described by den Hartog (1970). Among the sampling sites *H. ovalis* collected at Cu Mong Lagoon, which has been stored as *H.* cf *johnsonii*, showed different leaf shape (elliptic) (Fig. 2g). However, our detailed analysis of leaf morphology indicated that there are distinctive leaf morphotypes (narrow-leaf type). Morphotypes were also found at different *H. nipponica* populations in Japan (Shimada et al., 2012). Recently, putative *H. johnsonii* collected at Salt Pond, Antigua, was identified as *H. ovalis* based on leaf morphology and molecular analysis (Short et al., 2010). Another specific case is *H. ovalis* population at Nha Trang Bay: leaf morphology shows significant difference to the remaining populations by the trait of number of cross veins and the ratio of the distance between intra-marginal vein and lamina margin. It seems to be morphologically more similar to *H. major* as described for Japanese populations (Kuo et al., 2006; Uchimura et al., 2008).

In the present study, single analysis of *rbc*L and *mat*K sequences and the concatenated sequences of the two plastid markers were used to resolve the phylogenetic relationships within the *Halophila* complex. We used short *rbc*L (553 bp) and *mat*K (812 bp) sequences instead of complete sequences that are about 1400 and 1500 bp, respectively (Les et al., 1993; Tanaka et al., 1997; Kato et al., 2003). The shorter sequences meet three main criteria including (i) significant genetic variability and divergence on the species level, (ii) a suitable short sequence length so as to facilitate DNA extraction and amplification, and (iii) the presence of conserved sites (Kress and Erickson, 2007; Ford et al., 2009; Hollingsworth et al., 2009). Based on single and the concatenated sequences of the two plastid markers, tree-based analysis depicts three distinct clades that represent three species collected in Viet Nam including *H. beccarii, H. decipiens*, and *H. ovalis*. Topologies based on *rbc*L, *mat*K and the combined dataset are similar but not equal including higher bootstrap support. The comparison between the single loci revealed that *mat*K showed better species resolution with respect to *H. beccarii* and *H. decipiens* collected in Viet Nam while only *H. beccarii* was resolved when *rbc*L sequences were used. Finally, topology of *H. ovalis* is clearer when the combined dataset of *rbc*L and *mat*K sequences is used. However, the analyses do not support clear boundaries among described species or subspecies, such as *H. ovata*, *H. ovalis* or *H. ovalis* subsp. *ramamurthiana*. Results of additional marker systems or DNA fingerprint techniques might sharpen or challenge the taxonomic borders of *H. ovalis* taxa.

**Table 5**
Diagnosis of Character Attributes (CAs) for the *rbc*L and *mat*K fragments. Diagnostic characters for each species are listed with position and respective nucleotide. The analysis was carried out using CAOS software. TT: Thuy Trieu; CM: Cu Mong; TN: Thi Nai; NT: Nha Trang; VP: Van Phong.

| Species | Genes | Character attributes |
|---|---|---|
| *H. ovalis* VP, TT | *rbc*L | |
| | *mat*K | |
| *H. ovalis* CM | *rbc*L | |
| | *mat*K | 368(C) |
| *H. ovalis* NT | *rbc*L | |
| | *mat*K | 476(T) |
| *H. ovalis* TN | *rbc*L | |
| | *mat*K | 290(A) |
| *H. stipulacea* | *rbc*L | 336(G), 513 (G) |
| | *mat*K | 347(T), 671(G) |
| *H. decipiens* NT | *rbc*L | 132 (A) |
| | *mat*K | 30(T), 69(T), 181(C), 211(C), 315(A), 386(T), 496(A), 548(A), 644(A), 668(C), 758(C), 774(C) |
| *H. beccarii* TT | *rbc*L | 228(C), 240(C), 309(T) |
| | *mat*K | 2(T), 15(T), 90 (G), 111(G), 121(G), 234(A), 276(A), 279(T), 320(C), 333(C), 345(T), 438(A), 537(T), 544(G), 625(T), 676(C) |

In the genus *Halophila, H. beccarii* is resolved basal to the clade containing *H. decipiens*, *H. stipulacea*, and *H. ovalis*. Our results are supported by previous studies (Waycott et al., 2002; Uchimura et al., 2006, 2008; McMahon and Waycott, 2009; Short et al., 2010; Lucas et al., 2012) that *H. beccarii* is closer to the ancestor of *Halophila* species than *H. decipiens* and *H. ovalis*. The results of the concatenated sequences of the two plastid markers of *H. decipiens* collected at Nha Trang Bay show that it forms a single clade basal to the clade containing *H. stipulacea* and *H. ovalis* specimens. Actually, there are two *rbc*L sequences of *H. decipiens* found in NCBI (Table 1). However, above results indicate that *H. decipiens* is not resolved from *Halophila* complex when only *rbc*L sequences are used (Fig. 4a). *mat*K or the concatenated sequences of the two plastid markers can be used to resolve *H. decipiens* from the *Halophila* complex (Figs. 4b and 5). On the other hand, no difference or little variation were detected in comparisons of sequences among *H. decipiens* specimens collected from a wide geographical distribution (McDermid et al., 2002; Uchimura et al., 2008; Waycott et al., 2008; McMahon and Waycott, 2009). Morphologically *H. decipiens* can be distinguished from other *Halophila* species including *H. ovata, H. ovalis* and *H. minor* by several characteristics including present marginal serrations of leaves, leaf hairs (Fig. 2c) and being monoecious. Our direct comparisons of the tree-based results and divergence of *H. decipiens* collected in Viet Nam and India support the conclusion of Lucas et al. (2012) that *H. decipiens* collected in India was misidentified.

The character-based approach is based on the analysis of unique sequence sites. Comparing the results of the character-based (Table 5) and tree-based approach for the *rbc*L gene (Fig. 4a) indicates that the two approaches show similar results when low numbers of CAs are found, for example from *H. beccarii, H. stipulacea* and *H. decipiens* in comparison to the *Halophila* complex in agreement with low bootstrap values between species above (Tree-based approach). However, the number CAs found in the *mat*K gene is higher than in the *rbc*L gene (Table 5), especially in the case of *H. beccarii* and *H. decipiens*. Additionally, three *H. ovalis* sub-populations including Nha Trang Bay, Thi Nai and Cu Mong are detected by the character-based approach. The character-based approach shows advantages due to its correctness; therefore very accurate alignment and bioinformatic knowledge of the user are required (Lucas et al., 2012).

Our results on *rbc*L indicated that there was no nucleotide difference among *H. ovalis* populations and very low nucleotide differences on *mat*K although significant differences of leaf morphology were observed among the collection sites. Significant variation in leaf morphology was detected, but only low levels of the variation in genetic markers that were also found in other species of *Halophila* such as *H. hawaiiana* (McDermid et al., 2003), *H. nipponica* (Shimada et al., 2012) and *H. stipulacea* (Procaccini et al., 1999).

## 5. Conclusion

Our results show that single locus *rbc*L analysis can resolve only *H. beccarii* from the *Halophila* complex. *mat*K sequence show better resolution because *H. beccarii* and *H. decipiens* could be identified. The concatenated sequences of the two plastid markers is the best way to resolve all species analyzed in the *Halophila* genus. Moreover, putative *H.* cf *johnsonii* collected at Cu Mong Lagoon should be treated as *H. ovalis*. The analysis of the ratio of the distance between intra-marginal vein and lamina margin of *H. ovalis* reveal significant differences among populations. Moreover, the parameters of *H. ovalis* leaf morphology at Nha Trang Bay indicate that it is closer to *H. major* than to *H. ovalis*. The molecular analysis of the leaf samples reveals that morphological parameters are not sufficient to differentiate among closely related *H. ovalis* individuals. However, also the applied plastidic *rbc*L and *mat*K sequences did not resolve in the *H. ovalis* complex. Molecular marker systems which can differentiate lower taxa levels than the current genus and species level need to be applied, such as the ITS or DNA fingerprint approaches to challenge and delineate the boundary between species.

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# CHAPTER 3

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# New record of the seagrass species *Halophila major* (Zoll.) Miquel in Vietnam: evidence from leaf morphology and ITS analysis

**Abstract:** The seagrass *Halophila major* (Zoll.) Miquel is reported for the first time from Vietnam. It was found growing with other seagrass species nearshore, 4–6 m deep at Tre Island, Nha Trang Bay. Leaf morphology and phylogenetic analysis based on ribosomal internal transcribed spacer sequences confirmed the identification. There was very little sequence differentiation among samples of *H. major* collected in Vietnam and other countries in the Western Pacific region. A very low evolutionary divergence among *H. major* populations was found.

**Keywords:** *Halophila major*; internal transcribed spacer; new record; seagrass; Vietnam.

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## Introduction

In Vietnam, two species of *Halophila* were recorded in 1885: *Halophila ovalis* (R. Br.) H. *f.* and *H. beccarii* Ascherson (Tien 2008). *Halophila minor* (Zollinger) den Hartog and *H. decipiens* Ostenf. were added to the list of *Halophila* occurring in Vietnam during a later study (Dai et al. 1998). However, the taxonomic diversity of the genus *Halophila* has been given little attention until recently. Defining taxonomic boundaries within the genus *Halophila* has represented a real challenge due to leaf morphological traits that overlap among species (Kuo et al. 2006, Uchimura et al. 2008, Short et al. 2011, Shimada et al. 2012). *Halophila major* (Zoll.) Miquel [formerly known in Japan as *H. euphlebia* Makino but recently classified as *H. major* by Kuo et al. (2006)] was distinguished from closely related species, such as *H. ovalis*, *H. minor*, *H. ovata* Gaudichaud, and *H. nipponica* J. Kuo by two main characteristics: (i) the number of cross veins, which ranges from 18 to 22, and (ii) the ratio of the distance between the intramarginal vein and the lamina margin at the half-way point along the leaf length, which is 1:20–1:25 (Kuo et al. 2006). Recently, genetic markers, including plastid and nuclear sequences, have been used to reveal the genetic relationships among members of the genus *Halophila*. Among the molecular markers used, neither single sequence analysis of the plastid gene encoding the large subunit of ribulose-1,5-bisphosphate-carboxylase-oxygenase (*rbc*L) and of the plastid maturase K (*mat*K) nor analysis of the concatenated sequences of the two plastid markers has resolved the two closely related species *H. ovalis* and *H. ovata* (Lucas et al. 2012). In contrast, using phylogenetic analyses of the nuclear ribosomal internal transcribed spacer (ITS1-5.8S-ITS2) region showed that some specimens identified as *H. ovalis* belonged to different clades, and this clearly points to the need for critical taxonomic revision of *Halophila* material across the entire geographical range of this genus (Waycott et al. 2002). A reassessment of *Halophila* species from Japan based on ITS sequences indicated that *H. major* and *H. ovalis* are distinct species (Uchimura et al. 2008), which supported investigations by Kuo et al. (2006). Studies of ITS sequences have reported little or no nucleotide difference between individual *Halophila* species, such as *H. nipponica* (Shimada et al. 2012), *H. hawaiiana* Doty and B. C. Stone (McDermid et al. 2003), and *H. stipulacea* (Forss.) Ascherson (Ruggiero and Procaccini 2004), although the species varied considerably in leaf morphology. However, the ITS marker was a useful tool to reveal new records of *H. decipiens* for regions, such as the Hawaiian Islands (McDermid et al. 2002) and Kenya (McMahon and Waycott 2009). ITS sequences also indicated that *H. johnsonii* Eiseman and *H. ovalis* were synonyms (Short et al. 2010). The results of Uchimura et al. (2008) based on ITS sequences suggest that *H. gaudichaudii* J. Kuo, *H. okinawensis* J. Kuo, and *H. nipponica* may be conspecific.

Our initial studies based on morphology and analysis of genetic markers (*rbc*L and *mat*K) indicated that *H. ovalis* collected at Nha Trang Bay showed different traits in comparison with other *H. ovalis* populations

in Vietnam, although there were no nucleotide differences among *rbc*L sequences and only one different base pair among *mat*K sequences of the collections (Nguyen et al. 2013). This led to the hypothesis that phylogenetic analysis based on ITS sequences would resolve the taxonomic uncertainties among specimens of *Halophila* from Nha Trang, and clarify the status of *Halophila* species in Vietnam. There have been no previous records of *H. major* in Vietnam (Tien et al. 2002), although it occurs in neighboring countries, such as Indonesia and Thailand (Uchimura et al. 2008). This study documents a new record for *H. major* in Vietnam. The morphology, location, and habitats of *H. major* are described, and a molecular phylogeny is presented showing the position of *H. major* from Vietnam in a Western Pacific context.

## Materials and methods

Plants of *Halophila* species (*H. beccarii*, *H. decipiens*, and *H. ovalis*) were collected from five different locations: Thi Nai Lagoon, Cu Mong Lagoon, Van Phong Bay, Nha Trang Bay, and Thuy Trieu Lagoon along the coastal central provinces in Vietnam (Figure 1) by SCUBA diving or snorkeling in depths of 1–6 m. Sections of plants about 10–12 cm long in a developmentally comparable state were collected haphazardly from 10 to 15 different plants, which were separated by 10–15 m to avoid collecting from the same clone. These plant sections consisting of intact roots, rhizome, and leaves were washed with seawater in the field to remove epiphytes and debris that were attached to the plants. Each plant sample was sorted by species, placed in a single plastic bag, kept on ice, and transferred to the laboratory on the same day. In the laboratory, samples were rewashed with deionized water to remove seawater. Each plant was divided into two parts, one was pressed as an herbarium voucher specimen and the other was stored in high-salt cetyltrimethylammonium bromide (CTAB) buffer (Štorchová et al. 2000) for later DNA extraction and morphological analysis. Herbarium voucher specimens are currently deposited at the Institute of Oceanography, Nha Trang City, Vietnam. Material stored in CTAB buffer was brought to the Institute of Botany, Leibniz University Hannover, Germany, for further analysis. Ten mature leaves were selected from 10 different plants of each species for morphological measurements, including lamina width, distance from intramarginal vein to lamina margin, cross-vein angle, and the number of cross veins. The ratio between intramarginal veins and the width at the half-length of the lamina was calculated. Specimens were identified using the keys of den Hartog (1970) and Kuo et al. (2006).

Leaves were washed with deionized water to remove CTAB buffer completely. Eight to 10 young leaves from one individual of each species were homogenized by mortar and pestle in liquid nitrogen, and 100 mg of the finely powdered plant material was used for DNA extraction. DNA extraction was carried out using the Plant Nucleospin II Kit (Macherey & Nagel, Düren, Germany) following manufacturer's instructions with slight modifications according to Lucas et al. (2012). The region selected for PCR amplification was the nuclear ITS region including the 5.8S sequence. Primer pairs used in this study were P674 5′-CCTTATCATTTAGAGGAAGGAG-3′ (ITS5a) (Stanford et al. 2000) and P675 5′-TCCTCCGCTTATTGATATGC-3′ (ITS4) (White et al. 1990) to amplify a sequence of 700–720 bp consisting of ITS1, 5.8S, and ITS2. The total volume of 25 µl included 1× Dream *Taq* Green buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1 U *Taq* polymerase (MBI Fermentas, St. Leon-Rot, Germany), 10–30 ng template DNA, and 1 pmol of each primer. PCR was performed in a PTC 200 thermocycler (Biozym-Diagnostik GmbH, Hess Oldendorf, Germany) with a heated lid under the following conditions: initial denaturation for 4 min at 95°C followed by 30 cycles of denaturation for 25 s at 95°C, primer annealing for 30 s at 52°C, and extension for 35 s at 72°C, terminated by a final hold at 10°C. All PCR reactions were repeated two to four times independently with the same individual to keep errors (possibly created by the *Taq* polymerase) in the final consensus sequence to a minimum. Direct sequencing of PCR product was done by GATC Biotech (Konstanz, Germany) from both directions. Consensus sequence was achieved by Clone Manager 9 (Sci-Ed, Cary, NC, USA). For comparison, known ITS sequences of other *Halophila* species were added to the dataset (Table 1). These sequences were aligned by CLUSTAL X (Thompson et al. 1997), and the alignment was further modified by eye. Gaps were considered as missing data. Identical sequences within each species were excluded from the alignment. Additional in-group sequences were obtained from GenBank (Table 1), and included in the alignment. *Halophila angelmannii* Ascherson (AF366404) and *H. beccarii* Ascherson (AF366441) were used as outgroup (Waycott et al. 2002). The program jModelTest 0.1.1 (Posada 2008) was used to find the model of sequence evolution that fitted the data set best. Phylogenetic analyses were performed using maximum likelihood, neighbor joining (Saitou and Nei 1987) with the model Tamura 3-parameter + G, maximum parsimony (Felsenstein 1992) in MEGA5 (Tamura et al. 2011), and Bayesian analysis (metropolis-coupled Markov chain Monte Carlo method)

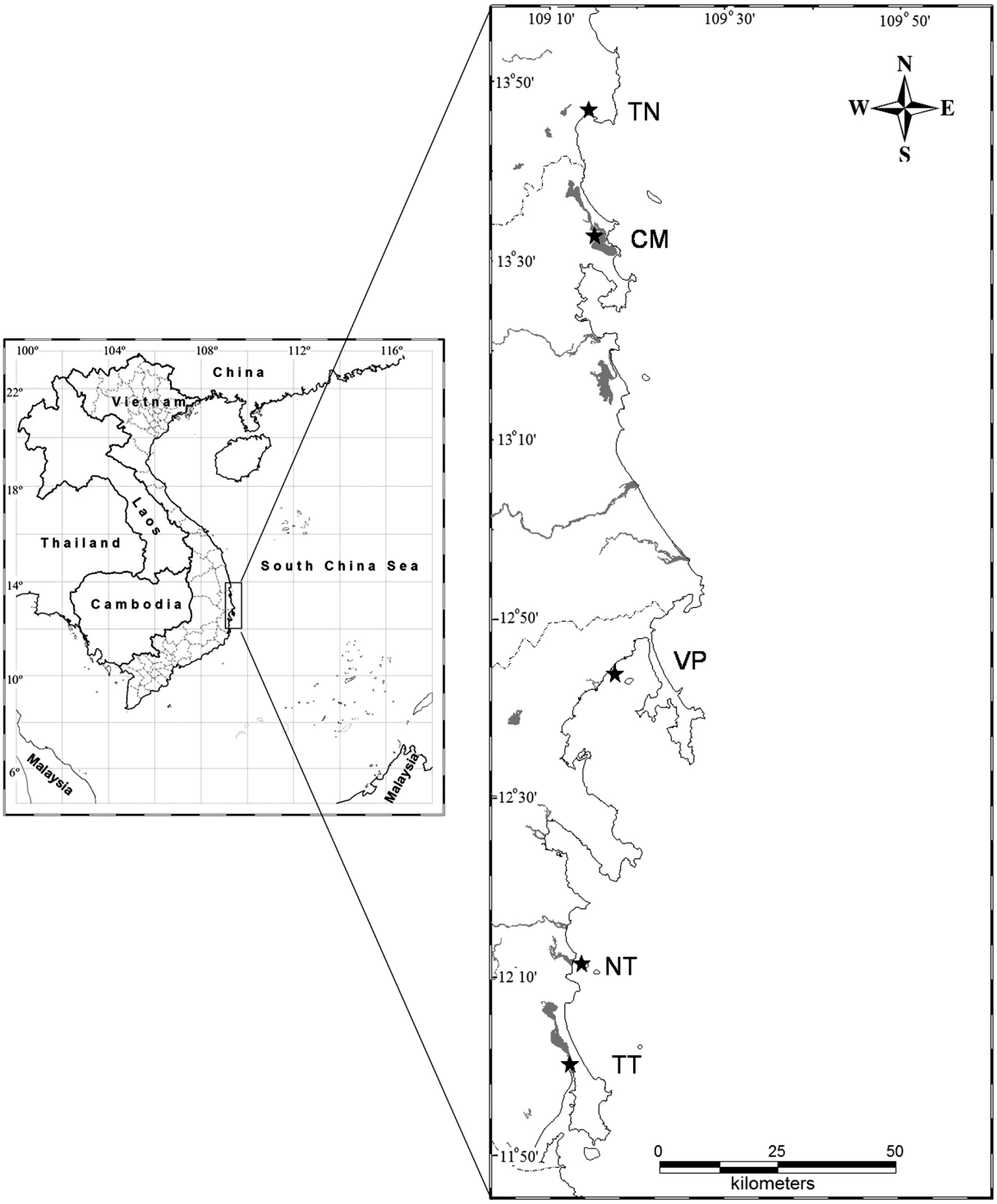


**Figure 1** Sample collection sites (★) for *Halophila* surveys in Vietnam.
TN, Thi Nai Lagoon; CM, Cu Mong Lagoon; VP, Van Phong Bay; NT, Nha Trang Bay; TT, Thuy Trieu Lagoon. (Source: Digital map, Department of Survey and Mapping, Ministry of Natural Resources and Environment, Vietnam.)

performed in MrBayes v.3.1.2 (Ronquist et al. 2011). In the Bayesian analysis, the two parallel runs with four chains each (three heated and one cold) were run for 1 million generations, sampling a tree every 100 generations. Only trees sampled after convergence were used to make inferences about the phylogeny and to compute a 50% majority-rule consensus tree. In the analyses, trees were tested by the bootstrapping method with 1000 replications.

**Table 1** List of the *Halophila* taxa included in the molecular analysis done in this study.

| Taxa | Geographic source | Citation | GenBank accession number |
|---|---|---|---|
| *H. beccarii* Ascherson | Gia Luan, Vietnam | Waycott et al. (2002) | AF366441 |
| ***H. beccarii* Ascherson** | **Thuy Trieu, Vietnam** | **This study** | **KC175914**[a] |
| *H. engelmannii* Ascherson | Florida, USA | Waycott et al. (2002) | AF366404 |
| *H. spinulosa* (R. Brown) Ascherson | Malaysia | Waycott et al. (2002) | AF366440 |
| *H. tricostata* Greenway | Australia | Waycott et al. (2002) | AF366438 |
| *H. decipiens* Ostenfeld | Florida, USA | Waycott et al. (2002) | AF366407 |
| *H. decipiens* Ostenfeld | Costa Rica | Waycott et al. (2002) | AF366409 |
| *H. decipiens* Ostenfeld | Malaysia | Waycott et al. (2002) | AF366412 |
| *H. decipiens* Ostenfeld | Nakagusuku, Japan | Uchimura et al. (2006) | AB243979 |
| *H. decipiens* Ostenfeld | Nakagusuku, Japan | Uchimura et al. (2006) | AB243980 |
| *H. decipiens* Ostenfeld | Oaura, Japan | Uchimura et al. (2006) | AB243984 |
| *H. decipiens* Ostenfeld | Izena Island, Japan | Uchimura et al. (2006) | AB243982 |
| ***H. decipiens* Ostenfeld** | **Nha Trang, Vietnam** | **This study** | **KC175913**[a] |
| *H. stipulacea* (Forsskäl) Anderson | Italy | Waycott et al. (2002) | AF366436 |
| *H. major* (Zoll.) Miquel | Bali, Indonesia | Uchimura et al. (2006) | AB436928 |
| *H. major* (Zoll.) Miquel | Sumbawa, Indonesia | Uchimura et al. (2006) | AB436926 |
| *H. major* (Zoll.) Miquel | Kagoshima, Japan | Uchimura et al. (2006) | AB436929 |
| ***H. major* (Zoll.) Miquel**[b] | **Nha Trang, Vietnam** | **This study** | **KC175910**[a] |
| *H. nipponica* J. Kuo | Odawa Bay, Japan | Uchimura et al. (2006) | AB436931 |
| *H. nipponica* J. Kuo | Mutsu Bay, Japan | Uchimura et al. (2006) | AB436932 |
| *H. nipponica* J. Kuo | Suou-Ohshima, Japan | Uchimura et al. (2006) | AB436933 |
| *H. minor* (Zollinger) den Hartog | Philippines | Waycott et al. (2002) | AF366405 |
| *H. minor* (Zollinger) den Hartog | Guam | Waycott et al. (2002) | AF366406 |
| *H. ovalis* (R. Brown) Hooker *f.* | Flores Island, Indonesia | Uchimura et al. (2008) | AB436940 |
| *H. ovalis* (R. Brown) Hooker *f.* | Dingo Beach, Australia | Waycott et al. (2002) | AF366431 |
| *H. ovalis* (R. Brown) Hooker *f.* | Trang, Thailand | Uchimura et al. (2008) | AB436939 |
| *H. ovalis* (R. Brown) Hooker *f.* | Trang, Thailand | Uchimura et al. (2008) | AB436938 |
| *H. ovalis* (R. Brown) Hooker *f.* | Nakagusuku, Japan | Uchimura et al. (2008) | AB243973 |
| *H. ovalis* (R. Brown) Hooker *f.* | Kayou, Japan | Uchimura et al. (2008) | AB243974 |
| *H. ovalis* (R. Brown) Hooker *f.* | Kabila, Japan | Uchimura et al. (2008) | AB243975 |
| *H. ovalis* (R. Brown) Hooker *f.* | Taketomi Island, Japan | Uchimura et al. (2008) | AB243976 |
| ***H. ovalis* (R. Brown) Hooker *f.*** | **Thuy Trieu, Vietnam** | **This study** | **KC175908**[a] |
| ***H. ovalis* (R. Brown) Hooker *f.*** | **Van Phong, Vietnam** | **This study** | **KC175909**[a] |
| ***H. ovalis* (R. Brown) Hooker *f.*** | **Thi Nai, Vietnam** | **This study** | **KC175911**[a] |
| ***H. ovalis* (R. Brown) Hooker *f.*** | **Cu Mong, Vietnam** | **This study** | **KC175912**[a] |

[a]Accession number was deposited in GenBank.
[b]First identification as *H. ovalis*.
Bold: Samples collected in the present study.

Sequence divergences and nucleotide differences were also calculated by Tamura 3-parameter model with gamma distribution in MEGA5 (Tamura et al. 2011).

## Results

This study shows for the first time that *Halophila major* (Figure 2), formerly identified as *H. ovalis* (Dai et al. 1998, Tien 2008), grows at Tre Island, Nha Trang Bay, at a depth of 4–6 m in patches within a mixed meadow of *Halodule uninervis* (Forssk.) Boiss, *Halodule pinifolia* (Miki) Hartog, and *Syringodium isoetifolium* (Asch.) Dandy. This is a new record for this seagrass species in Vietnam and expands its known geographical range northward. Both morphological and genetic analyses of specimens distinguished it as *H. major*.

The following leaf morphology was observed: lamina bright to dark green, oblong, paired leaves, without serrated leaf margins, lamina width 9–11 mm, length of mature leaves 15–18 mm, number of cross veins 18–22, and distance from intramarginal vein to lamina margin 0.20–0.25 mm. At the half-length point of the leaf, the ratio of the distance between intramarginal vein


**Figure 2** Fragment of wet living specimen of *Halophila major* collected from Nha Trang.

and lamina margin was 1:20–1:25. Cross-vein branching was very common, and cross-vein angle ranged from 45° to 60°.

On the basis of our data on leaf morphology, we conclude that only *H. major* and no *H. ovalis* was collected at Nha Trang Bay. Comparisons of the leaf morphology of *H. major* collected at Nha Trang Bay (Figure 3A) and that of *H. ovalis* collected at other locations (Figure 3B–E) showed differences in two leaf morphological traits: (i) the ratio of the distance between the intramarginal vein and the lamina margin (1:20–1:25 in *H. major*; 1:12–1:17 in *H. ovalis*) and (ii) the number of cross veins (<16 in *H. ovalis*; >16 in *H. major*). A detailed comparison of the leaf morphology of the two species is presented in Table 2 and Figure 3A–E. Direct comparison among specimens of *H. ovalis* collected from Thi Nai, Cu Mong, Van Phong, and Thuy Trieu, and *H. major* (originally identified as *H. ovalis*) collected in Nha Trang clearly indicated that *H. ovalis* specimens collected at Nha Trang were *H. major* (Table 2, Figure 3A–E).

## Genetic analyses

A set of 36 ITS sequences and 620 characters (nucleotides and gaps) covering ITS1, 5.8S, and ITS2 from the genus *Halophila* were included in our analysis. Only five nucleotides were different between *Halophila major* collected in Vietnam and the published sequence data for *H. major* (Uchimura et al. 2008; for collection locations, see Table 1; data not shown), whereas 24–30 nucleotide differences (data not shown) were found between *H. major* collected at Nha Trang Bay and *H. ovalis* collected in other locations in Vietnam (Table 1). In addition, evolutionary divergence between our sequence data and published *H. major* sequences is very low (0.009), while

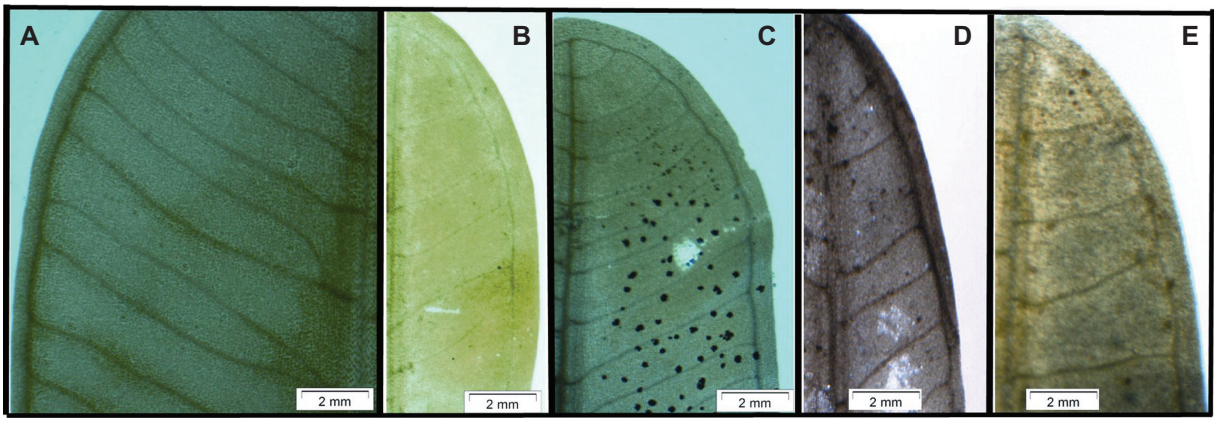


**Figure 3** Comparison of leaf morphology of *H. major* and *H. ovalis* specimens collected in Vietnam.
(A) *H. major*, (B) *H. ovalis* (TT), (C) *H. ovalis* (VP), (D) *H. ovalis* (CM), and (E) *H. ovalis* (TN). Abbreviations as in Figure 1.

**Table 2** Comparisons of leaf morphology of *H. major* and additional *H. ovalis* species collected in Vietnam and previous studies.

| Characteristic | Species | | | | | | |
|---|---|---|---|---|---|---|---|
| | ***H. ovalis*** | ***H. ovalis* TT** | ***H. ovalis* VP** | ***H. ovalis* CM** | ***H. ovalis* TN** | ***H. major* NT** | ***H. major*** |
| Lamina width (mm) | 5–20 | 5–7 | 6–7 | 3.7–4.7 | 6–7 | 9–12 | 9–11 |
| Lamina length (mm) | 10–40 (-70) | 9–12 | 8–11 | 8–11 | 8.5–11.7 | 10–18 | 15–25 |
| No. of paired cross veins | 10–25 | 8–12 | 12–16 | 8–10 | 12–16 | 16–22 | 14–17 |
| Space between intramarginal veins (mm) | 0.1–0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2–0.25 | 0.2 |
| Cross-vein branching | Common | Common | Occasional | Rarely | Common | Common | Common |
| Cross-vein angles | 45°–60° | 50°–70° | 60°–80° | 45°–50° | 60°–80° | 60°–80° | 45°–60° |
| Half lamina width: distance between intramarginal veins and lamina margin ratio | n/a | 1:12–17 | 1:15–17 | 1:9–11 | 1:15–17 | 1:24–25 | 1:20–25 |
| Source | den Hartog (1970) | This study | This study | This study | This study | This study | Kuo et al. (2006) |

n/a, not available.
The abbreviations are explained in the legend to Figure 1.

evolutionary divergence between our data and *H. ovalis* is much higher (0.043–0.051; data not shown). In this study, four methods were used to construct the phylogenetic trees. There was no difference in the topology of the phylogenetic trees based on these different methods except for small differences in the bootstrap values. The tree-based approaches showed that *H. major* collected in Vietnam grouped with *H. major* collected from other locations (Figure 4).

## Discussion

The highlight of this study is that *Halophila major*, a common species in the Western Pacific region, is recorded as a new species in Vietnam, increasing the known number of *Halophila* species in Vietnam to five. Variation of leaf morphology has been detected within several species of the *Halophila* genus, namely *H. ovalis* (Annaletchumy et al. 2005, Hedge et al. 2009), *H. hawaiana* (McDermid et al. 2003), and *H. nipponica* (Shimada et al. 2012). Molecular markers, especially ITS, were shown to be a valuable tool in resolving genetic relationships among the species of *Halophila*. For instance, *Halophila euphlebia* was once treated as synonym for *H. ovalis* (Miki 1934, den Hartog 1970); then, this species was transferred to *H. major* (Kuo et al. 2006). Results of Uchimura et al. (2008) and Shimada et al. (2012) supported the conclusion of Kuo et al. (2006) that *H. major* and *H. ovalis* are distinct species. In this study, leaf morphological parameters, including the distance between intramarginal veins, the lamina-to-margin ratio, and the number of cross veins, indicated that *Halophila* specimens collected at Nha Trang Bay in 2011 were much closer to *H. major* as described by Kuo et al. (2006) than to *H. ovalis*. Direct morphological comparison among specimens of *H. ovalis* collected from Thi Nai, Cu Mong, Van Phong, and Thuy Trieu, and the *Halophila* species collected in Nha Trang, which was originally identified as *H. ovalis*, clearly indicated this species is *H. major*. Our phylogenetic analysis also clarified the evolutionary relationships between Vietnamese *H. major* and other populations of *H. major* collected in the Western Pacific region. The topology of the phylogenetic tree derived from four methods does not reveal any differences, except slightly different bootstrap values at some nodes. All methods indicated that *H. major* and *H. ovalis* are distributed in two distinct clades. Moreover, nucleotide differences and evolutionary divergence within the *H. major* clade, including *H. major* from Vietnam, are much lower than between the *H. major* clade and *H. ovalis*. Our leaf morphological and phylogenetic analyses support the evidence from previous studies (Kuo et al. 2006, Uchimura et al. 2008, Shimada et al. 2012) that *H. major* and *H. ovalis* are distinct species. In contrast, Short et al. (2007, 2011) argued that the taxonomy of *H. major* was unclear because of overlapping leaf characteristics between *H. ovalis* and *H. major*. However, Short et al. (2011) suggested that the species should be accepted if supported by genetic data.

In terms of morphology, Waycott et al. (2002) stated that the basal group [*H. engelmanii*, *H. beccarii*, *H. tricostata* Greenway, and *H. spinulosa* (R. Brown) Ascherson] in this genus belongs to the more structurally complex species. Greater diversity of morphological and genetic traits was found in the simple phyllotaxy group

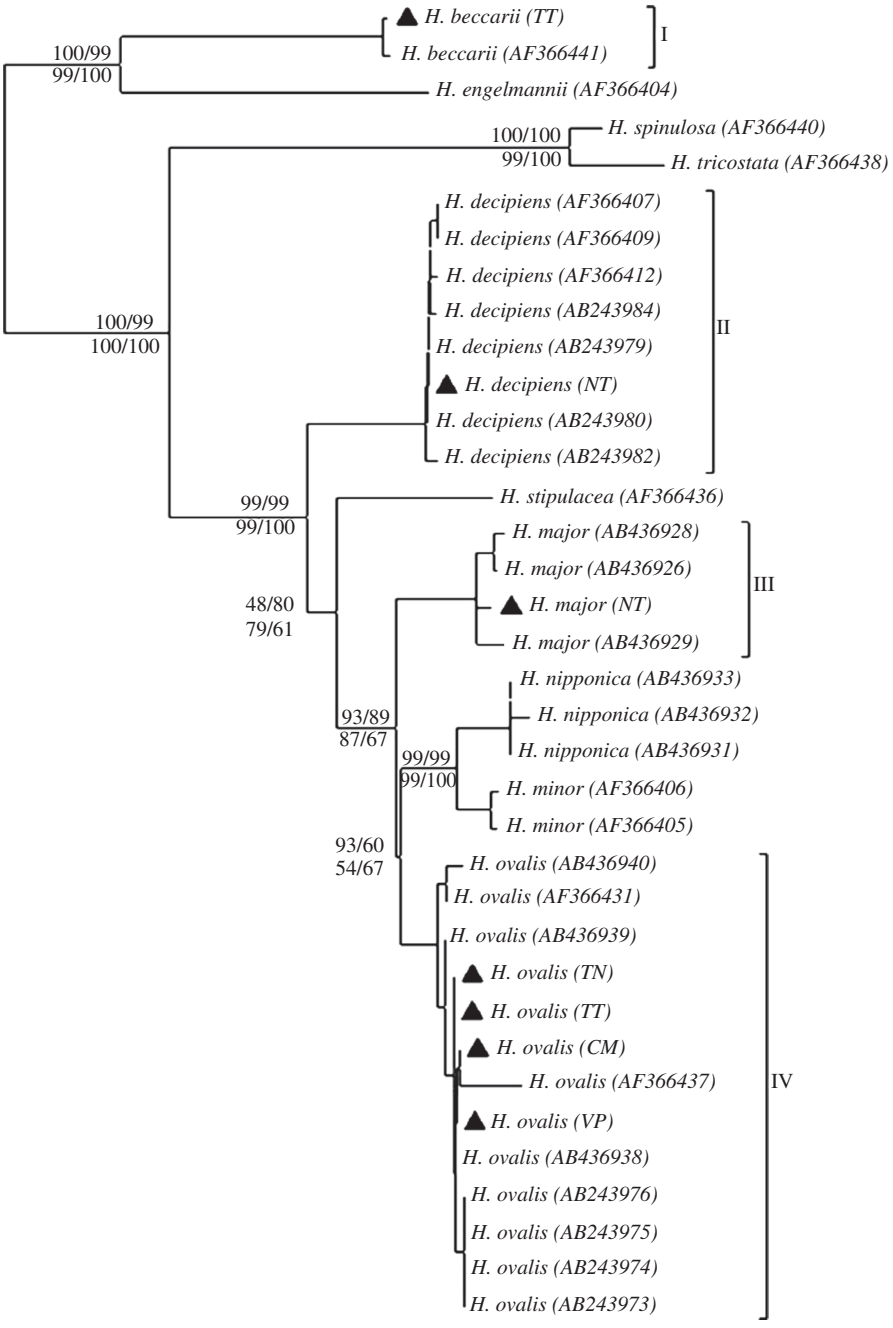


**Figure 4** Phylogeny of *Halophila* inferred from maximum likelihood, neighbor joining, maximum parsimony, and Bayesian analysis based on 620 bp (including gaps) of nrDNA sequences comprising ITS-1, 5.8S rDNA, and ITS-2.
The specimens collected from Vietnam are marked by bold triangles. The bootstrap value of each method is shown in each node: above nodes, left: maximum likelihood, right: neighbor joining; below nodes, left: maximum parsimony, right: Bayesian analysis. Abbreviations as in Figure 1.

(two leaves per shoot). Recently, a study conducted on generic phylogeny, historical biogeography, and evolution of the Hydrocharitaceae indicated that *Halophila* possibly originated in Southeast Asia 15.9–41.3 million years ago (Chen et al. 2012). The *H. ovalis* complex can be found in different environmental conditions with high morphological variability (den Hartog 1970). Waycott et al. (2002) suggested that the variation may be genotypically determined, as for *H. major*. Further molecular marker studies may show whether *H. major* is a recent immigrant to Vietnam or was just not recognized as this species by previous collectors.

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# CHAPTER 4

*Systematics and Biodiversity* (2013), **11(4)**: 467–476



## Research Article

# Genetic variation among *Halophila ovalis* (Hydrocharitaceae) and closely related seagrass species from the coast of Tamil Nadu, India – an AFLP fingerprint approach

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Among the seagrass species, *Halophila ovalis*, *H. ovalis* subsp. *ramamurthiana*, *H. ovata*, *H. major* and *H. minor* complex, leaf morphological characters overlap. Previously, plastic and nuclear sequences were successfully applied to resolve genetic variation among all the members within this genus. However, *Halophila ovalis*, *H. ovalis* subsp. *ramamurthiana* and the *H. ovata* complex have not yet been fully resolved. This leads to the hypothesis that the genetic differences of *H. ovalis* and *H. ovata* are not at the loci we were studying. In this study, AFLP analysis based on 49 demes of four *Halophila ovalis*, one *H. ovalis* subsp. *ramamurthiana* and two *H. ovata* populations collected along the coast of Tamil Nadu, India, were carried out to find the genetic distance within and among populations. Results from a band-based approach showed that *H. ovalis* and *H. ovata* are definitely clustered into two clades with 100% bootstrap value. Principal Coordinate Analysis (PCoA) clearly depicts the genetic relationship among the subspecies, lagoon clone and open sea clone of *H. ovalis*. Results from allele frequency-based approaches including matrix of genetic distances ($F_{ST}$) and AMOVA showed significant differences among populations. Based on the results it is concluded that *H. ovalis* and *H. ovata* are distinct species. *Halophila ovalis* collected at lagoon, estuary and open sea and *H. ovalis* subsp. *ramamurthiana* are genetically different.

**Key words**: AFLP, genetic diversity, *Halophila ovalis*, seagrass



## Introduction

Among 14 species of seagrass found along the coast of Tamil Nadu State, India, *Halophila ovalis* (R. Br.) H. *f., H. ovalis* subsp. *ramamurthiana* Ravikumar & Ganesan and *H. ovata* Gaud. occur in different habitats including lagoons, river mouths, open sea and offshore islands (Athiperumalsami *et al.*, 2008). Defining taxonomic boundaries within the *Halophila* section has represented a real challenge due to leaf morphological traits which overlap among species (Uchimura *et al*., 2008; Short *et al*., 2011; Shimada *et al*., 2012). *Halophila ovata* was distinguished from *H. ovalis* by the number of pairs of cross veins: 3–9 pairs of cross veins in *H. ovata* and 10–25 in *H. ovalis* (Kuo & Den Hartog, 2001). The morphological difference between *Halophila ovalis* and *H. ovalis* subsp. *ramamurthiana* is the number of seed per fruit: 18–27 seeds/fruit found in *H. ovalis*, but only 6–12 seeds/fruit found in *H. ovalis* subsp. *ramamurthiana* (Kannan & Thangaradjou, 2006).

Using phylogenetic analyses of the nuclear ribosomal internal transcribed spacer (ITS1-5.8S-ITS2) region showed that some specimens identified as *H. ovalis* belong to different clades, and this clearly points to the need for critical taxonomic revision of *Halophila* material from the entire geographic distribution of this genus (Waycott *et al.*, 2002). Several genetic markers such as ribulose-1,5-bisphosphate-carboxylase-oxygenase (*rbc*L), chloroplast maturase K (*mat*K) (Lucas *et al.*, 2012) or ITS regions including the 5.8S (Uchimura *et al*., 2008; Short *et al*., 2010; Shimada *et al*., 2012) were applied to show the phylogenetic relations of the members of *Halophila*. However, single sequence analysis of *rbc*L and *mat*K, or the analysis of the concatenated sequences of the two plastid markers did not resolve the relationship between the two closely

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**Table 1.** Sites, coordinates, species and species description of materials used in this study. Abbreviations as in Fig. 1.

| $N_0$ | Location | Coordinates | Date | Species | Species description |
|---|---|---|---|---|---|
| Pop. 1 | ML | 12°13′56″N; 79°58′42″E | 10.2012 | *H. ovalis* | Lamina width 6–8 mm, length of mature leaves 10–12 mm, number of cross veins 7–17. 18–27 seed/fruit |
| Pop. 2 | ML | 12°14′00″N; 79°58′45″E | 10.2012 | *H. ovalis* subsp. *ramamurthiana* | Lamina width 6–8 mm, length of mature leaves 10–12 mm, number of cross veins 7–17. 6–12 seed/fruit |
| Pop. 3 | VE | 11°29′21″N; 79°45′58″E | 10.2012 | *H. ovalis* | Lamina width 6–8 mm, length of mature leaves 10–12 mm, number of cross veins 7–17. 18–27 seed/fruit |
| Pop. 4 | PK | 10°15′56″N; 79°18′35″E | 10.2012 | *H. ovalis* | Lamina width 9–11 mm, length of mature leaves 15–18 mm, number of cross veins 7–17. 18–27 seed/fruit |
| Pop. 5 | PK | 10°07′40″N; 79°14′32″E | 10.2012 | *H. ovata* | Lamina width 6–8 mm, length of mature leaves 15–18 mm, number of cross veins 3–9. 18–27 seed/fruit |
| Pop. 6 | PK | 10°06′35″N; 79°14′32″E | 10.2012 | *H. ovata* | Lamina width 6–8 mm, length of mature leaves 15–18 mm, number of cross veins 3–9. 18–27 seed/fruit |
| Pop. 7 | KC | 08°06′00″N; 77°33′50″E | 10.2012 | *H. ovalis* | Lamina width 9–11 mm, length of mature leaves 15–18 mm, number of cross veins 7–17. 18–27 seed/fruit |



related species, *H. ovalis* and *H. ovata* (Lucas *et al*., 2012; Nguyen *et al*., 2013). Our initial studies with ITS markers also showed that *H. ovata* collected in India was not resolved from *H. ovalis* as distinct species (unpublished data). There are several techniques to assess genetic variation among and between seagrass populations including isozyme analyses (McMillan, 1982; Laushman, 1993; Capiomont *et al*., 1996; Reusch, 2001), Random Amplified Polymorphic DNA (RAPD) (Kirsten *et al*., 1998; Procaccini *et al*., 1999; Angel, 2002; Jover *et al*., 2003), and microsatellites (Reusch, 2002; Reynolds *et al*., 2012). Amplified Fragment Length Polymorphism (AFLP) is a DNA fingerprinting technique that is based on selective PCR amplification of restriction fragments from a total digest of genomic DNA, and is considered as a useful approach to resolve closely related species and genetic diversity of populations (Vos *et al*., 1995). Such an AFLP-based approach to test genetic diversity of *H. ovalis* has not been applied so far. Hence, this study was carried out to answer the following research questions: (i) Are *H. ovalis* and *H. ovata* distinct species and (ii) are *H. ovalis* subsp. *ramamurthiana*, *H. ovalis* in the estuary–lagoon and the open sea genetically different at the loci used?

# Materials and methods

## Sample collection

Samples of *Halophila ovalis* and closely related species (*H. ovalis* subsp. *ramamurthiana* and *H. ovata*) were collected from seven different populations along the Tamil Nadu Coast, India (Fig. 1) at depths of 1–3 m. Details of collection sites, coordinates, species and brief description of the leaf morphology are shown in Table 1. Seven demes were collected per population. Plants consisting of intact roots, rhizome, and leaves were selected and washed with seawater in the field to remove epiphytes and debris that were attached to the plants. Each plant sample was sorted by species, placed in a single plastic bag, stored on ice, and transferred to the laboratory on the same day. In the laboratory, samples were re-washed with de-ionized water to remove seawater. Each plant was divided into two parts; one part was pressed as herbarium voucher specimen and the remaining part was dried completely in between blotting paper for later DNA extraction. Herbarium voucher specimens are currently deposited at the Institute of Botany, Leibniz University Hannover, Germany. Specimens were identified using the keys of den Hartog (1970), Kuo & Den Hartog (2001), Kuo *et al*. (2006) and Kannan & Thangaradjou (2006). Eight to 10 young dry leaves of each plant were homogenized using a bead mill (22 Hz, 2 min), and 100 mg of the fine powdered plant material was used for DNA extraction

## DNA extraction and AFLP analysis

DNA extraction was carried out using the Plant Nucleospin II Kit (Macherey & Nagel, Düren, Germany) following the manufacturer's instructions with slight modifications according to Lucas *et al*. (2012). DNA qualities were checked on agarose gels stained with ethidium bromide, and concentration was measured by a microplate reader with micro-volume plates (Synergy Mx Multi-Mode, BioTek,

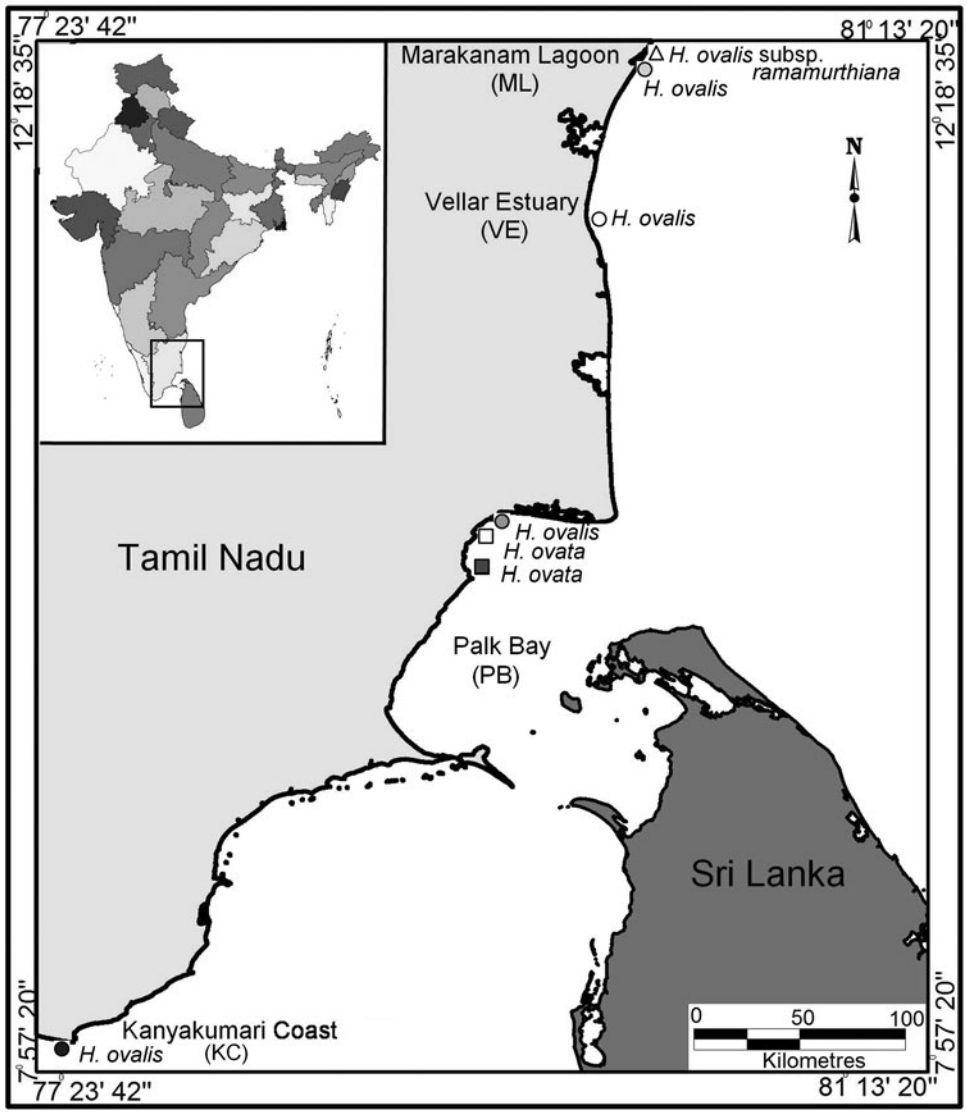


**Fig. 1.** The map shows India and seven sample collection sites along the Tamil Nadu coast, India (Source: Faculty of Marine Sciences, Annamalai University, India). Circle symbols represent *H. ovalis* (empty = Vellar Estuary; 25% solid = Marakanam Lagoon; 75% solid = Palk Bay; 100% solid = Kanyakumari Coast). Triangle symbols represent *H. ovalis* subsp. *ramamurthiana*. Square symbols represent *H. ovata* (empty = Palk Bay site 1; 50% solid = Palk Bay site 2).



Germany). The AFLP procedure was carried out as reported by Vos *et al*. (1995) with a few modifications. In brief, genomic DNA (250 ng) was digested with two restriction enzymes in a total volume of 25 $\mu$L including 5 U *Eco*RI, 3 U *Mse*I, 1× RL buffer (10 mM Tris/HCl, 10 mM MgAc, 50 mM KAc, 5 mM DTT, pH 7.5) overnight at 37 °C. Adapters (Table 2) were prepared in a total volume of 5 $\mu$L including 50 pmol of *Mse*I adapters, 5 pmol of *Eco*RI adapters, 0.5 mM ATP, 1.2 U of T4 DNA ligase, and 1× RL buffer. The mix of digested DNA and adapters were incubated at 37 °C for 3.5 h and then used as a template for PCR. The pre-selective PCR contained 5 $\mu$L of template, 1 U of *Taq* polymerase (MBI Fermentas, St. Leon-Rot, Germany), 0.25 mM of each of the four dNTPs, 1× Williams buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatine) and 50 ng of *Eco*RI and *Mse*I primers with one selective nucleotide (A) (Table 2) in a total volume of 50 $\mu$L. The PCR programme consisted of 20 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C, followed by 10 min at 72 °C. An aliquot of the reaction mix was diluted 1 : 20 with 1× TE Buffer (10 mM Tris/HCl pH 7.5, 1 mM EDTA). The selective PCR contained 2.5 $\mu$L of the diluted (1 : 20) product of the pre-selective PCR, 2 mM dNTPs, 5 U *Taq* polymerase in a total volume of 10 $\mu$L. Four primer combinations, *Eco*RI+ACA/*Mse*I+ATC,

**Table 2.** Sequences of adaptor and primers used in this study.

| Adapter/Primer | | Sequences |
|---|---|---|
| *Eco*RI adaptors | | 5'-CTCGTAGACTGCG TACC-3' |
| | | 5'-AATTGGTACGCAGT CTAC-3' |
| *Mse*I adaptors | | 5'-GACGATGAGTCC TGAG-3' |
| | | 5'-TACTCAGGAC TCAT-3' |
| Pre-selective primers (*Eco*RI+A) | | 5'-GACTGCGTACCAA TTCA-3' |
| Pre-selective primers (*Mse*I+A) | | 5'-GATGAGTCCTGAG TAAA-3' |
| Selective primer (*Eco*RI + ACA) | Set 1 | 5'-GACTGCGTACCAAT TCACA-3' |
| Selective primer (*Mse*I + ATC) | | 5'-GATGAGTCCTGAGTA AATC-3' |
| Selective primer (*Eco*RI + ACC) | Set 2 | 5'-GACTGCGTACCAATT CACC-3' |
| Selective primer (*Mse*I + ATC) | | 5'-GATGAGTCCTGAGTA AATC-3' |
| Selective primer (*Eco*RI + ACA) | Set 3 | 5'-GACTGCGTACCAATT CACA-3' |
| Selective primer (*Mse*I + ACA) | | 5'-GATGAGTCCTGAGT AAACA-3' |
| Selective primer (*Eco*RI + ACC) | Set 4 | 5'-GACTGCGTACCAAT TCACC-3' |
| Selective primer (*Mse*I + ACA) | | 5'-GATGAGTCCTGAG TAAACA-3' |

*Eco*RI+ACC/*Mse*I+ATC, *Eco*RI+ACA/*Mse*I+ACA and *Eco*RI+ACC/*Mse*I+ACA (Eurofins MWG Operon, Ebersberg, Germany) (Table 2) were used for the selective amplification. The first amplification cycle was carried out for 30 s at 94 °C, 30 s at 65 °C and 1 min at 72 °C. In each of the following 11 cycles, the annealing temperature was reduced by 0.7 °C. The last 24 cycles were carried out at an annealing temperature of 56 °C, and the final extension step was carried out at 72 °C for 10 min. To each sample, 20 $\mu$L of AFLP dye (98% formamide, 10 mM EDTA, 0.05% pararosaniline) was added. Mixtures were heated up to 72 °C for 5 min before loading onto 6% AFLP gels on the 4300 DNA Analyzer (LI-COR, Biosciences, Germany) by following the manufacturer's instructions.

## Data analysis

Only polymorphic fragments were scored as binary data (1, band present; 0, band absent). The binary scores were manually compared with the pictures to re-confirm presence or absence of bands. A presence/absence binary matrix of 49 individuals and 119 polymorphic loci was used as the basis for the analysis. In this study, band-based (for individual level) and allele frequency-based approaches (for population level) (Bonin *et al*., 2007) were followed. At the individual level, the similarity among 49 individuals was calculated by the Dice coefficient (Dice, 1945). A cluster analysis was performed using unweighted pair group method with arithmetic mean (UPGMA) based on the Dice index (Nei & Li, 1979). Bootstrap values (based on 1000 re-samplings) were used to estimate the reliability of the clustering pattern. This analysis was carried out in FreeTree (Hampl *et al*., 2001), and the dendrogram was edited and displayed in Mega 5.0 software (Tamura *et al*., 2011). Principal Coordinates Analysis (PCoA) of the correlation matrix was used to further investigate the relationship between individuals using NTSYSpc version 2.20 (Rohlf, 2005). At the population level, the allelic diversity at each locus was calculated as $H = 1-\sum p_i^2$, where $p_i$ is the frequency of the $i^{th}$ allele (Nei, 1973). Allelic diversity within each population was the mean allelic diversities among the 119 loci. Nei's $G_{ST}$ (Nei, 1973; Nei & Chesser, 1983) was used as a value of genetic differentiation. $G_{ST}$ was calculated using the formula $G_{ST} = (H_T - H_S)/H_T$ (Nei, 1973), where $H_T$ represents the total gene diversity and $H_S$ represents the gene diversity within populations. Those values and the dendrograms (UPGMA) were assessed by POPGENE 3.2 (Yeh *et al*., 2002) and Mega 5.0 software (Tamura *et al*., 2011). In addition, pairwise genetic distances were calculated and used in AMOVA (Analysis of MOlecular VAriance; Excoffier *et al*., 1992) by using Arlequin version 3.5 software (Excoffier & Lischer, 2010).

# Results

The number of polymorphic fragments for each primer varied from 18 (*Eco*RI + ACA/*Mse*I + ATC) to 41 (*Eco*RI + ACC/*Mse*I + ACA). The average number of polymorphic loci (DNA band/fragment) detected was about 30 per primer combination (Table 3). Genetic similarities among the 49 individual *Halophila* plant samples were estimated based on the number of common fragments. Similarity values among individual samples ranged from 0.695 to 0.933 on

**Table 3.** Total band, polymorphic band and per cent of polymorphic band gained from the analysis.

| Set | Pre-amplification | Final amplification | Total band | Polymorphic bands | Polymorphic bands (%) |
|---|---|---|---|---|---|
| 1 | *Eco*RI+A/*Mse*I+A | *Eco*RI + ACA/*Mse*I + ATC | 153 | 18 | 11.8 |
| 2 | *Eco*RI+A/*Mse*I+A | *Eco*RI + ACC/*Mse*I + ATC | 172 | 35 | 20.4 |
| 3 | *Eco*RI+A/*Mse*I+A | *Eco*RI + ACA/*Mse*I + ACA | 149 | 25 | 16.8 |
| 4 | *Eco*RI+A/*MseI*+A | *Eco*RI + ACC/*Mse*I + ACA | 207 | 41 | 19.8 |

the Dice index (data not shown). It also showed that the similarity values among populations of *H. ovalis* and *H. ovalis* subsp. *ramamurthiana* along the coast of Tamil Nadu is high (0.755–0.933); whereas, the similarity values between *H. ovata* and the *H. ovalis–H. ovalis* subsp. *ramamurthiana* group was lower (0.695–0.887). The cluster analysis (Fig. 2) revealed better results than the similarity matrix: *Halophila* populations were divided into two subgroups, *H. ovalis* and *H. ovalis* subsp. *ramamurthiana* as one group, and *H. ovata* (100% bootstrap value) as second subgroup. The Eigen vectors analysis of PCoA (Fig. 3) indicated that the contributions of the first three factors were 14.46, 9.02 and 6.63, respectively (explaining 30.11% of total variability). In order to explain 100% of the variation observed, 46 factors were required, thereby indicating the smaller contributions of each of the variables towards total variability. Results of PCoA also indicated that the taxa in the *H. ovalis*, *H. ovalis* subsp. *ramamurthiana* and *H. ovata* complex are clearly distributed in two main clades: the *H. ovalis* and *H. ovalis* subsp. *ramamurthiana* clade, and the *H. ovata* clade.

Based on the above results, the entire samples were organized in two groups: the *H. ovalis–H. ovalis* subsp. *ramamurthiana* and the *H. ovata* group. Gene diversity (H) of the entire samples (Nei, 1973) calculated using POPGENE was $0.333 \pm 0.126$ while the value of $G_{ST}$ (Nei, 1973), was 0.411. Results of AMOVA analysis revealed that genotypic variation was attributable to differentiation between the two groups. The majority of variation was within populations (71.73%), while the remaining was among populations within groups (11.55%) and among groups (16.72%) (Table 4). The matrix of genetic distance ($F_{ST}$) among populations of *H. ovalis* and closely related species indicated that genetic distance between *H. ovalis* populations (0.083–0.218, $P < 0.01$) was lower than between *H. ovalis* and *H. ovata* (0.234 – 0.329, $P < 0.01$). In detail, a significant difference was found between *H. ovalis* collected at Marakanam and *H. ovalis* collected at Vellar Estuary ($F_{ST} = 0.083$, $P < 0.01$). A significant difference also was found between *H. ovalis* subsp. *ramamurthiana* and *H. ovalis* (Marakanam, Vellar Estuary, Palk Bay and Kanyakumari) with $F_{ST}$ values of 0.192, 0.106, 0.269 and 0.267 respectively, $P < 0.01$ in all the cases. Interestingly, a significant difference was found between lagoon (Marakanam) – estuary (Vellar) clones and open sea clones (Palk Bay and Kanyakumari) ($P < 0.01$). In contrast, there are no significant differences between the *H. ovalis* population at Palk Bay and at Kanyakumari ($F_{ST} = 0.052$, $P = 0.135$), nor between *H. ovata* collected at two sites ($F_{ST} = 0.023$, $P = 0.297$) (Table 5). The dendrogram based on Nei's (1978) genetic distance showed that seven populations divided into two main clades: (1) *H. ovata* and (2) *H. ovalis–H. ovalis* subsp. *ramamurthiana* complex in which *H. ovalis* subsp. *ramamurthiana* is closer to *H. ovalis* lagoon–estuary populations than to *H. ovalis* open sea populations (Fig. 4).

# Discussion

The *Halophila* genus is one of the most speciose among seagrasses (Waycott *et al*., 2002). *Halophila ovata* (3–9 cross vein pairs) is distinguished from *H. ovalis* (10–25 cross vein pairs) by fewer cross vein pairs (Den Hartog, 1970; Den Hartog & Kuo, 2006). *Halophila ovalis* subsp. *ramamurthiana* (6–12 seeds) can also be distinguished from *H. ovalis* (18–27 seeds) by the number of seeds per fruit (Kannan & Thangaradjou, 2006). Our initial studies on plastid (*rbc*L, *mat*K) and nuclear (ITS) molecular markers did not resolve among the three species *H. ovata*, *H. ovalis* subsp. *ramamurthiana* and *H. ovalis* (Lucas *et al*., 2012; Nguyen *et al*., 2013). Després *et al*. (2003) stated that AFLP fingerprints were very useful in resolving phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. In this study, genetic variation in populations of *H. ovalis* and two other closely related species in the South of India were analysed based on AFLP fingerprint.

Initially, there were about 10–15 individuals per population collected. Unfortunately, DNA extracted from some plants showed degradation. It may be caused by the humid and hot weather in collection sites in South India. Meudt & Clarke (2007) indicated that use of degraded qualities of DNA can result in a poor quality profile with low reproducibility. Hence, the samples retrieving high-quality DNA were subjected to further testing. For the genetic population studies, a sample size of 20–30 individuals is the best selection (Pruett & Winker, 2008). However, five to six samples are sufficient to obtain a standard error equal to 10% of the diversity in the population of the species (Singh *et al*., 2006). In this study, seven samples per population were chosen to balance the sample size in all cases.

Among a total of 681 bands, 119 (17.5%) were polymorphic bands in nature. This contrasts with a level of variability of 30% using AFLP in land plant species, such as rice (Mackill *et al*., 1996), although results vary among studies (Mueller & Wolfenbarger, 1999). Significant population differentiation was found in our study; however, high level between-population differentiation has previously been reported in *Thalassia testudinum* (Waycott & Barnes, 2001) and *Zostera marina* (Li *et al*., 2012) using AFLP. This comparison indicated that the per cent of polymorphic bands varied from species to species, geographic distribution, and perhaps primer combination. Such increase in polymorphism within the species probably has been introduced over time by genetically fixing the eco-morphological variations resulting in the species. In the band-based approach, the similarity coefficients (Dice index) within *H. ovalis*, *H. ovalis* subsp. *ramamurthiana* and *H. ovata* are higher than among three taxa. The similarity index found in this study is higher than the similarity index based on RAPD data of *H. decipiens* and *H. johnsonii*, which were 0.6 and 0.66, respectively (Jewett-Smith *et al*., 1997). Comparison

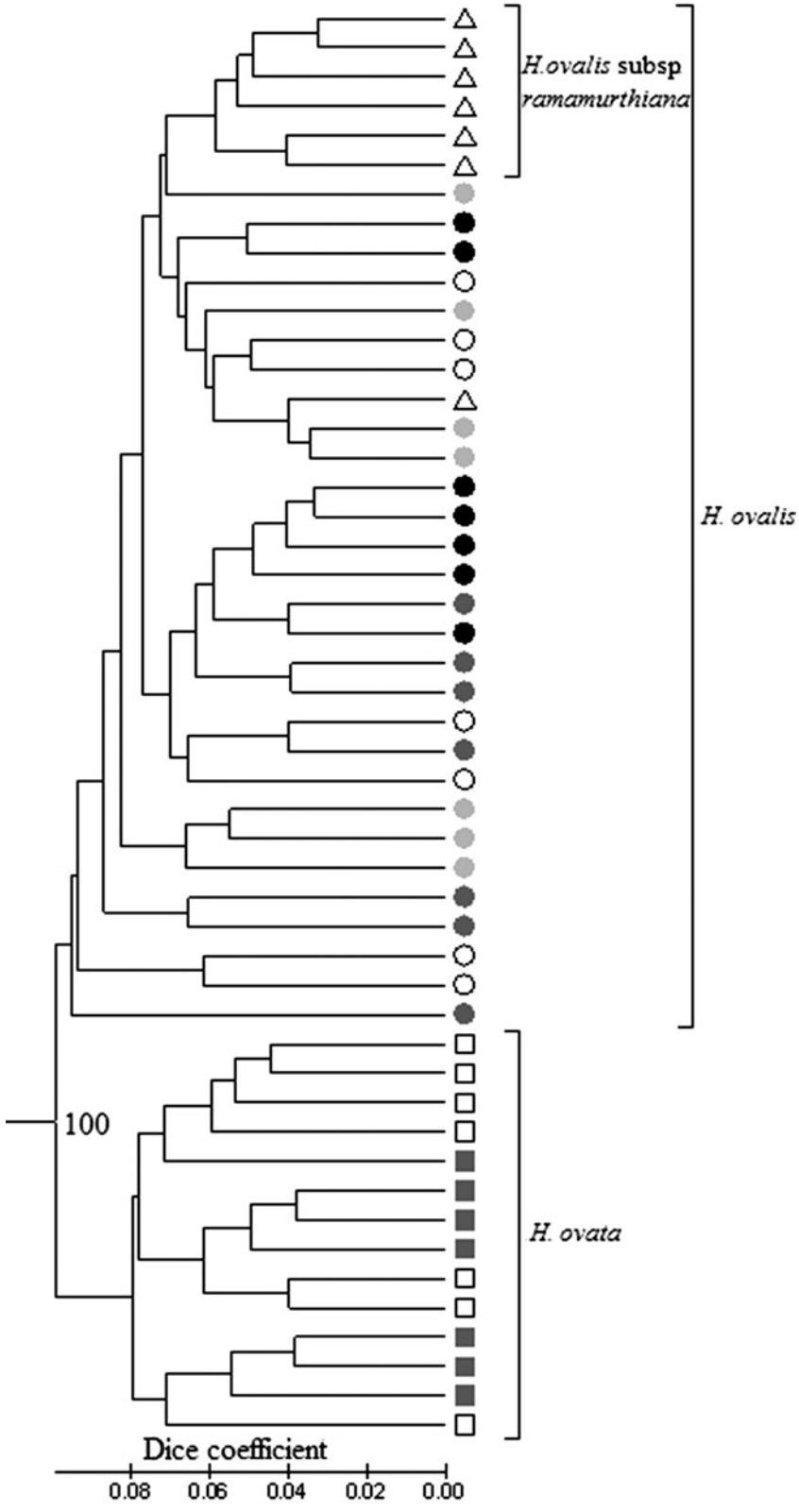


**Fig. 2.** UPGMA-based dendrogram of *Halophila ovalis* and closely related species generated from 119 AFLP markers. Confidence limits for the dendrogram are based on 1000 bootstrap replications. Bootstrap values were cut off at 50% and presented at the nodes. Dendrogram was assessed by FreeTree (Hampl *et al*., 2001), edited by Mega 5 (Tamura *et al*., 2011). Abbreviations as in Fig. 1.

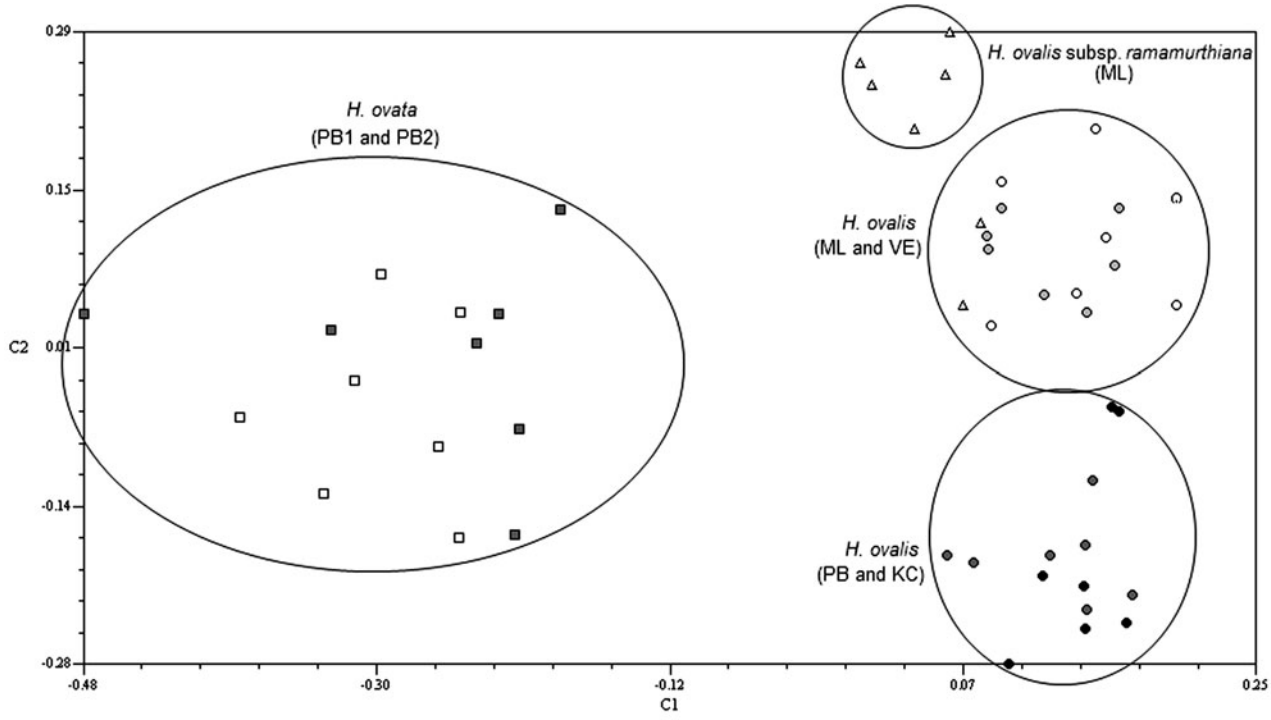


**Fig. 3.** PCoA based on 119 AFLP markers. The matrix plot is processed by NTSYSpc, 2.20 (Rohlf, 2005). There are three groups including *Halophila ovata*, *H. ovalis* lagoon–estuary and *H. ovalis* open water. Clustering of *H. ovalis* and *H. ovalis* subsp. *ramamurthiana* is not clear. The first three factors were 14.46, 9.02 and 6.63, respectively. Abbreviations as in Fig. 1.

between clustering analysis (UPGMA) and PCoA showed that the pattern of clustering of the taxa was similar with both analyses when *H. ovata* stands as single clade, whereas *H. ovalis* subsp. *ramamurthiana* groups with *H. ovalis*. However, cluster analysis based on the UPGMA dendrogram (Fig. 2) did not resolve distinct subgroups of *H. ovalis* and *H. ovalis* subsp. *ramamurthiana* because the similarity coefficient is very high among populations. In the PCoA, *H. ovalis* populations are clearly divided into two subgroups based on different clones of *H. ovalis* in the lagoon–estuary and the open sea (Fig. 3). Different clones were also found in other seagrass species based on DNA sequence comparison, such as *Zostera marina* (Lucas *et al*., 2012) and *H. ovalis* (Waycott *et al*., 2002), or based on RAPD (Procaccini *et al*., 1999). Close similarity between *H. ovalis* lagoon–estuary populations with *H. ovalis* subsp. *ramamurthiana* is good evidence that this sub-species probably originated from lagoon–estuarine *H. ovalis* rather than the open sea *Halophila ovalis*. This could be the reason for the restricted distribution of *H. ovalis* subsp. *ramamurthiana*

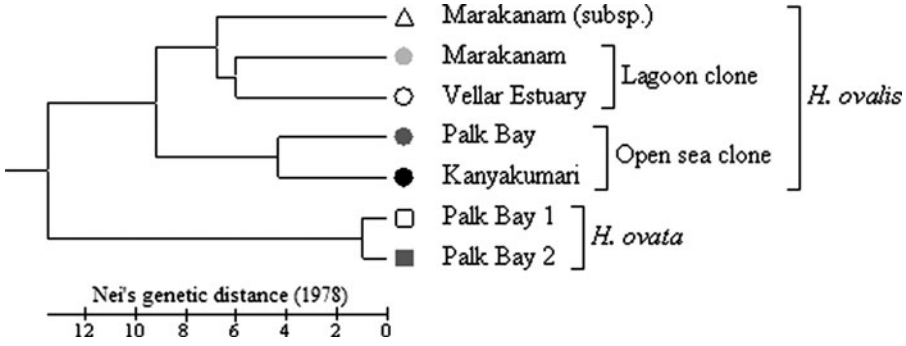


**Fig. 4.** Dendrogram of genetic distances among seven populations of *H. ovalis* and closely related species. Dendrogram was assessed by POPGENE 3.2 (Yeh *et al*., 2002), edited by Mega 5.0 (Tamura *et al*., 2011). Branch lengths were calculated by Nei (1978). Abbreviations as in Fig. 1.

**Table 4.** AMOVA (Analysis of MOlecular Variation; Excoffier *et al*., 1992) result for AFLP variation at seven collection sites of *H. ovalis* and of closely related species: four sites of *H. ovalis*, one site of *H. ovalis* subsp. *ramamurthiana* and two sites of *H. ovata*. Group 1 is *H. ovalis* and *H. ovalis* subsp. *ramamurthiana* population. Group 2 is *H. ovata* population. Calculations were conducted in Arlequin 2.2 (Excoffier & Lischer, 2010).

| Source of variation | d.f. | Sum of squares | Variance of components | Percentage of variation | Probability |
|---|---|---|---|---|---|
| Among groups | 1 | 87.437 | 3.00212 | 16.72 | $P < 0.05$ |
| Among populations within groups | 5 | 136.971 | 2.07382 | 11.55 | $P < 0.01$ |
| Within populations | 42 | 540.857 | 12.87755 | 71.73 | $P < 0.01$ |
| Total | 48 | 765.265 | 17.95349 | | |

at Marakanam Lagoon of Tamil Nadu and Theetapuram of Andhra Pradesh coasts (Kannan & Thangaradjou, 2006).

Results from the allele frequency-based approach demonstrate that populations of *H. ovalis* and closely related species vary considerably in their genetic diversity (H). Wide variation in genetic diversity among populations has been noted for other seagrass species such as *Halodule wrightii* (Travis & Sheridan, 2006), *Thalassia testudinum* (Waycott & Barnes, 2001), *Zostera marina* (Reusch, 2002; Olsen *et al*., 2004) and *Posidonia oceanica* (Procaccini *et al*., 1996). Among *Halophila* members, *H. ovalis* showed an unusual range of morphological variability that differs across the range of the species (Den Hartog, 1970). Waycott *et al*. (2002) added notes that some of the variation of *H. ovalis* may be genotypically determined. In fact, fluctuation of environmental conditions, such as light intensity, depth, salinity, nutrients and water motion in the lagoon and estuary, are quite different to those of open sea habitats (Jayaraman *et al*., 2007), and are capable of causing ecomorphological variations within the same species. In this study, pairwise genetic distance ($F_{ST}$) among populations (Table 4, Fig. 4) supports the hypothesis that *H. ovalis* in the estuary–lagoon and the open sea are genetically different. In addition, studies of Travis & Sheridan (2006) documented the effects of disturbance on genetic variation of *Halodule wrightii* populations, and concluded that the lowest population-level clonal diversity may have experienced the lowest levels of disturbance. Moreover, the distance between the Marakanam Lagoon and the Kanyakumari Coast is more than 500 km, which may cause evolution of specific clones by reducing the gene flow. In that sense, the geographic distances were responsible for the genetic differentiation among populations, which was earlier reported for *Z. marina* from China, Japan and Korea (Li *et al*., 2012).

Up to date, there are no reports on genetic variation of *H. ovalis* based on AFLP fingerprints. However, studies on genetic variation of different *H. ovalis* populations by other DNA fingerprint techniques including RAPD (May & Othman, 2002) and microsatellites (Xu *et al*., 2010) indicate that there are several clones found in different environmental habitats. Waycott & Barnes (2001) clearly stated that zero or high genetic diversity of seagrass populations depended on the respective species and their geographic distribution. High level of similarity at the intraspecific level has also shown that all the samples of each species are monophyletic. It can be inferred from the present investigation that the AFLP technique is a useful tool for the analysis of genetic diversity among seagrass populations, and can be used as a tool to resolve complex taxonomic issues of seagrasses at species and subspecies level. However, more samples from different populations, as well as ocean systems, are necessary to add more datasets for a better understanding of the genetic variation of this complex genus. Moreover, more collection sites of *H. ovalis* along a long latitude gradient should be investigated by other genetic markers, such as microsatellites, for a better understanding of genetic diversity within and among populations of this species.

**Table 5.** Matrix of genetic distances ($F_{ST}$) among populations of *H. ovalis* and closely related species calculated by using Arlequin 3.5 (Excoffier & Lischer, 2010). Ho = *H. ovalis*, Hos = *H. ovalis* subsp. *ramamurthiana*, Hv = *H. ovata*. Abbreviations as in Fig. 1. 1: site 1, 2 = site 2.

| | HoVE | HoML | HoPB | HoKC | HosML | HvPB1 | HvPB2 |
|---|---|---|---|---|---|---|---|
| HoVE | 0.000 | | | | | | |
| HoML | 0.083* | 0.000 | | | | | |
| HoPB | 0.180* | 0.139* | 0.000 | | | | |
| HoKC | 0.218* | 0.134* | 0.042 | 0.000 | | | |
| HosML | 0.192* | 0.106* | 0.269* | 0.267* | 0.000 | | |
| HvPB1 | 0.329* | 0.244* | 0.275* | 0.301* | 0.316* | 0.000 | |
| HvPB2 | 0.285* | 0.209* | 0.236* | 0.263* | 0.234* | 0.023 | 0.000 |

* Significant difference, $P < 0.01$.

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# CHAPTER 5

BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

# Genetic species identification and population structure of *Halophila* (Hydrocharitaceae) from the Western Pacific to the Eastern Indian Ocean

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## Abstract

**Background:** The Indo-Pacific region has the largest number of seagrass species worldwide and this region is considered as the origin of the Hydrocharitaceae. *Halophila ovalis* and its closely-related species belonging to the Hydrocharitaceae are well-known as a complex taxonomic challenge mainly due to their high morphological plasticity. The relationship of genetic differentiation and geographic barriers of *H. ovalis* radiation was not much studied in this region. Are there misidentifications between *H. ovalis* and its closely related species? Does any taxonomic uncertainty among different populations of *H. ovalis* persist? Is there any genetic differentiation among populations in the Western Pacific and the Eastern Indian Ocean, which are separated by the Thai-Malay peninsula? Genetic markers can be used to characterize and identify individuals or species and will be used to answer these questions.

**Results:** Phylogenetic analyses of the nuclear ribosomal internal transcribed spacer region based on materials collected from 17 populations in the Western Pacific and the Eastern Indian Ocean showed that some specimens identified as *H. ovalis* belonged to the *H. major* clade, also supported by morphological data. Evolutionary divergence between the two clades is between 0.033 and 0.038, much higher than the evolutionary divergence among *H. ovalis* populations. Eight haplotypes were found; none of the haplotypes from the Western Pacific is found in India and vice versa. Analysis of genetic diversity based on microsatellite analysis revealed that the genetic diversity in the Western Pacific is higher than in the Eastern Indian Ocean. The unrooted neighbor-joining tree among 14 populations from the Western Pacific and the Eastern Indian Ocean showed six groups. The Mantel test results revealed a significant correlation between genetic and geographic distances among populations. Results from band-based and allele frequency-based approaches from Amplified Fragment Length Polymorphism showed that all samples collected from both sides of the Thai-Malay peninsula were clustered into two clades: Gulf of Thailand and Andaman Sea.

**Conclusions:** Our study documented the new records of *H. major* for Malaysia and Myanmar. The study also revealed that the Thai-Malay peninsula is a geographic barrier between *H. ovalis* populations in the Western Pacific and the Eastern Indian Ocean.

**Keywords:** Eastern Indian Ocean, Evolution, Genetic distance, *Halophila ovalis*, Western Pacific Ocean

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## Background

The Indo-Pacific Ocean – the origin of seagrass - has the largest number of seagrass species worldwide with huge meadows of mixed species stands, but the taxonomy of *Halophila* members is still unclear and genetic variation has not been much investigated so far [1]. In comparison to other seagrass species in the meadows, *Halophila ovalis* (R. Br.) Hooker is the dominant species and very commonly found in the region. Recently, some new records of *Halophila* members such as *Halophila major* (Zoll.) Miquel, were documented in Southeast Asian countries including Indonesia, Viet Nam and Thailand [2,3]. Additionally, *H. sulawesii* J. Kuo was found and described for the first time in Indonesia [4]. Traditional classification of *H. ovalis* and closely related species based on leaf morphological data is very challenging, and species misidentification among *Halophila* members is reported in various studies [1,5,6]. Genetic markers are considered as helpful tools to resolve boundaries between species as well as the genetic variation among populations within species [6-8].

The Indo-Pacific Ocean also shows a high diversity of landscapes, habitats as well as several existing geographic barriers. Geographic isolation refers to a situation where a species, or a population of a species, becomes separated by a physical barrier, allowing each group to diverge along separate evolutionary paths [9]. The effect of geographic isolation is that the two populations are subjected to different selection pressures, since the conditions in the two areas are different [10]. Thus different alleles will be selected and genetic differences will gradually accumulate between the populations. In general, halophytes such as mangroves, marine algae, and seagrass grow in the coastal zone, which is connective between land and sea [1,11]. Currents along the coast or ocean currents play an important role for the migration of species from one coastal area to another [7]. Recently, there were several studies published on mangroves [12,13] and animals [14,15] from this region revealing the genetic variation isolated by barriers.

Among the members of *Halophila*, *H. ovalis* is widespread in the Indo-Pacific Ocean. In the Pacific, it occurs from southern Japan throughout Southeast Asia, many islands of the western Pacific, and through all but the southern coast of Australia, as well as Lord Howe and Norfolk Islands, and as far east as Tonga and Samoa. In the Indian Ocean, *H. ovalis* is found from southwestern Australia to East Africa and the Red Sea, including Madagascar, with the exception of islands or coastlines with no records. Recently, *H. ovalis* has been also discovered in the Atlantic Ocean on the Island of Antigua [16]. The plant is diminutive and lacks strongly lignified tissue, making it flexible, but vulnerable to physical disturbances [7]. *Halophila ovalis* grows on a variety of substrates and is often the first to colonize newly available sediments [5,17]. The species can grow at a range of temperatures and is distributed from tropical to warm-temperate waters [16,18]. This species has a wide depth distribution as well, with individuals growing from the intertidal up to a depth of 30 m [19]. Like other seagrass species, *H. ovalis* reproduces vegetatively by branching of rhizomes and the formation of new shoots, and sexually through seeds [11]. Due to high variation of leaf morphology and adaptation, Den Hartog [11] emphasized the need for detailed studies of this species to better understand the link between morphological variability and environmental parameters.

Leaf morphology is used as the main key to identify and name *Halophila* species [11,20]. However, traits of leaf morphology are overlapping among members of this genus [1]. Recently, genetic markers of plastid sequences have been used to reveal the genetic relationships among the members of the *Halophila* genus [2,21]. However, the species boundaries could not be fully resolved. Using phylogenetic analyses of the nuclear ribosomal internal transcribed spacer (ITS1-5.8S-ITS2) region showed that some specimens identified as *H. ovalis* belonged to different clades, and this clearly points out the need for critical taxonomic revision of *Halophila* material from the entire geographic distribution of this genus [7]. This nuclear sequence was also used to identify the genetic relation of *H. ovalis* and closely related species namely *H. major, Halophila nipponica* J. Kuo, *Halophila minor* (Zoll.) den Hartog and *Halophila hawaiana* Doty and B. C. Stone [6,7,22,23].

There are several techniques including isozyme analyses [24,25], Random Amplified Polymorphic DNA (RAPD) [26-29], Amplified Fragment Length polymorphism (AFLP) [30-33] and microsatellites [34,35] to access genetic variation among and between seagrass populations. The major advantage of the AFLP technique is the large number of polymorphisms that the method generates compared with other markers. However, the methodology of AFLP experiments and post-run data analysis are complex and time consuming compared with other markers [36,37]. Microsatellites are simple sequence repeats (SSRs) with advantages like locus-specificity, co-dominance, high degree of polymorphism, and it is also possible to work with partially degraded DNA [38]. So far there is only little information of DNA fingerprinting techniques applied for *H. ovalis*.

It is hypothesized that (i) taxonomic uncertainty among different populations of *H. ovalis* persists and (ii) geographic distance, differentiation of habitats or the geographic barrier of the Indo-Malay peninsula may affect the genetic variation of *H. ovalis* from the Western Pacific to the Eastern Indian Ocean. The aims of this study are (i) to identify *Halophila* species collected in Hong Kong, Thailand, Malaysia and India based on the molecular marker (ITS1-5.8S-ITS2) and (ii) to search for the genetic

structure of *H. ovalis* from the Western Pacific to the Eastern Indian Ocean based on microsatellite and AFLP approaches.

## Results

### Species identification based on the nuclear ITS sequences and morphology

Nineteen ITS sequences (Additional file 1) achieved from haplotypes collected at 17 populations of *Halophila* spp. in the study sites shown in Figure 1 and listed in Table 1 were used for the phylogenetic analysis. The alignment of the sequences received from three independent PCRs revealed that there were no nucleotide differences among replications. Fragments of 18S and 28S were removed to gain only the sequence of ITS1-5.8S-ITS2 (620–624 bp). A final alignment of 628 bp (including nucleotides and gaps) was generated for ITS1-5.8S-ITS2, of which 43 (6.8%) were parsimony informative characters, 75 (11.9%) were variable sites, 549 (87.4%) were conserved sites, and 32 (5.1%) were singleton sites. Results of the four algorithms applied (maximum likelihood (ML), neighbor joining (NJ), maximum parsimony (MP) and Bayesian analysis (BA)) showed that all samples collected from the 17 populations were distributed into two clades consisting of *H. major* (clade I) and *H. ovalis* (clade II) with 98, 100, 96 and 99% bootstrap values, respectively. There was no difference in the topology of the phylogenetic trees based on these different methods except for small differences in the bootstrap values. In clade I, haplotypes (Hap.) 4, 5, 13, and 16 clustered with known *H. major* sequences. In clade II, the remaining haplotypes clustered with known sequences from *H. ovalis*. None of the samples clustered with known sequences from *H. minor* (Figure 2). The results also showed that nucleotide differences among individuals of the *H. major* clade and among individuals of the *H. ovalis* clade were zero to six nucleotides and zero to three nucleotides, respectively. However, the counts of different nucleotides between the two clades were 19 to 23. In addition, evolutionary divergence among individuals of the *H. major* clade and among individuals of the *H. ovalis* clade was 0.000 to 0.010 and 0.000 to 0.005, respectively. Evolutionary divergence between the two clades was 0.033 to 0.038. The results clearly indicate that haplotypes 4, 5, 13, and 16 need to be classified as *H. major* and samples collected at TH-tr (Hap. 9) need to be grouped into the *H. ovalis* clade instead with *H. minor* sequences. For both countries, Malaysia and Myanmar, it is the first time that *H. major* was recorded.

The morphological data also supported the results obtained from the molecular ITS data. For the samples identified as *H. major* based on ITS, five characters of leaf morphology including lamina width, lamina length,

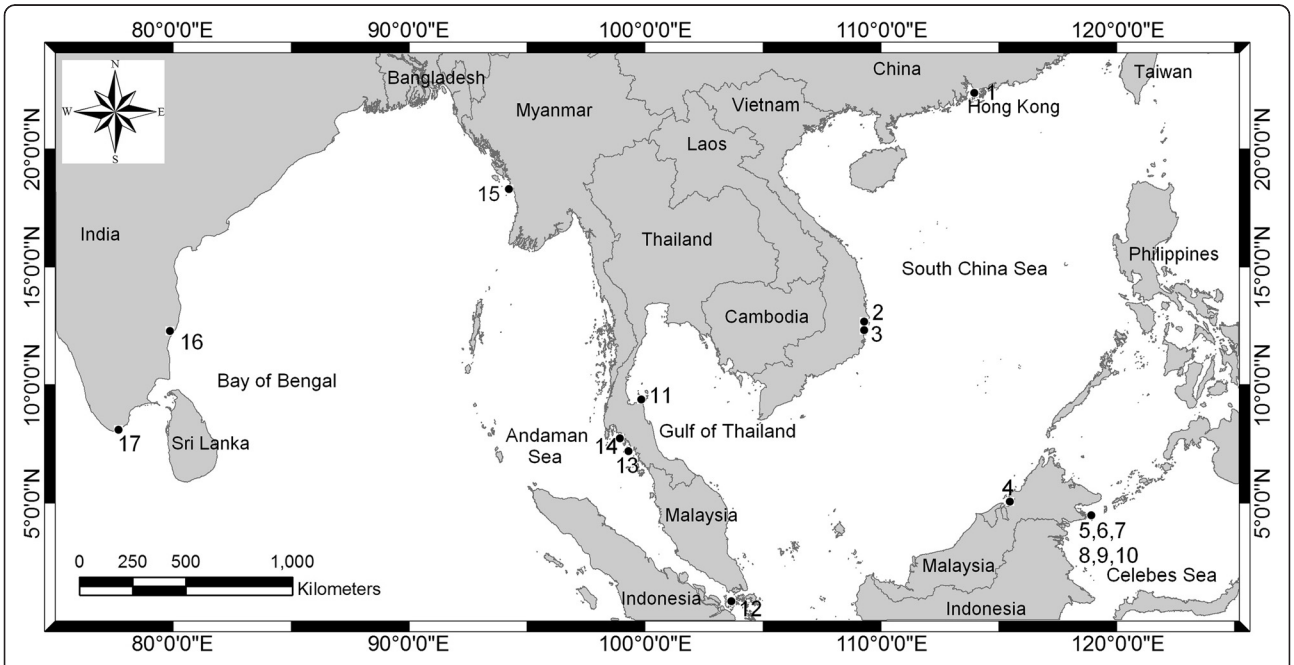


**Figure 1 The map shows the Western Pacific (South China Sea, Celebes Sea and Gulf of Thailand), the Eastern Indian Ocean (Andaman Sea and Bay of Bengal) and the respective countries (Source: The National Oceanic and Atmospheric Administration (*NOAA*), USA, public domain data).** Seventeen sample collection sites are represented as numbers. 1. Tung Chung Bay, Hong Kong (HK-tc), 2. Van Phong Bay, Viet Nam (VN-vp), 3. Thuy Trieu lagoon, Viet Nam (VN-tt), 4. Sarawak, Malaysia (MY-sr), 5. Tiga Island, Malaysia (MY-tg), 6. Mabul Island, Malaysia (MY-mb), 7. Gusungan Island, Malaysia (MY-gs), 8. Sibangat Island, Malaysia (MY-sb), 9. Bodgaya Island, Malaysia (MY-bd), 10. Maiga Island, Malaysia (MY-mg), 11. Kanom, Thailand (TH-kn), 12. Johore, Malaysia (MY-jo), 13. Satun, Thailand (TH-sa), 14. Trang, Thailand (TH-tr), 15. Gyeiktaw, Myanmar (MM-gy), 16. Marakanam, India (IN-ma) and 17. Kanyakumari, India (IN-ka).

**Table 1 Locations/abbreviations, regions, coordinates, sample size and taxa used in this study**

| | | | | | Kind of analysis | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **No.** | **Location** | **Coordinates (degree)** | **Sample size** | **Taxon** | **ITS** | **AFLP** | **SSRs** | **Citations** | **GB number** |
| 1 | HK-tc[1] | 113.9249°E; 22.2889°N | 6 | *H. ovalis* | X, Hap.1 | | X | This study | KF620337[+] |
| 2 | VN-vp[1] | 109.3445°E; 12.1289°N | 10 | *H. ovalis* | X | | X | [23] | KC175909 |
| 3 | VN-tt[1] | 109.3222°E; 12.1278°N | 10 | *H. ovalis* | X | | X | [23] | KC175908 |
| 4 | MY-sr[1] | 115.4652°E; 04.9825°N | 5 | *H. ovalis* | X, Hap. 2 | | X | This study | KF620338[+] |
| 5 | MY-tg[1] | 118.6006°E; 04.3750°N | 5 | *H. ovalis* | X, Hap. 3 | | X | This study | KF620339[+] |
| 6 | MY-mb[1] | 118.6265°E; 04.2479°N | 5 | *H. major** | X, Hap. 4 | | | This study | KF620340[+] |
| 7 | MY-gs[1] | 118.5458°E; 04.3161°N | 5 | *H. major** | X, Hap. 5 | | | This study | KF620341[+] |
| 8 | MY-sb[1] | 118.6626°E; 04.5546°N | 5 | *H. ovalis* | X, Hap. 6 | | X | This study | KF620342[+] |
| 9 | MY-bd[1] | 118.7208°E; 04.6016°N | 5 | *H. ovalis* | X, Hap. 7 | | X | This study | KF620343[+] |
| 10 | MY-mg[1] | 118.6868°E; 04.6080°N | 5 | *H. ovalis* | X, Hap. 8 | | X | This study | KF620344[+] |
| 11 | TH-kn[1] | 099.8802°E; 09.2128°N | 4 | *H. major*** | X, Hap. 9 | X | X | This study | KF620345[+] |
| 12 | MY-jo[1] | 103.1333°E; 01.3322°N | 5 | *H. ovalis* | X, Hap. 10 | | X | This study | KF620346[+] |
| 13 | TH-sa[2] | 099.7586°E; 06.7824°N | 9 | *H. ovalis* | X, Hap. 11 | X | X | This study | KF620347[+] |
| 14 | TH-tr[2] | | | | | | | | |
| | Site 1 | 099.3159°E; 07.3745°N | 5 | *H. ovalis* | X, Hap. 12 | X | X | This study | KF620348[+] |
| | Site 2 | 099.3159°E; 07.3745°N | 6 | *H. ovalis*** | X, Hap. 13 | X | X | | KF620349[+] |
| | Site 3 | 099.3389°E; 07.3829°N | 5 | *H. ovalis* | X, Hap. 14-15 | | | | KF620350-1[+] |
| 15 | MM-gy[2] | 094.3393°E; 18.3650°N | 7 | *H. major** | X, Hap. 16 | | | This study | KF620352[+] |
| 16 | IN-ma[2] | 079.9790°E; 12.2330°N | 10 | *H. ovalis* | X, Hap. 17-18 | | X | This study | KF620354-5[+] |
| 17 | IN-ka[2] | 077.5640°E; 08.1001°N | 10 | *H. ovalis* | X, Hap. 19 | | X | This study | KF620353 |
| | | | | *H. decipiens* | X | | | [23] | KC175913 |
| | | | | *H. minor* | X | | | [7] | AF366405[+] |
| | | | | *H. minor* | X | | | [7] | AF366406[+] |

There are 122 individuals collected from 17 populations in the Western Pacific and the Eastern Indian Ocean. X: genetic marker used for the populations. *, **, ***: First identification as *H. ovalis, H. minor* and *H. major*, respectively. Hap. 1–19: Haplotypes 1–19. Abbreviations as in Figure 1. [1]Pacific Ocean, [2]Indian Ocean. [+]Accession number for sequences deposited in GenBank.

number of cross-veins, space between intra-marginal veins, and especially the ratio of the distance between intra-marginal vein (*r*) and lamina margin (*R*) showed clear differences in comparison to *H. ovalis*. The ratio of the distance between the intra-marginal vein and the lamina margin was 1:20.8 to 1:25.6. In contrast, this ratio was 1:12 to 1:16 in *H. ovalis* [4]. Moreover, the number of paired cross veins of *H. major* was 18 to 20 and therefore higher than the number of paired cross veins in *H. ovalis* (14 to 17) [4]. The p-values obtained from Levene's test of lamina width, lamina length, number of paired cross veins were lower than 0.05 (heteroscedasticity). In contrast, the p-values obtained from Levene's test of the ratio *r*/*R* was higher than 0.05 (homoscedasticity). Single factor ANOVA shows that for the ratio (*r*/*R*) significant differences can be observed among the collection sites ($F = 77.82 > F_{crit.}$, $p < 0.001$). Details resulting from multiple comparisons of each trait obtained by the Tukey test showed that there were significant differences of the ratio (*r*/*R*) between populations at MY-mb, MY-gs, MM-gt (*H. major*) and the remaining populations (*H. ovalis*). Details of comparisons of the leaf morphology of *H. major* and *H. ovalis* are presented in Table 2 and Figure 3.

## Genetic diversity and population structure of *H. ovalis* from the Western Pacific to the Eastern Indian Ocean

### *Genetic diversity*

Data on observed heterozygosity ($H_o$), expected heterozygosity ($H_e$) and allelic richness (A) are presented in Table 3. Among populations, the highest expected heterozygosity ($H_e$) or genetic diversity in the Western Pacific and the Indian Ocean were found at MY-jo and TH-sa, respectively. The lowest expected genetic diversity in the Western Pacific and Indian Ocean were observed at HK-tc and IN-ma, respectively. Genetic diversity of the populations in the Western Pacific Ocean was slightly higher than of the populations in the Indian Ocean (0.306 vs 0.289). However, there was no significant difference between the oceanic systems (*t*-test, $p = 0.78$). Likewise, observed heterozygosity and allelic richness in the Western Pacific

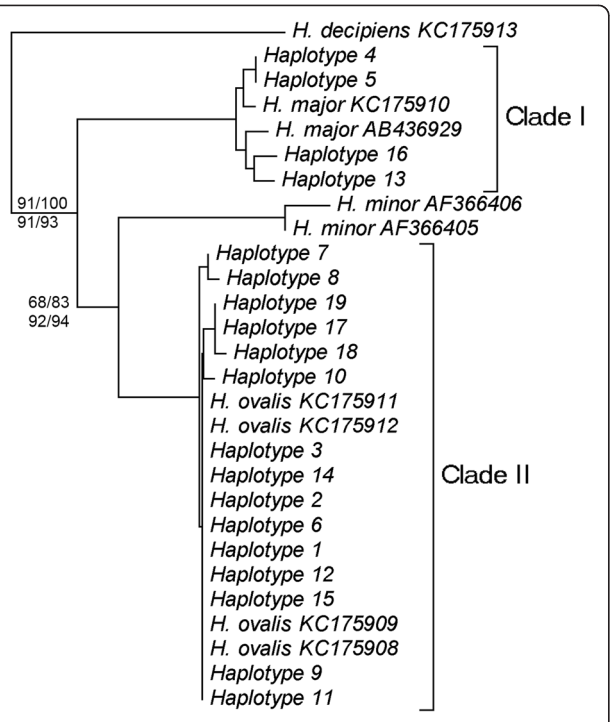


**Figure 2 Phylogeny of members of the *Halophila* genus inferred from Maximum Likelihood, Neighbour Joining, Maximum Parsimony and Bayesian Analysis.** The dataset based on 628 bp (including gaps) of nrDNA sequences comprising ITS-1, 5.8S rDNA, and ITS-2. The bootstrap value of each method is shown in each node: above nodes, left: Maximum Likelihood, right: Neighbor Joining; bellow nodes, left: Maximum Parsimony, right: Bayesian Analysis. See Table 1 for locations of the haplotypes.

were slightly higher than in the Indian Ocean (0.552 vs 0.542 and 1.560 vs 1.550, respectively). For the observed heterozygosity and allelic richness, there was no statistically significant difference between both oceanic systems (*t*-test, p = 0.926 and 0.929, respectively).

### *Population structure*

*Halophila ovalis* populations were markedly differentiated from each other in the Western Pacific and the Eastern Indian Ocean (Table 4). For the Western Pacific region (South China Sea, Celebes Sea and Gulf of Thailand), a significant genetic differentiation among investigated populations was observed.

For the Western Pacific Ocean, genetic distances among populations in regions I, II, III, IV, V and VI (see Table 1 and Figure 1 for abbreviations) were very high. In detail, the genetic distance between region I and III was the highest (2.636). There were lower genetic distances between region II and region V (0.288 to 0.377). However, the genetic distance between II and VI was lower than the genetic distance between VI and III (0.327 to 0.337 vs 0.444). Within region II, the genetic distance between VN-vp and VN-tt (see Table 1 for abbreviations) was 0.221. In contrast, genetic distances among populations greatly varied from population to population, ranking from 0.05 to 0.818, in which the genetic distance between MY-mg and MY-bd was the lowest and the genetic distance between MY-sb and MY-tg was the highest (Table 4). Results of AMOVA for SSRs variation of *H. ovalis* populations in the Western Pacific Ocean showed significant differentiation among groups ($p < 0.01$), among populations within groups ($p < 0.01$) and within populations ($p < 0.01$) (Table 5). Hence, high genetic distance and statistical differences were not only found among regions, but also among populations in the Western Pacific Ocean. The overall genetic variation from the *Halophila* populations in the Western Pacific Ocean was 0.438 calculated from FSTAT.

For the Eastern Indian Ocean, a very high genetic distance between the two regions VII and IX, ranking from 0.731 to 1.296 was observed (Table 4). For the Andaman Sea, the genetic distance between two populations, TH-sa and TH-tr, was determined as zero and non-significant ($p = 0.53$). In contrast, the genetic distance between IN-ma and IN-ka was very high and significantly different (1.280, $p < 0.001$). The results of AMOVA for SSRs variation of *H. ovalis* populations in the two regions VII and IX (see Table 1 for abbreviations) indicated that the percentage of variations among groups, among populations within groups and within populations were 20.85, 28.74 and 50.41%, respectively. Significant difference was just

**Table 2 Comparisons of leaf morphology characteristics of *H. major* collected in this study and published data from *H. ovalis***

| Characteristic | Species | | | | |
|---|---|---|---|---|---|
| | ***H. ovalis*** | ***H. major* MY-mb** | ***H. major* MY-gs** | ***H. major* MM-gy** | ***H. major*** |
| **Lamina width (mm)** | 5-20 | 12 – 15 | 12 – 15 | 13 | 9-11 |
| **Lamina length (mm)** | 10-40 (–70) | 18 – 22 | 18 – 22 | 22 | 15 – 25 |
| **No. of cross veins** | 10 – 25 | 18 – 20 | 18 – 20 | 20 | 14 - 17 |
| **Space between intramaginal vein (mm)** | 0.1 – 0.3 | 0.25 – 0.3 | 0.25 – 0.3 | 0.3 | 0.2 |
| **Half lamina width: distance between intramarginal veins and lamina margin ration** | 1:12-16 | 1:21 – 22 | 1:21 – 22 | 1:20 | 1:20-25 |
| **Source** | [5] | This study | This study | This study | [5] |

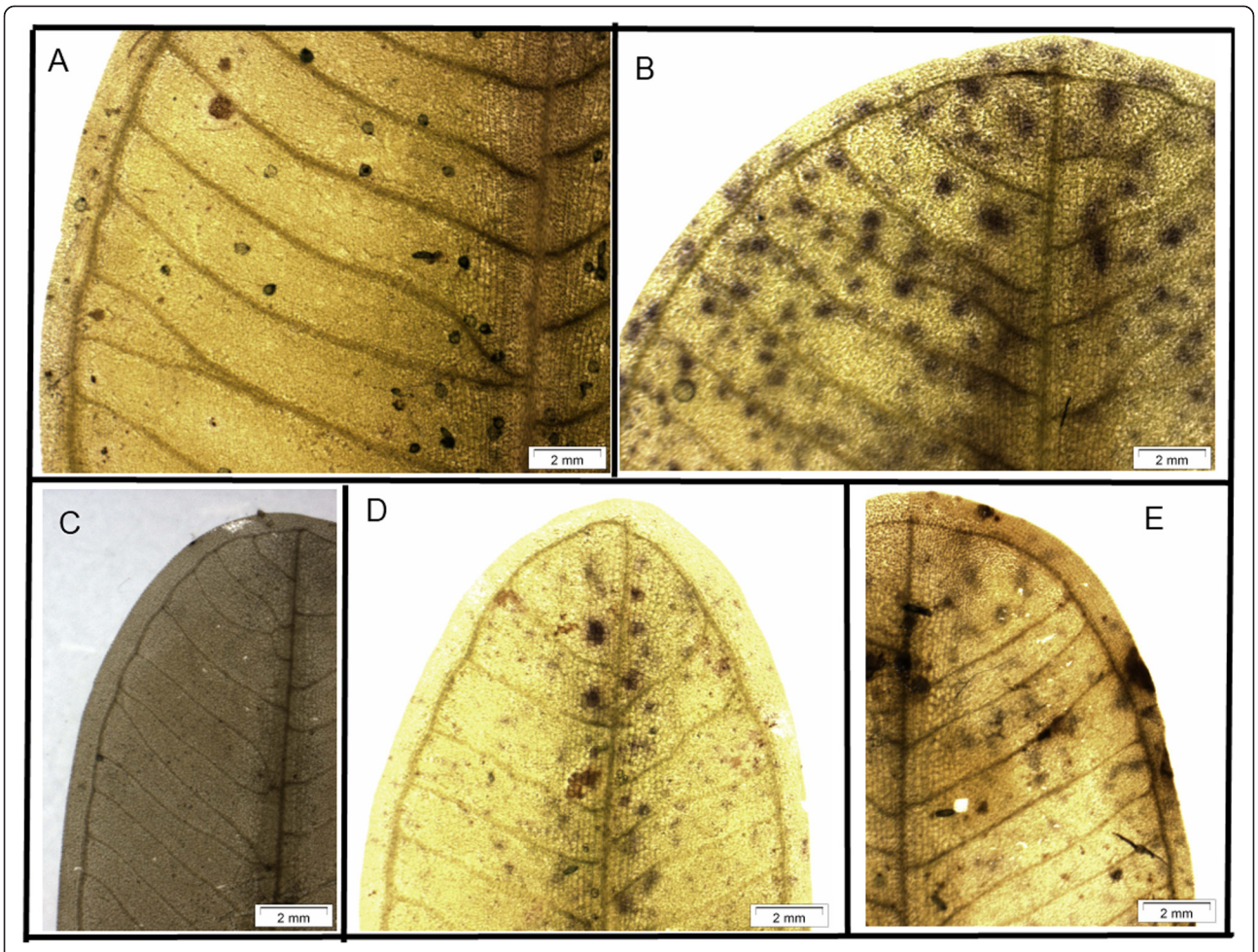


**Figure 3 Leaf morphology of *Halophila ovalis* and *Halophila major* collected at various sites in Malaysia. A** and **B**: *Halophila major* samples collected at MY-gs and MY-mb respectively. **C**, **D** and **E**: *Halophila ovalis* samples collected at MY-bd, MY-tg and MY-sb respectively. The scale bar is 2 mm.

found among groups and among populations within groups ($p < 0.01$) and there were no significant differences within populations ($p = 0.5$). Moreover, the results of AMOVA for SSRs variation of *H. ovalis* populations in both oceanic systems (Western Pacific vs Eastern Indian) showed significant differences among groups ($p < 0.01$), among populations within groups ($p < 0.01$), and within populations ($p < 0.01$). The overall genetic variation from the *Halophila* populations in the Eastern Indian Ocean was 0.485 calculated from FSTAT.

The unrooted neighbor-joining tree among 14 populations from eight regions in the Western Pacific and Eastern Indian Ocean showed six main groups including group 1 - Region I: Northern part of the South China Sea (HK), group 2 - Region II and V: Western part of the South China Sea and the Gulf of Thailand (VN-vp, VN-tt and TH-kn), group 3 – Region III and IV: Eastern part of the South China Sea and the Celebes Sea (MY-sr, MY-sb, MY-mg, MY-db and MY-tg), group 4 – Region 6: Southern part of the South China Sea (MY-jo), group 5 – Region VII: Andaman Sea (TH-tr and TH-sa) and group 6 – Region IX: Bay of Bengal (IN-ma and IN-ka) (Figure 4). The multi-locus estimate of spatial differentiation among 14 populations relative to the whole sampled distribution was large ($F_{ST} = 0.679$). The correlation between geographic and genetic distances in the study area is presented in Figure 5. The result of the Mantel test showed that the geographic distance was linearized and plotted against the geographic distances between populations ($r = 0.578$, $P_{Mantel} < 0.0001$, the significance level $\alpha = 0.05$). An approximately linear increase in Slatkin's genetic distance with increasing geographic distance between all pairs of populations confirmed a simple model, namely differentiation-by-distance.

Based on 15 ITS sequences from *H. ovalis*, there are eight distinct haplotypes found in 14 populations (populations from MM-gy, MY-mb, and MY-gs were rejected because these samples were classified as *H. major*).

**Table 3 Comparison of genetic diversity among *H. ovalis* populations**

| Oceanic system | Population | Observed heterozygosity ($H_o$) | Expected heterozygosity ($H_e$) | Allelic richness (A) |
|---|---|---|---|---|
| | HK-tc | 0.200 | 0.109 | 1.2 |
| | VN-vp | 0.800 | 0.421 | 1.8 |
| | VN-tt | 0.600 | 0.316 | 1.6 |
| | MY-sr | 0.200 | 0.111 | 1.2 |
| | MY-tg | 0.600 | 0.333 | 1.6 |
| Pacific | MY-sb | 0.520 | 0.316 | 1.6 |
| | MY-bd | 0.600 | 0.333 | 1.6 |
| | MY-mg | 0.600 | 0.333 | 1.6 |
| | TH-kn | 0.600 | 0.343 | 1.6 |
| | MY-jo | 0.800 | 0.444 | 1.8 |
| | Mean (SE) | 0.552 (0.206) | 0.306 (0.112) | 1.560 (0.207) |
| | TH-sa | 0.600 | 0.320 | 1.6 |
| | TH-tr | 0.567 | 0.310 | 1.6 |
| Indian | IN-ma | 0.400 | 0.211 | 1.4 |
| | IN-ka | 0.600 | 0.316 | 1.6 |
| | Mean (SE) | 0.542 (0.096) | 0.289 (0.053) | 1.550 (0.100) |

Genetic diversity gained from 14 populations in the Western Pacific ($N = 10$) and the Indian Ocean ($N = 4$). Abbreviations as in Figure 1. Calculation was carried out by the excel microsatellite toolkit [39] and FSTAT [40].

Haplotype I (including Hap. 1, 2, 3, 6, 9, 11, 12 and 14) commonly occurred in the South China Sea, Celebes Sea and Andaman Sea. Haplotypes II (Hap. 7) and III (Hap. 8) were found in MY-bd and MY-gm (Celebes Sea), respectively, and haplotype IV (Hap. 10) in MY-jo only. In the Andaman Sea, there was one more haplotype present – haplotype V (Hap. 15). Three haplotypes (VI, VII and VIII) that did not occur in the South China Sea, Celebes Sea and Andaman Sea were found in the Bay of Bengal. Haplotype VI (Hap. 19) was identified in IN-ka, while haplotypes VII (Hap. 17) and VIII (Hap. 18) were detected in IN-ma (Figure 6).

For the AFLP analysis, the genetic similarities (Dice index) among the 24 individual *H. ovalis* samples were estimated based on the number of common fragments ranged from 0.560 to 0.928. It also showed that the

**Table 4 Pairwise comparison of population differentiation among *H. ovalis* populations**

| | HK-tc | VN-vp | VN-tt | MY-sr | MY-tg | MY-sb | MY-bd | MY-mg | TH-kn | MY-jo | TH-sa | TH-tr | IN-ma | IN-ka |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HK-tc | - | 0.912 | 1.183 | 2.636 | 1.100 | 1.879 | 1.100 | 1.100 | 1.146 | 0.870 | 0.794 | 0.691 | 2.529 | 1.183 |
| VN-vp | 0.477** | - | 0.221 | 0.858 | 0.434 | 0.591 | 0.434 | 0.561 | 0.288 | 0.508 | 0.741 | 0.791 | 1.375 | 1.036 |
| VN-tt | 0.542** | 0.181** | - | 1.325 | 0.547 | 0.900 | 0.547 | 0.858 | 0.377 | 0.485 | 1.046 | 1.078 | 1.850 | 1.375 |
| MY-sr | 0.725** | 0.462** | 0.56** | - | 0.800 | 0.594 | 0.350 | 0.350 | 1.535 | 0.800 | 0.953 | 0.918 | 2.725 | 1.725 |
| MY-tg | 0.524** | 0.303** | 0.354** | 0.444** | - | 0.818 | 0.200 | 0.350 | 0.332 | 0.157 | 0.688 | 0.723 | 1.525 | 0.703 |
| MY-sb | 0.653** | 0.372** | 0.474** | 0.373** | 0.450** | - | 0.356 | 0.356 | 1.103 | 0.816 | 1.169 | 1.211 | 2.168 | 1.502 |
| MY-bd | 0.524** | 0.303** | 0.354** | 0.259* | 0.167* | 0.263** | - | 0.050 | 0.480 | 0.286 | 0.688 | 0.723 | 1.525 | 1.014 |
| MY-mg | 0.524** | 0.360** | 0.462** | 0.259** | 0.259* | 0.263** | 0.048** | - | 0.628 | 0.414 | 0.688 | 0.723 | 1.525 | 1.014 |
| TH-kn | 0.534** | 0.223** | 0.274** | 0.605** | 0.249* | 0.524* | 0.324** | 0.386** | - | 0.388 | 0.976 | 1.022 | 1.538 | 0.686 |
| MY-jo | 0.465** | 0.337** | 0.327** | 0.444* | 0.136** | 0.449** | 0.222** | 0.293** | 0.279** | - | 0.464 | 0.508 | 1.150 | 0.765 |
| TH-sa | 0.443** | 0.425** | 0.511** | 0.488** | 0.408** | 0.539** | 0.408** | 0.408** | 0.494** | 0.317** | - | 0.000 | 1.296 | 0.731 |
| TH-tr | 0.409** | 0.442** | 0.519** | 0.479** | 0.42** | 0.548** | 0.420** | 0.420** | 0.506** | 0.337** | $-0.05^{ns}$ | - | 1.280 | 0.731 |
| IN-ma | 0.717** | 0.579** | 0.649** | 0.732** | 0.604** | 0.684** | 0.604** | 0.604** | 0.606** | 0.535** | 0.564** | 0.561** | - | 1.280 |
| IN-ka | 0.542** | 0.509** | 0.579** | 0.633** | 0.413** | 0.600** | 0.503** | 0.503** | 0.407** | 0.433** | 0.422** | 0.422** | 0.561** | - |

Genetic differentiation $F_{ST}$ (below diagonal) and Slatkin's genetic distance [41] derived from 14 populations. Statistical significance based on a comparison-wise error rate of $\alpha = 0.05$ (below diagonal). ns = non-significant, * $0.05 \geq p > 0.01$, ** $p < 0.01$. Abbreviations as in Figure 1. Data was implemented by Arlequin version 3.5 [42].

**Table 5 AMOVA (Analysis of Molecular Variance) [43] results for SSR variation at 14 collection sites of *H. ovalis***

| Source of variation | d.f. | Sum of squares | Variance of components | Percentage of variation | Probability |
|---|---|---|---|---|---|
| Among groups | 1 | 46.1 | 0.27 | 17.25 | $p < 0.01$* |
| Among populations within groups | 12 | 98.4 | 0.54 | 34.34 | $p < 0.01$* |
| Within populations | 186 | 140.9 | 0.76 | 48.41 | $p < 0.01$* |

Group 1 are the populations from the Western Pacific Ocean and group 2 from the Eastern Indian Ocean. Calculations were conducted in Arlequin 3.5.1.3 [42].
*Significantly different.

similarity values of the populations within the Andaman Sea (TH-tr and TH-sa) and within the Gulf of Thailand (TH-kn) were 0.565 to 0.928 and 0.624 to 0.822, respectively. The similarity values between the populations of TH-tr and TH-sa were higher than between the population of TH-tr and TH-kn (0.634 to 0.820 vs 0.582 to 0.731).

The cluster analysis (Figure 7) revealed that *H. ovalis* populations were divided into two groups, either collected in the Gulf of Thailand or in the Andaman Sea (100% bootstrap value). However, results of clustering individuals of TH-tr and TH-sa were not significant. The plot of a principal coordinate analysis (PCoA), based on individual genetic distances calculated with 208 AFLP markers, is presented in Additional file 2. The first two axes explained 71.8% and 3.0% of the variation, respectively (explaining 74.8% of total variability). As axis two explained 3% of variance only, it is evident that the remaining axes contribute poorly to explain the variance. Results of PCoA also indicated that *H. ovalis* was clearly distributed in two main clades: Gulf of Thailand clade and Andaman Sea clade.

Based on the above results, the entire samples were organized in two groups: Gulf of Thailand and Andaman Sea group. Gene diversity (H) [49] of the entire sample set calculated using POPGENE was 0.272 ± 0.172, while the value of $G_{ST}$ [49] was 0.190. Results of AMOVA analysis revealed that genotypic variation was attributable to

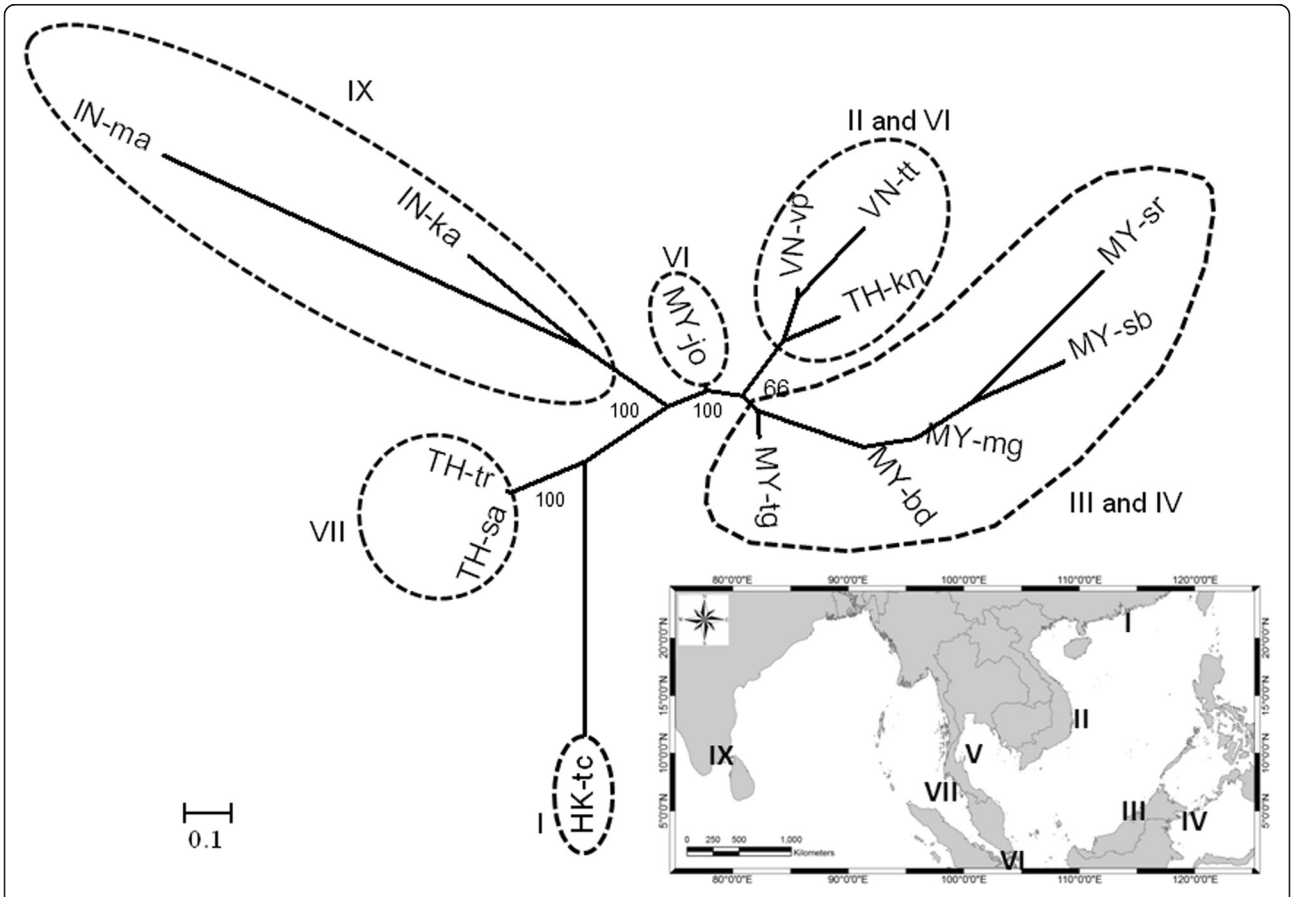


**Figure 4 Unrooted neighbor joining tree illustrating the relationship among *H. ovalis* populations in the Western Pacific and the Indian Ocean.** The tree was based on pair wise Slatkin's distances [41] and implemented in package Phylip version 3.5 [44]. The consensus tree was created using FigTree version 1.3.1 [45], edited and displayed in MEGA5.2 [46]. Abbreviations as in Table 1.

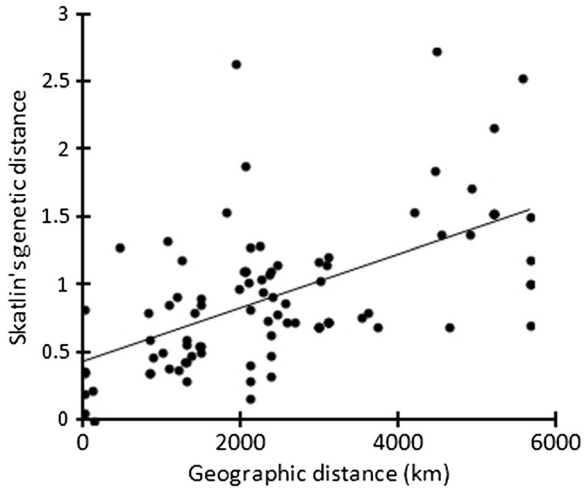


**Figure 5 Correlation between geographic and genetic distance in the study area.** The Mantel test shows significant isolation by distance at significance level of 95%. The Mantel *r* statistic of 0.578 indicates that there is a relatively strong positive correlation between genetic and geographic distance. The p-value < 0.001 indicates that our results are statistically significant at α = 0.05. The chart was implemented by Genepop'007 [47].

differentiation between the two groups. The majority of variation among groups was 20.47% ($p < 0.01$) (Table 6). The matrix of genetic differentiation ($F_{ST}$) among populations of *H. ovalis* revealed that the genetic distance between TH-tr and TH-sa populations (0.137, $p < 0.01$) was lower than between TH-tr and TH-kn populations (0.335, $p < 0.01$). The dendrogram based on Nei's genetic distance also showed that three populations were divided into two main clades: 1) Gulf of Thailand and 2) Andaman Sea (Additional file 3). All data are stored in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S15597).

## Discussion

The present study is the first report of genetic diversity, as well as genetic differences, within and among populations of *H. ovalis* collected from the Western Pacific Ocean to the Eastern Indian Ocean using nuclear sequence comparison (ITS) and two DNA fingerprinting approaches: AFLP and SSRs. Conformation of new records for *H. major* in Malaysia and Myanmar and detection of high levels of polymorphism underlined impressively that genetic markers are powerful tools for species identification and assessing genetic diversity in seagrass.

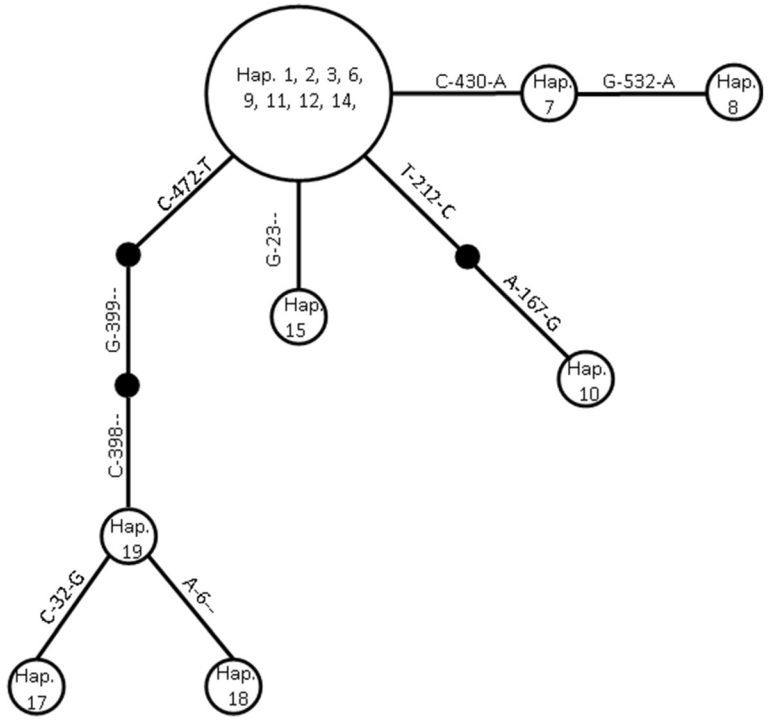


**Figure 6 Haplotype network of eight distinct haplotypes and their distribution detected for *Halophila ovalis* in both Western Pacific and Indian Ocean.** Haplotypes are presented by abbreviations in the circles. Abbreviations as in Table 1. Small solid circles are missing haplotypes. Nucleotide position and differences of nucleotide between two haplotypes are presented in each node. The dendrogram was implemented by software TCS version 1.21 [48].

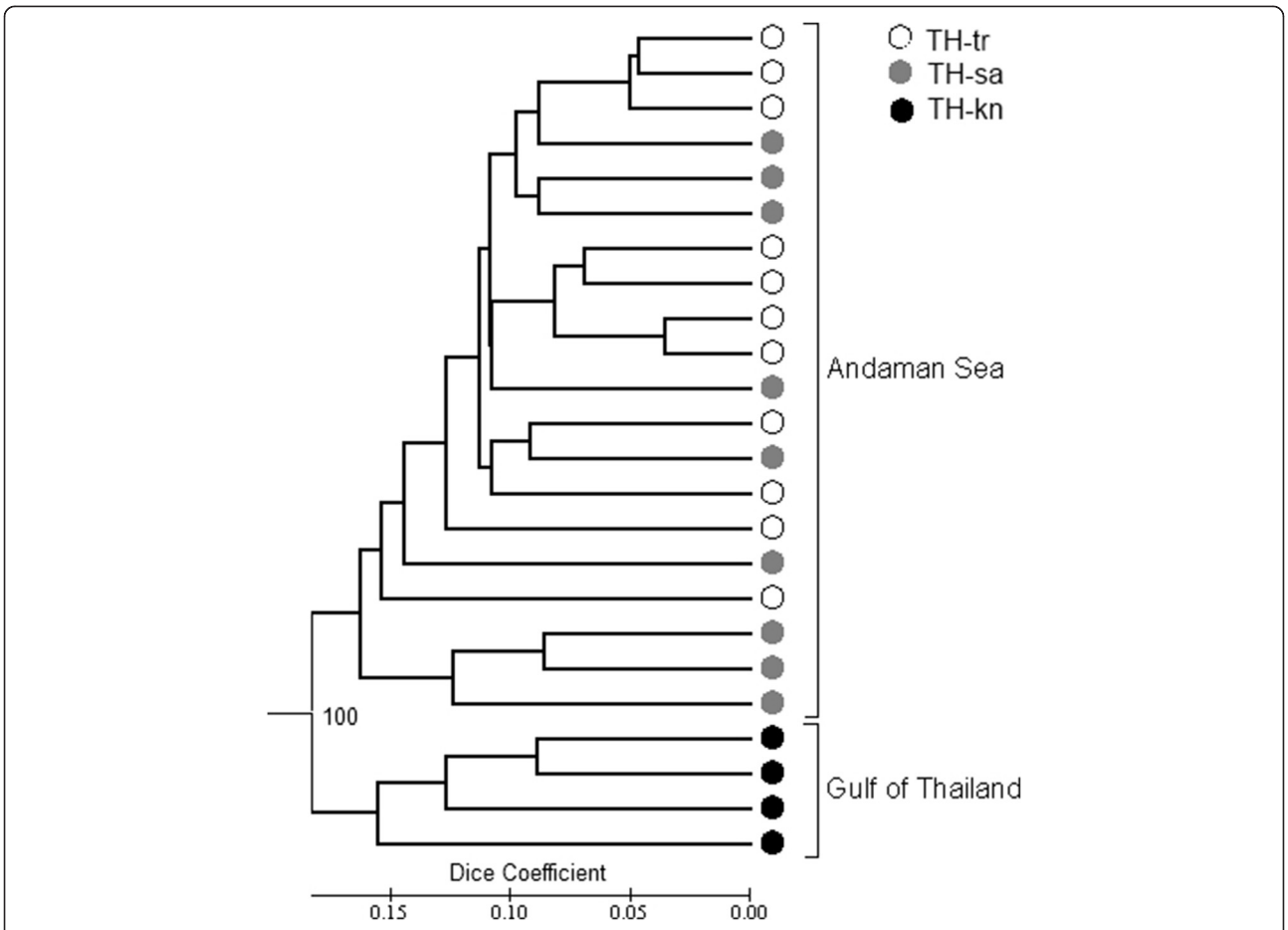


**Figure 7 UPGMA-based dendrogram of *H. ovalis* and closely related species generated from 208 AFLP markers.** The individuals collected in the Andaman Sea and the Gulf of Thailand were divided into two groups with a 100% bootstrap value. There are no significant differences between TR and SA populations. Abbreviations as in Figure 1. The dendrogram was assessed by FreeTree [45] and edited by MEGA5.2 [46].

### New records of *Halophila major* for Malaysia and Myanmar

Variation of leaf morphology has been detected within several species of the *Halophila* genus, namely *H. ovalis* [50,51], *H. hawaiana* [22], and *H. nipponica* [6]. Short et al. [1,52] argued that the taxonomy of *H. major* was unclear, because of overlapping leaf characteristics between *H. ovalis* and *H. major.* Molecular markers, especially ITS, were shown to be a valuable tool in resolving genetic relationships among the species of *Halophila*. For instance, *Halophila euphlebia* Makino was once treated as synonym for *H. ovalis* [11,53]; then, this species was transferred to *H. major* [8]. Results of Uchimura et al. [3] and Shimada et al. [6] supported the conclusion of Kuo et al. [5] that *H. major* and *H. ovalis* are distinct species based on ITS analysis and morphological data. Recently, Short et al. [1] suggested that species in general should be accepted as a new species only if a complete published taxonomic description existed, documenting unique sexual reproductive characters and significant genetic differences. There are three and six species of *Halophila* currently reported in Myanmar and Malaysia, respectively, [17,54] not including *H. major. Halophila major* has been found in the recent years along the coastlines of Southeast Asian countries including Indonesia, Thailand, Viet Nam and Japan [3,23]. As

**Table 6 AMOVA (Analysis of Molecular Variance) [43] results for AFLP variation at three collection sites of *H. ovalis***

| Source of variation | d.f. | Sum of squares | Variance of components | Percentage of variation | Probability |
|---|---|---|---|---|---|
| Among groups | 1 | 102.9 | 8.1 | 20.47 | $p < 0.01$* |
| Among populations within groups | 1 | 69.7 | 4.3 | 10.79 | $p < 0.01$* |
| Within populations | 21 | 572.8 | 27.3 | 68.74 | $p = 0.3$ |

Group 1 are the populations from the Gulf of Thailand and group 2 from the Andaman Sea. Calculations were conducted in Arlequin 2.2 [42].
*Significantly different.

it was demonstrated recently that *Halophila* members could not be fully resolved among closely related species such as *H. ovalis, H. major* and *H. ovata* Gaudich based on concatenated sequences of the two plastid markers *rbc*L and *mat*K [2,21]. In contrast, the phylogenetic analysis of the nuclear ITS sequence indicated that *H. ovalis, H. major* and *H. minor* are distinct species [3,7,23]. Hence, the use of the ITS marker to classify the entire set of samples collected for this study is the best choice based on the current knowledge. In this study, cluster analysis, direct comparison of nucleotide differences and evolutionary divergence between the two clades *H. ovalis* and *H. major* revealed that the materials collected in Mabul Island (MY-mb) and Gusungan Island (MY-gs), both in Malaysia, and one population (MM-gy) in Myanmar differ significantly from the *H. ovalis* clade. Moreover, four methods of constructing phylogenic trees also indicated that materials collected in Mabul Island and Gusungan Island (both in Malaysia) and Myanmar are *H. major*. Only the indications of the molecular methods initiated a detailed microscopic analysis of the leaf samples. The leaf morphology based on the ratio of the distance between the intra-marginal vein and the lamina margin confirmed the ITS analysis. Hence, morphological and nuclear sequence (ITS) analysis indicated that the materials collected in Mabul Island and Gusungan Island (both in Malaysia) and one population in Myanmar are actually *H. major*. In the field this kind of analysis is usually not possible, but our results suggest that careful analysis of seagrass samples need to be conducted before classifying them as *H. ovalis.*

The Indo-Pacific region has the largest number of seagrass species worldwide and this region was considered as the origin of the Hydrocharitaceae family [52,55]. Malaysia not only shows the highest number of *Halophila* species, summing up to seven species [17], this study, but also the highest diversity of *H. ovalis* haplotypes: there are four haplotypes found in six populations in Malaysia. In contrast, Nguyen et al. [23] found only one haplotype in four populations in Viet Nam. This finding is congruent with the hypothesis of Malaysia being the center of origin of the seagrasses.

### Genetic and geographic distance of *H. ovalis* based on SSRs

The genetic diversity indices showed relatively high values from 0.298 to 0.306. Compared to results reported from other studies on seagrass species including *Z. marina* (0.504 to 0.601) [34], (0.310 to 0.460) [56], *Zostera noltii* Hornemann (0.442 – 0.630) [57], *Posidonia oceanica* (L.) Delile (0.191 to 0.363) [58] and *Cymodocea nodosa* (U.) Ascherson (0.286 to 0.564) [59], (0.383 to 0.647) [60] using SSRs markers showed that the genetic diversity of *H. ovalis* is lower. Unfortunately, there are no studies on the genetic diversity of *H. ovalis* based on SSRs markers for direct comparison so far. In other AFLP approaches of seagrass species the genetic diversity such as *Thalassia testudinum* Banks ex König (H = 0.35, [32]) is slightly higher or much lower than in our results, such as for *Z. marina* (H = 0.007 to 0.072, [61]). Hence, the genetic diversity of seagrass varied indeed from species to species, geographic distribution, and different DNA fingerprinting approaches.

The present distribution of the genetic structure within species is influenced by evolutionary history [62]. In this study, genetic and AMOVA analyses indicated significant genetic differences among populations in the Western Pacific Ocean ($F_{ST}$ = 0.483), among populations in the Eastern Indian Ocean ($F_{ST}$ = 0.485), and larger significant differences among 14 populations surveyed in the Western Pacific Ocean and the Eastern Indian Ocean ($F_{ST}$ = 0.679). All above results indicated that great genetic differentiation among populations was detected and/or gene flow among populations is very low. For the Celebes Sea, pair wise genetic differentiation among populations showed genetic differentiation although the geographic distance among populations is about 30 to 40 km. However, genetic differentiation between MY-mg and MY-bd is very low (0.048) in contrast to other populations in the Celebes Sea. This could be explained by the diversity of the habitat such as substratum, currents, and time exposure to air during low tide etc., and those factors may affect the genetic differentiation. Japar et al. [17] stated that there are remarkable variations of *H. ovalis*, which grows in different substratum and depth. Significant genetic differences were also found in *Z. marina* between the Wadden Sea and the Baltic Sea where geographic distance among populations is within areas of 10 to 50 km [34]. Leaf morphology (small form) of *H. ovalis* collected in Tiga Island showed great differences in comparison to the other populations in the Celebes Sea (Prof. Japar, Malaysia, personal observation).

For the South China Sea, there are very great genetic differentiations among populations in the northern part of the South China Sea (HK-tc) and the remaining populations in the western, eastern and southern part of the South China Sea. Perhaps high latitude (or lower average temperature) in the northern part of the South China Sea may lead to the genetic differentiation. Both populations collected in Viet Nam also showed significant differences, although the geographic distance between two populations is less than 100 km. In fact, there are great differences between the environmental conditions from two populations, in the lagoon and in the open sea. It could be explained by the differentiation of salinity, with high salinity (open sea) and low salinity (lagoon). The genetic difference between *H. ovalis* populations in the open sea and the lagoon were also found in India based

on AFLP data [33]. For the population of the Gulf of Thailand, the results indicated low genetic differentiation between populations in Thailand and the western part of the South China Sea. Perhaps there was no geographic barrier found between the western part of the South China Sea and the Gulf of Thailand. A study of Morton and Blackmore [63] shows surface currents between the Gulf of Thailand and the western part of the South China Sea, that frequently occur in both summer and winter season.

The genetic differentiation between populations in the Andaman Sea and in the Bay of Bengal is significantly different. This could be explained by a very long geographic distance (more than 2,000 km) between the Bay of Bengal and the Andaman Sea. However, there is no significant genetic differentiation between populations of TH-tr and TH-sa in the Andaman Sea. Perhaps short geographic distances and the same habitat are the main causes that led to the high similarity between the two populations. Results from AFLP analysis also indicated that the genetic distance between populations from TH-tr and TH-sa within the Andaman Sea is much lower than between populations from the Andaman Sea and the Gulf of Thailand. Moreover, surface currents in the winter (from TH-tr to TH-sa) and in the summer (TH-sa to TH-tr) [63] support species dispersal between TH-tr and TH-sa. In contrast, genetic differentiation between populations from IN-ka and IN-ma was also high. It could be explained by the geographic distance as well as habitat differences (lagoon vs open sea).

The result from the unrooted neighbor-joining tree based on Slatkin's genetic distance showed the identified six main clusters corresponding to populations from different regions. Based on the genetic distance, the population in MY-jo seems to be in between the Western Pacific and the Eastern Indian Ocean, which corresponds to the geographic distribution of *H. ovalis* populations in the study. However, one of the most striking results is the unexpected result in the case of the HK-tc population. It showed no simple relationship between genetic differentiation and distance between pairs of population. The HK-tc population was genetically closer to the population in the Eastern Indian Ocean than to populations in the Western Pacific Ocean. At present we are unable to explain this puzzling result.

### Role of the Thai–Malay Peninsula as a geographic barrier to *H. ovalis* populations in Thailand based on AFLP analysis

Among a total of 231 bands, 208 (90.05%) were polymorphic bands. This contrasts with a level of variability of 30% using AFLP in land plant species, such as rice [64]. In a recent study by Nguyen et al. [33] it was shown that the 17.5% of polymorphic bands are presented in the *H. ovalis* – *H. ovata* complex. High level of polymorphic bands has previously been reported in *Thalassia testudinum* Banks ex König [32] and *Zostera marina* Linnaeus [61] using AFLP. The percentage of polymorphic bands varies from species to species, geographic distribution, and primer combinations. For the band-based approach performed in this study, the similarity index showed comparable values to the similarity index of *H. ovalis* populations found in India [33]. Comparison between clustering analysis (UPGMA) (Figure 4) and PCoA (Additional file 2) showed that the pattern of clustering the taxa was similar with both analyses: The individuals collected in the Gulf of Thailand clustered as single clade, whereas individuals collected in the Andaman Sea grouped together. AMOVA results (Table 6) also indicate this variation between two groups. In this study, pair wise genetic differentiation ($F_{ST}$) and genetic distance (Additional file 3) among populations support the hypothesis that *H. ovalis* in the Gulf of Thailand and the Andaman Sea are genetically different. The results from AFLP analysis are also in agreement with the results of ITS analysis when different haplotypes in the Gulf of Thailand and the Andaman Sea were classified. In addition, the previous studies on marine animals [12,13] and mangroves [14,15] also indicated that the Thai-Malay peninsula is an effective geographic barrier for populations of different organisms in the Gulf of Thailand and the Andaman Sea.

Based on ITS, AFLP and SSRs analysis of genetic variation of *H. ovalis*, results indicated that the genetic markers are powerful tools to assess the genetic differentiation on the broad sample collection sites. However, the sample size was still low and in the case of TH-kn that may affect the standard error of the diversity in the population of the species as discussed by Singh et al. [65]. According to all our results, missing haplotypes were made visible in the haplotype network, hence we recommend the collection of more samples from populations in the Philippines, somewhere between the two mainlands of Malaysia (Peninsular Malaysia and East Malaysia), and somewhere between the Andaman Sea and the Bay of Bengal (Myanmar and Nicobar Islands) to be included in future studies.

## Conclusion

Our study documented the new records of *H. major* for Malaysia and Myanmar. The study also revealed that the Thai-Malay peninsula is a geographic barrier of *H. ovalis* populations in the Western Pacific and the Eastern Indian Ocean. Characteristics of habitat are also an ecological barrier to the evolution of *H. ovalis* in the smaller scale area.

## Methods

### Sample collection, DNA extraction and morphological analysis

Samplings of *Halophila* species were carried out at the Pacific Ocean and the Indian Ocean. Samples were collected from 17 populations belonging to eight regions depending on the geographic distribution. Regions were determined by long geographic distance (more than 1,000 km in this study) or geographic barrier. Region I (northern part of South China Sea): 1-Hong Kong (HK-tc). Region II (western part of South China Sea): 2-Van Phong (VN-vp), 3-Thuy Trieu (VN-tt). Region III (eastern part of South China Sea): 4-Sarawak (MY-sr). Region IV (Celebes Sea): 5-Tiga Island (MY-tg), 6-Mabul Island (MY-mb), 7-Gusungan Island (MY-gs), 8-Sibangat Island (MY-sb), 9-Bodgaya Island (MY-bd), 10-Maiga Island (MY-mg). Region V (Gulf of Thailand): 11-Kanom (TH-kn). Region VI (southern part of South China Sea): 12-Johore (MY-jo). All above six regions belong to the Pacific Ocean. Region VII (eastern part of Andaman Sea): 13-Satun (TH-sa), 14-Trang (TH-tr). Region VIII (northern part of Andaman Sea): 15-Myanmar (MM-gy). Region IX (Bay of Bengal): 16-Marakanam (IN-ma), 17-Kanyakumari (IN-ka). Details of each sampling site are presented in Figure 1 and Table 1. At each sampling point, plants containing root, rhizome and leaf were selected, and washed with seawater in the field to remove the epiphytes and debris attached to the plants. Each plant sample was placed in a single plastic bag and kept on ice. Plant material was transferred to the laboratory at the same day. In the laboratory, materials were re-washed with de-ionized water to remove seawater. One plant was divided into two parts, one part was pressed as a herbarium voucher specimen and the remaining part was desiccated in silica gel [66] for later DNA extraction. Parts with a length of 10 to 12 cm in a developmentally comparable state from five to ten different plants were haphazardly collected across the beds with a distance of 10 to 15 m among individuals. Materials desiccated in silica gel were brought to the Institute of Botany, Leibniz University Hannover, Germany, for further analysis. Eight to ten young leaves of each individual were homogenized by a bead mill (22 Hz, 2 min), and 100 mg of the fine powdered plant material was used for DNA extraction. DNA extraction was carried out using the Plant Nucleospin II Kit (Macherey & Nagel, Düren, Germany) following manufacture's instruction with slight modifications according to Lucas et al. [21]. DNA quality was checked on agarose gels stained with ethidium bromide and the concentration was measured by a microplate reader with micro-volume plates (Synergy Mx Multi-Mode, BioTek, Germany).

For the morphological analysis, ten adult leaves collected from ten different individuals from each location were used for the analysis. The five most important and differentiating parameters of leaf morphology including lamina width, lamina length, number of paired cross veins, space between intra-marginal veins and the ratio of the distance between intra-marginal vein ($r$) and lamina margin ($R$) were measured under the microscope Olympus SZ (Olympus, Tokyo, Japan). Photographs were taken using a U-TV1X-2 digital camera (Olympus) connected to a computer. The test for equal variances of each data set of leaf morphology among groups was checked by Levene's test for homoscedasticity. Levene's test, one-way analysis of variance (ANOVA), Tukey test was carried out by Minitab software (State College, PA, USA). Specimens were identified using the keys of Kuo et al. [5].

### ITS amplification procedure and sequencing

In this analysis, three individuals per population randomly selected from 15 populations (45 samples in total) described above were used for ITS amplification (Table 1). The region selected for PCR amplification was the nuclear ITS region including the 5.8S sequence. Primer pairs used in this study were (ITS5a) [67] and (ITS4) [68] (Table 7) to amplify a sequence of 700 to 710 bp consisting of ITS1, 5.8S, and ITS2. The total volume of 25 μl included 1x Dream *Taq* Green buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1 U *Taq* polymerase (MBI Fermentas, St. Leon-Rot, Germany), 10 to 30 ng template DNA, 1 pmol primer each. The PCR was performed in a PTC 200 thermocycler (Biozym-Diagnostik GmbH, Hess. Oldendorf, Germany) with a heated lid under the following conditions: initial denaturation for 4 min at 95°C followed by 30 cycles of denaturation for 25 s at 95°C, primer annealing for 30 s at 52°C and extension for 35 s at 72°C, terminated by a final hold at 10°C. All PCR reactions were repeated two to four times independently with the same individual to reduce errors, possibly created by the *Taq* polymerase, in the final consensus sequence to a minimum. Direct sequencing of PCR products was done by GATC Biotech (Konstanz, Germany) from both directions. Consensus sequence was achieved by Clone Manager 9 (Sci-Ed, Cary, NC, USA).

### SSRs procedure

One hundred individuals (data given from Table 1) collected from 14 populations in the Pacific and the Indian Ocean were used for the analysis. Details of sample size, names of locations and coordinates are presented in Table 1. Among 10 primer pairs suggested by Xu et al. [70], we used five primer pairs resulting in highly polymorphic bands (HO5, HO8, HO36, HO48 and HO51) (Table 7) for PCR. Thirty ng of template DNA was used in each 15 μl PCR including 1x Williams buffer, 0.2 mM dNTPs, 1 U *Taq* polymerase (MBI Fermentas), and 1 pmol primer each. The PCR was performed in a PTC 200 thermocycler (Biozym-Diagnostik GmbH under the following conditions: initial denaturation for 5 min at

**Table 7 Sequence of primers/adaptors used for ITS, AFLP and SSRs**

| Sequence of primers used for ITS | Name of primer | Ann. temp. (°C) | Motive | Length of PCR product (bp) | Source |
|---|---|---|---|---|---|
| 5′-CCTTATCATTTAGAGGAAGGAG-3′ | ITS5a | 52 | | 700 | [67] |
| 5′-TCCTCCGCTTATTGATATGC-3′ | ITS4 | | | | [68] |
| Sequence of adaptors and primers used for AFLP | | | | | |
| 5′-CTCGTAGACTGCGTACC-3′ | *Eco*RI adaptors | | | | [69] |
| 5′-AATTGGTACGCAGTCTAC-3′ | | | | | |
| 5′-GACGATGAGTCCTGAG-3′ | *Mse*I adaptors | | | | |
| 5′-TACTCAGGACTCAT-3′ | | | | | |
| 5′-GACTGCGTACCAATTCA-3′ (*Eco*RI + A) | Pre-selective primers | | | | |
| 5′-GATGAGTCCTGAGTAAA-3′ (*Mse*I + A) | | | | | |
| *Eco*RI + ACA/*Mse*I + ATC (set1) | Final amplification | | | 50-500 | |
| *Eco*RI + ACC/*Mse*I + ATC (set2) | | | | | |
| *Eco*RI + ACA/*Mse*I + ACA (set3) | | | | | |
| *Eco*RI + ACC/*Mse*I + ACA (set4) | | | | | |
| Sequence of primers used for SSRs | | | | | |
| 5′-GAATGGGAAGGTGAAAGAG-3′ | HO5 | 59 | $(AT)_n(GA)_n$ | 260-296 | [70] |
| 5′-CACGGCACTGTTCATCTAC-3′ | | | | | |
| 5′-ATAACCAAAGCCTCCCAAGC-3′ | HO8 | 52 | $(GA)_n$ | 156-186 | |
| 5′-AAATATCAAACGCCCCTCAC-3′ | | | | | |
| 5′-CAACTAACCAAACGAGAAAC-3′ | HO36 | 59 | $(GA)_nGC(GA)_n$ | 220-240 | |
| 5′-AACCTTGACACCTGCTAATA-3′ | | | | | |
| 5′-ATCGAACCCAATAGACACCAG-3′ | HO48 | 59 | $(GA)_n$ | 196-246 | |
| 5′-CAGGCAACTTAGCAAGAAACT-3′ | | | | | |
| 5′-AGATAAGTTTCACTCCTGTG-3′ | HO51 | 46 | $(GA)_n$ | 141-175 | |
| 5′-ACCAGAACCAATCAAGAT-3′ | | | | | |

There are four primer pairs used for final amplification in AFLP and five primer pairs used to amplify five loci in SSRs. Ann. temp. Annealing temperature; bp, base pairs.

94°C followed by 25 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 52 to 59°C and extension for 35 s at 72°C, and terminated by a final hold at 10°C. To each sample, 200 μl of dye (98% formamide, 10 mM EDTA, 0.05% pararosaniline) was added. Reactions were heated up to 72°C for 5 min before loading onto 6% AFLP gels (Sequagel XR, National Diagnostics, Hull, England). For running an AFLP gel on the 4300 DNA Analyzer (LI-COR, Biosciences, Germany) manufacture's instruction were followed. Base pair lengths obtained from visual analysis was resolved with previously published allele lengths [70] and sequencing was performed when necessary.

### AFLP procedure

Samples were collected from three populations from the Andaman Sea and the Gulf of Thailand. Initially, 10 to 15 individuals per population were collected in Thailand for AFLP analysis. Unfortunately, DNA extracted from some plant samples was degraded. Degradation may have been caused by the humid and hot climate during the collection period in Thailand. Meudt et al. [71] indicated that use of degraded DNA could result in poor quality profiles with low reproducibility in AFLP analysis. Hence, only the samples retrieving high quality DNA were subjected for further experiments. According to Pruett and Winker [72], a sample size of 20 to 30 individuals is recommendable for genetic population studies. However, five to six samples are sufficient to obtain a standard error equal to 10% of the diversity in the population of the species [65]. In this study, there are four and twenty samples included from the Gulf of Thailand and the Andaman Sea, respectively, showing high quality of DNA.

Details of sample size, name of locations and coordinates are presented in Table 1. The AFLP procedure was carried out as reported by Vos et al. [69] with few modifications. In brief, genomic DNA (250 ng) was digested with two restriction enzymes in a total volume of 25 μl including 5 U *Eco*RI, 3 U *Mse*I, 1x Restriction Ligation (RL) buffer (10 mM Tris/HCl, 10 mM MgAc, 50 mM KAc, 5 mM DTT, pH 7.5) for overnight at 37°C. Adapters were

prepared in a total volume of 5 μl including 50 pmol of *Mse*I adapters, 5 pmol of *Eco*RI adapters, 0.5 mM ATP and 1.2 U of T4 DNA ligase, and 1x RL buffer. The mix of digested DNA and adapters were incubated at 37°C for 3.5 h and then used as a template for PCR. The pre-selective PCR contained 5 μl of template, 1 U of *Taq* polymerase (MBI Fermentas, St. Leon-Rot, Germany), 0.25 mM of each of the four dNTPs, 1x Williams buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatine) and 50 ng of *Eco*RI and *Mse*I primers with one selective nucleotide (A) in a total volume of 50 μl. The PCR program consisted of twenty cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, followed by 10 min at 72°C. An aliquot of the reaction mix was diluted 1:20 with 1x TE Buffer (10 mM Tris/HCl pH 7.5, 1 mM EDTA). The selective PCR contained 2.5 μl of the diluted (1:20) product of the pre-selective PCR, 2 mM dNTPs, and 5 U *Taq* polymerase in a total volume of 10 μl. Four primer pairs, *Eco*RI + ACA/*Mse*I + ATC, *Eco*RI + ACC/*Mse*I + ATC, *Eco*RI + ACA/*Mse*I + ACA and *Eco*RI + ACC/*Mse*I + ACA, (Eurofins MWG Operon, Ebersberg, Germany) were used for the selective amplification. The first amplification cycle was carried out for 30 s at 94°C, 30 s at 65°C and 1 min at 72°C. In each of the following 11 cycles, the annealing temperature was reduced by 0.7°C. The last 24 cycles were carried out at an annealing temperature of 56°C, and the final extension step was carried out at 72°C for 10 min. To each sample, 50 μl of dye (see above) was added. Running conditions and instruments were the same as for SSRs.

#### Bioinformatic analysis

The obtained ITS sequences and known sequence of *Halophila decipiens* Ostenfeld (KC175913) and *H. minor* (AF366405; AF366406) were aligned by CLUSTAL X [42] and the alignment was further modified by eye. Gaps were considered as missing data. Identical sequences within each species were excluded from the alignment. Additional in-group sequences were obtained from GenBank (Table 1), and included in the alignment. The program jModelTest 0.1.1 [73] was used to find the model of sequence evolution that fitted best with the data set. Phylogenetic analyses were performed using ML, NJ [74] with the model Tamura 3-parameter, MP [75] in MEGA5.2 [46], and BA (Metropolis-coupled Markov chain Monte Carlo method) performed in MrBayes v.3.2 [76]. *Halophila decipiens* was used as out-group, because it is closer to its ancestor than the *Halophila ovalis* complex [7]. In the analyses, trees were tested by the bootstrapping method with 1,000 replications. All phylogenetic trees achieved from analysis were analyzed and exactly constructed by the "tree of trees" approach [77]. Moreover, a network of relationships among haplotypes was constructed as well as a cladogram that showed the nested structure of the haplotypes. This analysis was conducted in software TCS version 1.21 [78]. Only populations determined as *H. ovalis* based on ITS analysis were used for AFLP and SSRs analysis.

For the AFLP analysis, only polymorphic fragments were scored as binary data (1, band present; 0, band absent). The binary scores were manually compared with the pictures to re-confirm presence or absence of bands. A presence/absence binomial matrix of 30 individuals and 201 polymorphic loci was used as basis for the analysis. In this study, the analysis with two approaches including band-based approach (for individual level) and allele frequency-based approach (for population level) [48] was carried out. In the individual level, the similarity among 30 individuals was calculated by the Dice coefficient [79]. A cluster analysis was performed using unweighted pair group method with arithmetic mean (UPGMA) based on the Dice index [79]. Bootstrap values (based on 1,000 re-samplings) were used to estimate the reliability of the clustering pattern. This analysis was carried out in FreeTree software [80]. The dendrogram was edited and displayed by MEGA5.2 [46]. Principal Coordinates Analysis (PCoA) of the correlation matrix was used to further investigate relationships between individuals using NTSYSpc version 2.20 [81]. At the population level, the allelic diversity at each locus was calculated as $h = 1 - \sum p_i^2$, where $p_i$ is the frequency of the $i^{th}$ allele [49]. Allelic diversity within each population was the mean allelic diversities among the 114 loci. Nei's $G_{ST}$ [82] was used as a value of genetic differentiation. $G_{ST}$ was calculated using the formula $G_{ST} = (H_T - H_S)/H_T$ [49], where $H_T$ represents the total gene diversity and $H_S$ represents the gene diversity within populations. Those values and the dendrograms (UPGMA) were assessed by POPGENE 3.2 [83] and MEGA5.2 [46]. In addition, pairwise genetic distances were calculated and used in AMOVA (Analysis of Molecular Variance, [43]). The analyses were conducted with the Arlequin version 3.5 [42].

For the SSRs, genetic diversity was measured for each site using the indices described by Williams and Orth [84]. These indices include: expected heterozygosity under Hardy-Weinberg equilibrium ($H_e$) = (Σ expected frequency of heterozygotes at each locus)/(total number of loci); observed heterozygosity ($H_o$) = (Σ frequency of heterozygotes at each locus)/(number of individuals); and allele richness (A) = (Σ number of alleles at each locus)/(total number of loci). All those parameters were assessed by Microsatellite Toolkit for Excel [39] and FSTAT version 2.9.3.1 [40]. Deviation from Hardy-Weinberg proportion was tested using a Markov-chain algorithm developed by Guo and Thompson [85] and implemented in the Genepop'007 [47]. Linkage disequilibrium among all pairs of loci for each population and all populations in the Western Pacific and the Indian Ocean was also tested by

Genepop'007 [47]. For the population structure, Wright's F-statistics ($F_{ST}$) was calculated. $F_{ST}$ measures the degree of inbreeding in the subpopulation relative to the total population, and is commonly used to estimate population differentiation. The software FSTAT version 2.9.3.1 [40] was also used for calculation. Significant differences among groups ($F_{ST}$), among populations within groups ($F_{SC}$) and within population ($F_{CT}$) were test by AMOVA (Analysis of Molecular Variance). This analysis was carried out by Arlequin 3.5 [42]. Pairwise distances were calculated from allele frequency data using the Slatkin's distance [41] in Arlequin 3.5 [42]. The unrooted neighbor joining tree was constructed using neighbor joining with bootstrap resampling (1,000 replications) in package Phylip version 3.5 [44] and a consensus tree was created using FigTree version 1.3.1 [45]. The tree was edited and displayed in MEGA5.2 [46]. Geographic distances (km) among populations were determined from NOAA digital map (Figure 1). The genetic-geographic distance matrix was statistically tested for correlation using the Mantel test [86]. This test was carried out by Genepop'007 [47].

## Availability of supporting data

The data sets supporting the results of this article are available in the TreeBASE repository, http://purl.org/phylo/treebase/phylows/study/TB2:S15597.

## Additional files

**Additional file 1: ITS sequences (ITS1-5.8S-ITS2) and their Genbank number (KF620337-KF620355).**

**Additional file 2: Principal Coordinate Analysis (PCoA) based on 208 AFLP markers.** There are two groups including the Gulf of Thailand and the Andaman Sea. Clustering of TH-tr and TH-sa is not significant. Abbreviations as in Figure 1. Symbols as in Figure 4. The matrix plot is processed by NTSYSpc, 2.20 [81].

**Additional file 3: Dendrogram of genetic distances among three populations of *H. ovalis*.** Branch lengths were calculated by Nei [87]. Abbreviations as in Figure 1. Symbols as in Figure 4. Dendrogram was assessed by POPGENE 3.2 [82], edited by MEGA5.2 [46].

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

JP, AP and NXV defined the research topic and the experimental design. NXV, MD, PT, USH, MHZ and JSP collected the materials. NXV and MD carried out the laboratory experiments and generated the data. JP and NXV analyzed the data and wrote the manuscript. All authors have contributed to, seen and approved the manuscript.

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# CHAPTER 6

# Distinctive Features and Role of Sulfur-Containing Compounds in Marine Plants, Seaweeds, Seagrasses and Halophytes, from an Evolutionary Point of View

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**Abstract**

Many seaweeds, seagrasses and many halophytes, grow in the tidal zone in similar environments. Their every-day-life and their life cycle are influenced by regular flooding during high tide and exposure to the air at low tide. Therefore they are confronted with similar daily changes in the osmotic potential and need to take up nutrients from the water and/or from the sediment. In addition, coastal zones and estuaries are often contaminated with high loads of some nutrients and heavy metals. Sulfur-deficiency is a major issue for land-based agriculture, whereas seawater act as a global sulfur reservoir and sulfur does not limit growth of marine plants. Sulfur-containing compounds and proteins seem to play a pivotal in the adaptation to these environmental conditions. This review highlights the putative roles of sulfur-containing compounds in a comparative way in seaweeds, seagrasses and halophytes. Can we observe similar metabolic and proteomic adaptations in regularly flooded coastal plants? The role of sulfur-containing proteins and of sulfur-containing secondary metabolites and their responsible set of enzymes will be analyzed from an evolutionary point of view. New strategies to increase salt-tolerance in higher plants based on sulfur-containing compounds are discussed.

## 1 Introduction

Although halophytes represent only 2 % of terrestrial plant species, they are present in about half the higher plant families and represent a wide diversity of plant forms [1]. The seagrasses developed three to four times from land plants within the order Alismatales to salt-adapted marine plants. Marine algae, we would like to

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focus here on seaweeds, belong even to very different higher taxa: the Phaeophyceae (brown algae) belong to the subgroup Stramenopiles or Heterokontophyta in the eukaryotic supergroup Chromalveolata, whereas Rhodophyta (red algae), Chlorophyta (green algae) and plants are divisions in the Archaeplastida [2].

Sulfur-deficiency is a major issue for land-based agriculture. Many soils become sulfur-deficient, especially when high nitrogen fertilizer is used. In freshwater and soil interstitial waters the sulfate concentration varies from 0.09 to 1.4 mM. Seawater contains about 28 mM or 8 % sulfate salts [3], equivalent to about 900 ppm sulfur, mainly as $MgSO_4^{2-}$. Thus seawater acts as a global sulfur reservoir whereas the nitrogen content accounts for only 16 ppm (http://ocean-plasma.org/). Sulfur is the element with the 6th highest concentration in marine environments. Therefore sulfur does not limit the growth of marine plants at all. Since 1.5 Bio years the composition of seawater has not changed much except local variations and anthropogenic contamination.

There are several examples that sulfur-containing compounds and proteins play a pivotal role in the adaptation to the marine environment. This review highlights the putative roles of sulfur-containing compounds in a comparative way in seaweeds, seagrasses and halophytes. One could assume that during evolution similar metabolic and proteomic adaptation mechanisms were established in marine plants. The role of sulfur-containing proteins and of sulfur-containing secondary metabolites and their responsible set of enzymes will be analyzed from an evolutionary point of view.

It will be interesting to see whether there are specific compounds in different taxonomic groups. These results could be used for chemotaxonomy in combination with genetic data. Some of the secondary compounds might be interesting from an economic point of view. Based to the availability of the complete genome sequence from the seaweed *Ectocarpus siliculosus* (Dillwyn) Lyngbye [4] comparative analysis became more meaningful, however, so far there are only fragmentary genome sequences of seagrasses and halophytes, except *Thellungiella salsuginea* (Pall.) O.E. Schulz and *Thellungiella parvula* (Schrenk) Al-Shehbaz and O'Kane available on the market. Unlike algae, seagrasses have roots and vascular tissue allowing them to absorb and translocate nutrients from soft sediment. In low nutrient environments this provides seagrass with a competitive advantage over algae as they can access the higher nutrient concentrations available in the sediment compared to the overlying water. On the other hand, in contaminated sediments seagrasses need good strategies to avoid accumulation of toxic compounds in their tissue. Halophytes form extensive root systems including tap roots which allows them to exploit also deeper sediment layers with different elemental composition.

## 2 Selected Sulfur-Containing Metabolites with Specific Functions in Salt-Tolerant Plants

### 2.1 Analysis of Sulfated Polysaccharides

#### 2.1.1 Abundance of Sulfated Polysaccharides

Sulfated polysaccharides (SP) comprise a complex group of macromolecules with a wide range of important biological functions. These anionic polymers are widespread in nature, occurring in a large variety of organisms. SP are found in vertebrates and invertebrates [5, 6] and in seaweeds [7]. Recently, they were also identified in halophytic angiosperms and in a salt-tolerant pteridophyte [8] and even in freshwater plants [9]. In this review we would like to focus on the occurrence of SP in seaweeds and angiosperm plant species.

The most well known SP in red algae are carragenans and agarans. Brown algae possess fucan and fucoidan SP. SP found in green algae are usually heteropolysaccharides. However, there is a predominance of one monosaccharide at the expense of others in several algal orders [10]. In a previous study, correlation between SP and salinity in plants was investigated [8]. Different

halophytic aquatic plants were used: marine angiosperms (*Ruppia maritima* L., *Halodule wrightii* Ascheson, *Halophila decipiens* Ostenfeld), mangrove angiosperms (*Rhizophora mangle* L., *Avicennia schaueriana* Stapf & Leechm. ex Moldenke), and the salt-tolerant pteridophyte *Acrostichum aureum* L.. The results reveal that in these halophytic aquatic plants exposed to different salinities the concentration of SP was increased. Also the degree to which they were sulfated correlated positively with the concentration of salt in the environment. The effect could not be found in the terrestrial angiosperms *Zea mays* L., *Oryza sativa* L. and *Phaseolus vulgaris* L. (glycophytes). Interestingly, *O. sativa* did not induce the biosynthesis of SP but increased the concentration of carboxylated polysaccharides [8]. Recently, a study revealed the presence of SP also in the three freshwater plants *Nymphaea ampla* L., *Hydrocotyle bonariensis* Comm. ex. Lam. and especially in *Eichhornia crassipes* (Mart) Solm, known as water hyacinth [9].

### 2.1.2 Description of Sulfated Polysaccharides

The most well known SP in red algae are carragenans and agarans, which are sulfated galactans. SP from brown algae are homo- and hetero-polysaccharides containing α-L-sulfated fucose called fucan and fucoidan, respectively. SP found in green algae are usually heteropolysaccharides containing a mixture of xylose, galactose, arabinose, mannose, glucuronic acid or glucose [11, 12]. SP of examined plants are built up differently. Those of seagrass species are composed of galactose units. SP of mangrove species contains arabinose and galactose, and the pteridophyte contains glucose units [8]. Galactose, glucose and arabinose are the main monosaccharides found in the SP from *E. crassipes* [9], comparable with the monosaccharide composition of green algae (Table 1).

### 2.1.3 Biosynthesis and Evolution of Sulfated Polysaccharides

The brown algal cell walls share some components with plants (cellulose) and animals (sulfated fucans), but they also contain some unique polysaccharides (alginates) (Fig. 1, Table 1). Analysis of the *E. siliculosus* genome failed to detect homologues of many enzymes known from other organisms involved in alginate biosynthesis and in remodeling of alginates, fucans and cellulose, indicating that brown algae have independently evolved enzymes to carry out many of these processes. The biosynthetic route for sulfated fucans is an ancestral pathway, conserved with animals [2]. A number of polysaccharide-modifying enzymes, such as mannuronan C5 epimerase, sulfotransferases and sulfatases, were identified [4]. Probably the biosynthesis of sulfated galactans starts with a precursor of lower molecular weight and degree of sulfation suggesting that glycosyltransferases and sulfotransferases may function simultaneously during the biosynthesis of sulfated galactans, at least in *R. maritima* [8].

Interestingly, green algae, the ancestor of higher plants [13], possess all units of SP as also found in all investigated halophytic aquatic plants (Table 1). This finding suggests that the production of SP is conserved throughout the plant evolution from green algae [8]. It is speculated that the activation and inhibition of glycosyltransferase genes alters the composition of SP among the different phyla [8].

**Table 1** The composition of sulfated polysaccharides of halophytic plants and algae

| Plant | Species | Units of sulfated polysaccharides |
|---|---|---|
| Marine angiosperm (seagrasses) | *R. maritima* | Galactose |
| | *H. decipiens* | |
| | *H. wrightii* | |
| Mangrove angiosperm | *R. mangle* | Galactose, arabinose |
| | *A. schaueriana* | |
| Freshwater angiosperms | *E. crassipes* | Galactose, glucose, arabinose |
| Pteridophyte | *A. aureum* | Glucose |
| Brown algae | *E. siliculosus* | Fucose |
| Green algae | | Galactose, arabinose, glucose |
| Red algae | | Galactose |

Modified after Ref. [8] with data from Ref. [9]

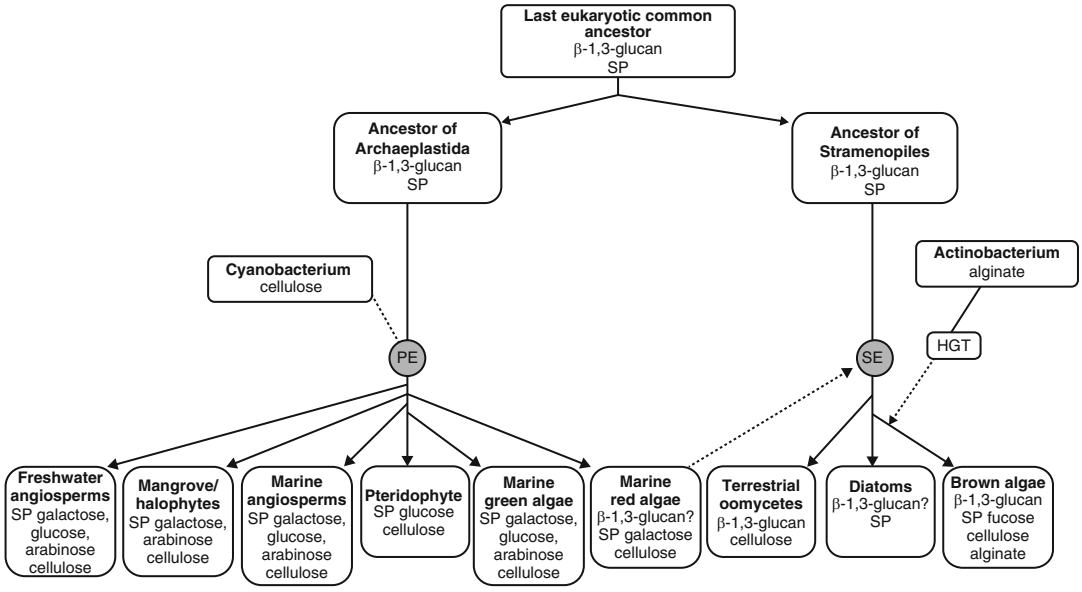



**Fig. 1** Scheme illustrating the origin and evolution of the main extracellular matrix polysaccharides in the Archaeplastida and Stramenopiles. Endosymbiotic events are indicated by dotted lines. PE, plastid primary endosymbiosis; SE, plastid secondary endosymbiosis; HGT, horizontal gene transfer; SP, sulfated polysaccharides (Modified after Ref. [8] with data from [9])

The current view about the origin and evolution of the main extracellular matrix polysaccharides in the Archaeplastida and Stramenopiles by [2] is extended including the newest published data (Fig. 1, Table 1). The recent evidence of SP in terrestrial plants can be further differentiated: In seagrasses SP with galactose, glucose and arabinose units, in halophytes and mangroves SP with galactose and arabinose units, and in freshwater angiosperms the same composition as in seagrasses and in green algae were determined.

### 2.1.4 Function of Sulfated Polysaccharides

In seaweeds, SP are found in the extracellular matrix. SP might protect against dehydration occurring at low tide, they are important both in terms of resistance to mechanical stresses and as protection from predators [4]. The function of SP in the plant cell wall in high salt environments is still unclear. It is speculated that SP increase the Donnan potential [14], supporting ion transport at high salt concentrations [8].

It was shown that in *E. siliculosus* enzymes involved in the desulfatation of SP were induced in low salt medium while two sulfotransferases involved in the synthesis of the cell wall SP were induced in seawater [15]. Therefore modifications of SP by enzymes like sulfatases and sulfotransferases are likely to modify the phytochemical properties of the cell wall, influencing rigidity, ion exchange and resistance to abiotic stress [4].

SP were found in 15 aquatic species of invertebrates [5] and in six halophytic aquatic plants [8] with a positive correlation between SP and water salinity. Obviously, the production of SP is correlated with salinity in the environment. If this is the case it remains an open question, what is the biological function of SP in freshwater plants [9].

Species being able to survive in both saline and freshwater conditions might be well suited study objects to analyze the function of SP. Salt-tolerance and adaptations to low salinities in a freshwater strain compared to the sequenced marine strain of *E. siliculosus* on physiological

and molecular levels was examined. It exhibited profound, but reversible, morphological, physiological, and transcriptomic changes when transferred to seawater. This indicates that for the colonization of freshwater, genomic alterations have occurred that produced permanent changes in the metabolite profiles, among them SP to stabilize the transition [15]. Also in the marine angiosperm *R. maritima* SP were not found when the plant was cultivated in freshwater [8].

In summary, the current state of knowledge suggests that the presence of SP in plants is an adaptation to high salt environments, which have been conserved during plant evolution from marine green algae. In future practical approaches to use the potential of SP in engineering salt-tolerant plants should be investigated in more detail.

### 2.2 Sulfur-Containing Compatible Osmolytes

Compatible osmolytes decrease the water potential of the cell, act as chemical chaperones and play a role in the solubilization of molecular complexes. It was shown that next to brown, red and green algae several angiosperms produce the compatible tertiary sulfonium osmolyte dimethylsulfoniopropionate (DMSP), derived from amino acids, being uncharged at neutral pH and of high solubility in water. DMSP is broken down by marine microbes to form two major volatile sulfur products, each with distinct effects on the environment. Its major breakdown product is methanethiol is assimilated by bacteria into protein sulfur. Its second volatile breakdown product is dimethyl sulfide (DMS). Atmospheric oxidation of DMS, particularly sulfate and methanesulfonic acid, is important in the formation of aerosols in the lower atmosphere. Probably these aerosols act as cloud nucleation sites. Therefore DMS is thought to play a role in the Earth's heat budget by decreasing the amount of solar radiation that reaches the Earth's surface [16].

However, the presence of high concentrations of DMSP in higher plants is limited to a few salt-tolerant species such as *Spartina* spp. (>50 μmol DMSP $g^{-1}$ fresh weight in the leaves) [17]. In seagrasses different DMSP concentrations have been found: *H. wrightii* 3.3 μmol $g^{-1}$ fresh weight, *Syringodium filiforme* Kutz. 0.10 μmol $g^{-1}$ fresh weight, *Thalassia testudinum* Banks ex. Konig between 0.18 and 4.0 μmol $g^{-1}$ fresh weight in epiphytized and non-epiphytized leaves and very low amounts in the rhizome [18]. These results indicate that the degree of epiphytization plays a major role in the contribution of seagrasses to the total DMSP production. In comparison, seaweeds contain between 0 and 85 μmol $g^{-1}$ fresh weight dependent on the region and conditions they have been collected [19]. The regulation of the biosynthetic pathway of DMSP in seagrasses and halophytes needs to be elucidated to clarify the overall contribution by salt-tolerant angiosperms to the DMSP production. Another sulfur-containing osmolyte is choline-*O*-sulfate. So far this compound has only been found in the family Plumbaginaceae, such as the genus *Limonium* [20]. It would be interesting to follow the distribution and roles of this sulfur-containing compound in more halophytic plant species.

## 3 Is Metal-Binding the Only Function of Phytochelatins and Metallothioneins?

### 3.1 Metals in Marine Tidal Environments

Phytochelatins (PCs) and metallothioneins (MTs) are Cys-rich metal chelators that represent the two principle groups of metal-binding molecules found across most taxonomic groups [21]. What is their particular role in plants grown in saline environments?

Both essential and non-essential transition metal ions can easily be toxic to cells. The physiological range for essential metals between deficiency and toxicity is therefore extremely narrow and a tightly controlled metal homeostasis network to adjust to fluctuations in micronutrient availability is a necessity for all organisms. Heavy metals are predominantly released into environment through anthropogenic activities

and farming, then accumulated in sediment with different levels [22]. These heavy metals in higher concentration which have contaminated large areas of land due to use of sludge, pesticides, fertilizers, residues from metalliferous mines and smelting industries are for plants the most toxic substances [23]. Coastal areas were considered as places which receive huge pollutants and heavy metals contribute massively [24]. Halophytes contribute a huge role in terms of heavy metal accumulation in their tissue [25]. Several results indicate that heavy metal concentration in the plant tissue is much higher than in their environmental ambient. Heavy metal concentration differs also from organ to organ as well as from species to species. The ability to respond to potentially toxic levels of heavy metal ions appears to be ubiquitous in biological systems. Heavy metals are taken up and accumulated by seagrasses [26], mangroves [27], marine algae [28] and other salt-tolerant plants [29].

There are at least three options to cope with heavy metals in salt-tolerant plants, dependent on the species compartmentation, metal excretion through salt glands or chelation in the extracellular space [30]. In all processes PCs and MTs might play a role. In seagrasses, oxygen is transported to rhizomes and roots of seagrasses during periods of light when photosynthesis releases oxygen into aerenchyma. Around the roots an oxidized zone is formed and oxygen diffuses into the anoxic sediment. By night almost all oxygen transport stops and alcoholic fermentation starts in roots [31]. The oxygenated rhizosphere of seagrasses during photosynthesis might create a special environment for the uptake of limiting nutrients with the help of nutrient/metal-binding and the detoxification of toxic elements by oxygenation and/or binding to chelating compounds.

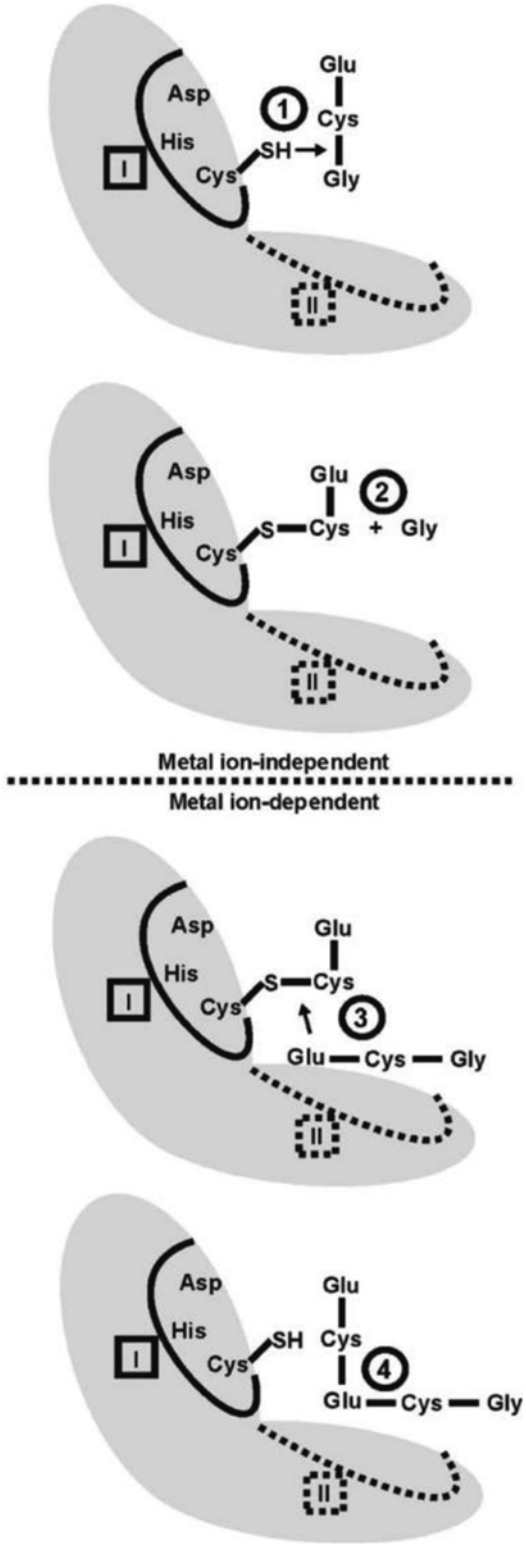
### 3.2 Abundance and Putative Function of Phytochelatins

PCs, glutathione-derived metal binding peptides, usually with the structure of $(1'\text{-Glu-Cys})_n$ – Gly (n = 2 – 11) are enzymatically synthesized peptides known to be involved in heavy metal detoxification, mainly Cd and As, which has been demonstrated in plants, algae and some yeast species grown at high heavy metal concentrations. PCs are translocated within the plant, transported to the vacuole as low molecular PC-metal complexes and are stored as high-molecular weight PC-metal complexes in the vacuole [32].

The current model of PC biosynthesis by phytochelatin synthase (PCS) starting from glutathione in a substituted enzyme mechanism is shown in Fig. 2. It has become apparent that PCS genes are far more widespread than ever anticipated. PCS expression can be found in representatives of all eukaryotic kingdoms and the presence of PCS-like proteins in several prokaryotes. The constitutive expression of PCS genes and PCS activity in all major plant tissues is still mysterious. It is unclear, how the extremely rare need to cope with an excess of Cd or arsenic ions could explain the evolution and distribution of PCS genes [32, 33].

In the last years substantial accumulation and tolerance to Pb and Cd by some aquatic plants have been reported [34–36]. Experiments done under Hg stress showed that PCs are synthesized in *Hydrilla verticillata* (L. f.) Royle and *Vallisneria spiralis* L. plants and play a role in Hg detoxification even though the accumulation of Hg was differently distributed in the plant [37].

*H. verticillata* plants also tolerated higher concentrations of AsV and AsIII than normally present in contaminated areas. Toxicity appeared only at the respective highest exposure concentrations of both As species after prolonged treatment. In view of their fast growth, high biomass and adequate As detoxification system, the authors propose that *H. verticillata* plants have great potential for remediation purposes [38]. The occurrence of the metal-complexing thiol peptides in natural populations of brown, red and green seaweeds was studied [39]. Concentrations of PCs and its precursor glutathione were measured. During heavy metal exposure PCs may also be limited by glutathione availability and, on the other hand, an excessive activity of PCs may lead to glutathione depletion causing oxidative stress to the cell [40]. The non-protein thiols




were identified and quantified in seaweed extracts and the molecular structures of PCs were confirmed. The authors concluded that for the first time PCs are reported in native brown algae (*Fucus* spp.), red algae (*Solieria chordalis* (C. Agardh) J. Agardh) and green algae (*Rhizoclonium tortuosum* (Dillwyn) Kützing) but not in thalli of *Ulva* spp. and *Codium fragile* (Suringar) Hariot (green algae) [39]. The results clearly showed that natural assemblages of seaweeds, belonging to disparate phylogenetic groups produced PCs when exposed to a mixture of metals in their environment. However, the involvement of thiol peptides in metal homeostasis, detoxification and resistance varies between seaweed species that are grown under the same environmental conditions [39].

The production of PC-like compounds by *Fucus vesiculosus* L. in response to Cd exposure suggests that marine macroalgae detoxify this metal by a similar mechanism as land plants, freshwater algae and yeasts. The response tended to the depletion of the intracellular glutathione pool but lead to a decreased ability to display other stress responses that depend on glutathione at higher heavy metal levels.

In the seagrass *T. testudinum* (Hydrocharitaceae) the heavy metal accumulation and thiol compound synthesis induced by Cd exposure were studied. Shoots were exposed to several $CdCl_2$ concentrations. Levels of Cd, Cys, glutathione, γ-glutamylcysteine (γ-EC), and PC-like peptides were determined in green blades, live sheaths and root/rhizomes tissues. The detected metal accumulation was dependent on the Cd

**Fig. 2** The hypothetical mechanism of phytochelatin synthesis. PCS probably carry two substrate binding sites (I and II). Acylation of binding site I (step 1) occurs at a conserved Cys which forms together with a His and an Asp the catalytic triad typical for Cys proteases. Gly is cleaved off (step 2) and the resulting γ-glutamylcysteine dipeptide is transferred onto another glutathione (or a PC molecule) (step 3). A new peptide bond is formed (step 4). Steps 1 and 2 are metal ion-independent. Acylation of site II and peptide transferase activity require metal ion activation and/or the binding of a metal–glutathione complex (The figure is taken from Ref. [33])

**Table 2** List of halophytic species and their phytochelatin synthases

| Plant | Species | Type | GB number | Sources |
|---|---|---|---|---|
| Marine algae | *E. paludosa* | n.k. | CBP94207 | –/–[a] |
| | *E. paludosa* | n.k | CBP94206 | –/– |
| | *N. palea* | n.k | CBP94204 | –/– |
| | *E. siliculosus* | n.k | CBJ32985 | [4] |
| | *M. pusilla* | n.k | EEH55879 | [42] |
| | *Micromonas* sp | n.k | ACO65712 | –/– |
| | *T. pseudonana* | n.k | EED94631 | [43] |
| | *T. pseudonana* | n.k | EED91948 | –/– |
| | *T. pseudonana* | n.k | EED89626 | –/– |
| | *M. vaginatus* | n.k. | EGK88687 | Unpublished |
| Mangrove species | *A. germinans* | 1 | ABA43317 | [44] |
| Other halophytes | *T. salsuginea* | n.k | BAJ34584 | [45] |
| Non halophytes | *B. juncea* | 1 | BAB85602 | –/–[a] |
| | *L. sativa* | 1 | AAU93349 | [46] |
| | *A. sativum* | n.k | AAO13809 | –/–[a] |
| | *A. thaliana* | n.k | NP_199220 | –/–[a] |
| | *A. thaliana* | 2 | NP_171894 | [47] |

*n.k.* not known
[a]Direct submission to GenBank, unpublished

concentration and the type of tissue in which green blades showing the highest content followed by live sheaths and root/rhizomes. All tissues experienced an increase in thiol-containing compounds resulted from Cd exposure. The lower glutathione content together with comparable higher PC-like Cd inducible peptides in green blades indicated the enhanced glutathione demand induced by Cd stress under which glutathione is directly used for handling the oxidative stress, and indirectly for thiol peptide synthesis [40]. Live sheaths showed the highest levels of Cys, glutathione and γ-EC [40].

Previous report indicated that plant tissue of *Posidonia oceanica* (L.) Delile and *Zostera marina* L. significantly accumulated high levels of heavy metals when growing on heavy metal-impacted water [41]. When treated plants were compared with control plants it was shown that foliage leaves and sheaths contained two to over six times higher amounts of Hg. It was not referred how these plants accumulated heavy metals.

Plants usually described which accumulate PCs are freshwater plants or halophytes living terrestrial. Few articles are published about PC (or PC-like compounds) in seagrasses or plants living in the tidal zone till up to now. The detailed way of metal accumulation has not been described.

The protein sequence for a putative PCS (Table 2) available from the halophyte *T. salsuginea* (BAJ34584) has been used for the search of additional PSC in halophytes (Fig. 3). Obviously, the PCS are abundant and functioning in salt-tolerant plants. Keeping in mind the high potential of halophytes for heavy metal accumulation one should think about the profit of phytoremediation in soils and sediments containing high salt contents. Genome sequencing of more salt-tolerance plants and parallel physiological analysis of their PCS could help to select the best species for phytoremediation processes.

### 3.3 Abundance and Putative Function of Metallothioneins

Although PCs have been shown to play an important role in the detoxification of certain heavy metals in both plants and animals [52; see Sect. 3.2], the role of MTs in this process has not been

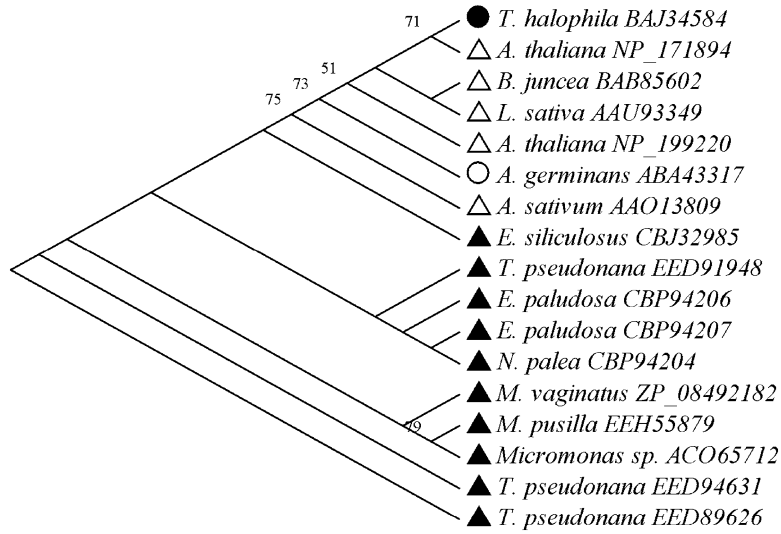



**Fig. 3** Molecular phylogenetic analysis of phytochelatin synthases. The evolutionary history was inferred using the Neighbor Joining method [48]. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed [49]. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown above the branches [49]. The evolutionary distances were computed using the Dayhoff matrix based method [50] and are in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1.92). The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 50 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [51]. ▲: Marine algae, ○: Mangrove species, △: Non halophytes; ●: Other halophytes

conclusively shown in plants [21]. Originally, MTs were identified in animals by their ability to protect against Cd toxicity but MTs have also been reported to play a role in other cellular processes, including the regulation of cell growth and proliferation, DNA damage repair, and scavenging of ROS but also in providing zinc [53]. Thus, although the importance of MTs throughout the life cycle of a plant has been demonstrated it is not clearly understood how they fulfill these roles [54].

### 3.3.1 Definitions, Classification and Functions

MTs are a group of proteins with low molecular mass and high Cys content that bind heavy metals and are thought to play a role in their metabolism and detoxification [32]. In recent studies, the criteria that define a protein or peptide as MT are: (i) low molecular weight (<10 kDa), (ii) high metal and sulfur content (>10 %), (iii) spectroscopic features typical of M–S bonds and (iv) absence or scarcity of aromatic amino acids [55]. However, often they are called MT-like proteins because one or more criteria do not apply.

Based on the arrangement of Cys residues, classes of MT proteins are grouped including class I: MTs contain 20 highly conserved Cys residues and are found in mammalian and vertebrates. MTs without this strict arrangement of Cys residues are referred to class II MTs and include all those from plants and fungi as well as non-vertebrate animals. In this MT classification system, PCs are, somewhat confusingly, described as class III MTs [32, 56]. Plant type II MTs are divided into four types, based on their Cys arrangement [32]. All four types were experimentally shown to be capable of acting as metal chelators [57]. Interestingly, the MT protein sequence from the marine alga *F. vesiculosus* does not fit easily into any of these four plant types [58, Table 3]. Hence, further studies

**Table 3** List of halophytic species and their class II metallothioneins

| Plant | Species | Type | GB number | Sources |
|---|---|---|---|---|
| Brown algae | *F. vesiculasus* | n.k. | CAA06729 | [58] |
| | *E. siliculosus* | n.k. | CBJ32637 | [4] |
| | *E. siliculosus* | n.k. | CBJ27567 | [4] |
| Seagrass | *P. oceanica* | 2 | AJ249602 | [59] |
| | *P. oceanica* | 2 | AJ249603 | –/– |
| | *P. oceanica* | 2 | AJ628138 | [60] |
| | *P. oceanica* | 2 | AJ628139 | –/– |
| | *P. oceanica* | 2 | AJ628140 | –/– |
| | *P. oceanica* | 2 | AJ628141 | –/– |
| | *P. oceanica* | 2 | AJ628142 | –/– |
| | *P. oceanica* | 2 | AJ628143 | –/– |
| | *P. oceanica* | 2 | AJ628144 | –/– |
| | *P. oceanica* | 2 | AJ628145 | –/– |
| | *P. oceanica* | 2 | AJ628146 | –/– |
| Mangrove species | *S. apetala* | 2 | ABQ42032 | [61] |
| | *S. ovata* | 2 | ABQ42031 | –/– |
| | *S. caseolaris* | 2 | ABQ42030 | –/– |
| | *S. alba* | 2 | ABQ42029 | –/– |
| | *B. gymnorrhiza* | 2 | ABF50984 | [62] |
| | *A. marina* | 2 | AAK11269 | –/–[a] |
| | *A. marina* | 2 | AAG61121 | –/–[a] |
| | *A. marina* | 2 | AAG50080 | –/–[a] |
| | *A. marina* | 2 | ABQ63078 | [63] |
| | *K. candel* | n.k. | ABD75757 | [64] |
| | *A. germinans* | 2 | AAY59706 | [44] |
| Other halophytes | *S. portulacastrum* | 2 | AEK87151 | –/–[a] |
| | *M. crystallinum* | n.k | AAC27531 | –/–[a] |
| | *M. crystallinum* | n.k. | AAB61212 | –/–[a] |
| | *L. bicolor* | n.k. | ABL10086 | –/–[a] |
| | *P. tenuiflora* | 2 | AFF18618 | –/–[a] |
| | *A. tripolium* | 1 | AB090882 | [65] |
| | *S. brachiata* | 2 | AEF01492 | [66] |
| | *T. salsuginea* | [b] | | [67] |

*n.k.* not known
[a]Direct submission to GenBank, unpublished
[b]Translated from DNA sequence: BQ060316

are needed to determine the diversity of the MT gene family.

Some data suggest a metal-binding capacity [57] and MTs may play a role in the homeostasis of essential metal ions and also the detoxification of heavy metals [21, 60, 68]. However, other studies showed that additional functions of MTs are still unknown and unclear [21, 55, 69]. Interestingly, MTs are highly expressed in seagrasses even in normal conditions when growing in uncontaminated sediments. In a heat stress experiment a MT with unknown function was the most abundant transcript but its expression was decreased by high temperature (3–15 %) [69, EST database http://drzompo.uni-muenster.de/].

### 3.3.2 Abundance of Metallothioneins in Halophytes

The seaweed *F. vesiculosus* is member of the brown algae family and it was shown that this species contains the gene for MT [58, Table 3]. The filamentous brown algae *E. siliculosus*

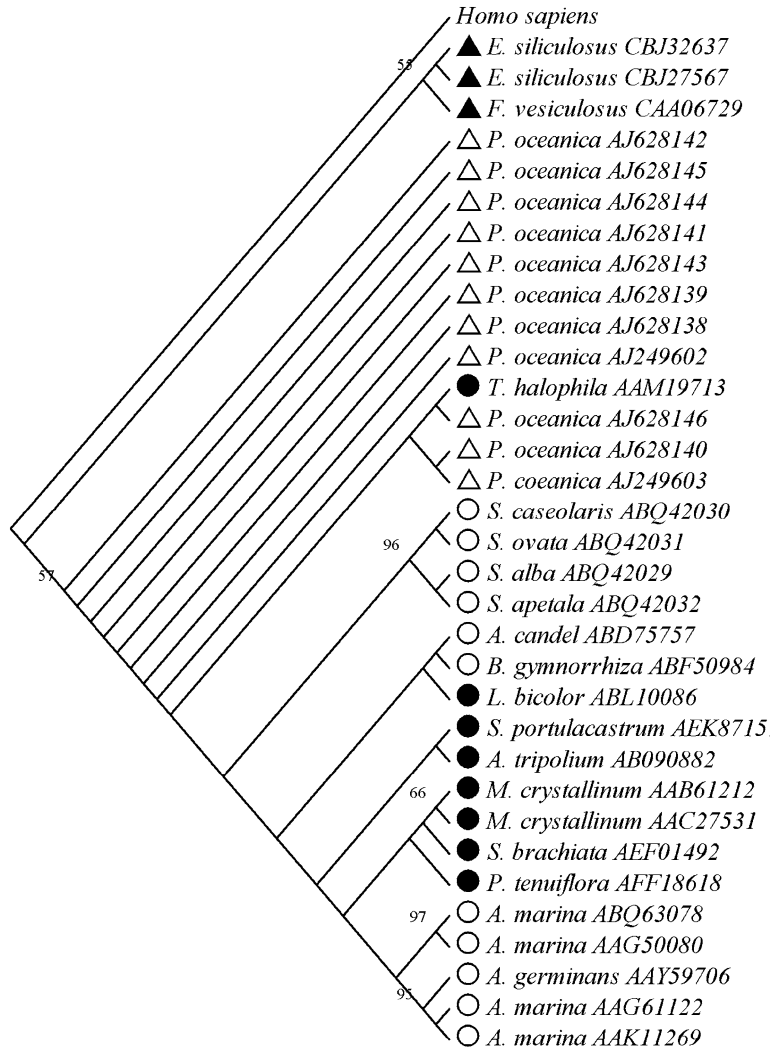



**Fig. 4** Molecular phylogenetic analysis of type II MT. For explanation see Fig. 3. ▲: Marine algae, ○: Mangrove species, △: Seagrasses; ●: Other halophytes. A MT sequence of *Homo sapiens* was used as out-group

contains at least two genes encoding MTs (own analysis). Nine MT-like sequences from Cu or Cd treated *P. oceanica* were isolated and classified into two subgroups [60]. Type II MT genes were also found in several mangrove species [27]. With respect to other halophytes, a type II MT gene was isolated from *Salicornia brachiata* L. [66]. This species is an extreme halophyte growing luxuriantly in the coastal marshes and is frequently exposed to various abiotic stresses including heavy metals. Expression of SbMT-2 gene was up-regulated concurrently with Zn, Cu, salt, heat and drought stress, down-regulated by cold stress while unaffected under Cd stress suggesting a role not only in metal binding. Type II MT also occurred in several other halophytes (Table 3). However, their function has not been analyzed so far. Phylogenetic relations of MTs isolated from halophytes so far are shown in Fig. 4. There is no clear pattern visible corresponding to the respective taxonomic group. The MT sequences from halophytes and mangroves have similarities with brown algae and also with seagrasses. One can assume that different types of MTs were taken for analysis. Therefore, as long as not all species included in the tree are

completely sequenced a final conclusion about the distribution of different groups of MTs in different taxa is not possible. In addition, more metal binding studies need to be done.

## 4 Conclusions

More and more data reveal the specific functions of sulfur-containing molecules in the marine environment. The high sulfate content of marine water bodies was obviously used as a positive selection pressure to develop adaptation to the high salt content. The algae use SP since a long time and the seagrasses remembered their old set of enzymes to produce new cell wall components during their way back to the sea. Probably in seagrasses the function of MTs was modified during adaptation. And the same is true for some halophytes which also contain in comparison to terrestrial plants unusual sulfate-containing polysaccharides which are interested from an applied point of view because they have some pharmacological activities among them they could be used as efficient anticoagulant with smaller side effects. These compounds constitute another treasure of the ocean. The same might be true for MTs because their unusual high abundance in seagrasses indicate a new role for MTs.

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# CHAPTER 7

# General Discussion

## Systematics and morphology of members of the *Halophila* genus

The *Halophila* genus belonging to the Hydrocharitaceae family is most interesting to the taxonomist due to the broad morphological plasticity. Among the members of the *Halophila* genus, *Halophila ovalis* and closely related species (also called the *Halophila ovalis* complex) shows overlapping morphological characteristics such as lamina size, number of cross-veins, cross-vein angle, ratio between the distance from the intra-marginal vein and to the lamina margin, number of seeds and fruits (Sachet and Fosberg 1973). Therefore the taxonomic classification was challenged and these facts provided arguments for establishing *H. minor* as the correct name for what has been called *H. ovata*. However, Kuo (2000) suggested that *H. ovata* and *H. minor* should be treated as distinct species based on identified characteristics. Also based on leaf morphology, Kuo (2006) distinguished seven distinct species from the *H. ovalis* complex including *H. minor, H. major, H. ovalis, H. mikii, H. nipponica, H. okinawensis* and *H. gaudichaudii*. However, Short et al. (2007, 2011) had argumented that the taxonomic classification of *H. major, H. ovalis, H. mikii, H. okinawensis* and

*H. gaudichaudii* was not clear. Great leaf variation was also found in *H. hawaiiana* and *H. nipponica* (McDermid et al. 2003; Shimada et al. 2012). Den Hartog (1970) emphasized the need for a detailed study of the *H. ovalis* complex as a whole to better understand the link between morphological variability and environmental parameters. Results of our study described in chapter 2 stated that there were wide differences of leaf morphology in seagrass materials collected in different habitats in Viet Nam. Especially, samples collected at Nha Trang Bay the leaf morphology showed significant differences in comparison to other populations by the trait of the number of cross veins and the ratio of the distance between the intra-marginal vein and the lamina margin. It seemed to be morphologically more similar to *H. major* as described for Japanese populations (Kuo et al. 2006; Uchimura et al. 2008). Hence, our morphological analysis indicated that materials collected at Nha Trang may be *H. major* which formerly identified as *H. ovalis.* Due to overlapping of leaf morphology among *H. ovalis* and closely related species, another approach: molecular markers can solve species boundaries in the proper way.

### Species separation by molecular means depends on the choice of the genetic marker

Analysis of single polymorphic nucleotides by DNA fingerprinting techniques is considered as a powerful tool that may resolve the boundaries among the species within a genus. For the species *Halophila ovalis* Waycott et al. (2002) added notes that some of the variation within *H. ovalis* may be of genetic origin challenging the current species definition of *H. ovalis.* Recently, the detailed study by Lucas et al. (2012) based on single plastid *rbc*L/*mat*K or the concatenated sequences of the two plastid markers indicated that *H. decipiens* collected in India was misidentified. The authors suggested the concatenated sequences of the two plastid markers (*rbc*L and *mat*K) may be used as DNA barcoding for seagrasses. Our initial work on identification based on DNA barcoding stated that there are no or very low nucleotide differences among individuals of *H. ovalis* collected in Viet Nam showing a broad variation in leaf morphology. However, results from tree-based and character-based approaches did not resolve the boundary between *Halophila* specimens collected in Nha Trang Bay and other locations in Viet Nam although our results on the distance between intra-marginal vein and lamina margin was similar to what had been described for *H. major* by Kuo (2006) (see also chapter 2). Hence, it is necessary to apply the third sequence – such as the nuclear ITS sequence.

The ITS region (ITS1-5.8S-ITS2) showed that some specimens identified as *H. ovalis* belonged to different clades, and this clearly points to the need for a critical taxonomic revision of *Halophila* species across the entire geographic range of this genus (Waycott et al. 2002). Authors indicated that *H. ovalis* and *H. minor* were distinct species based ITS analysis for members of *Halophila*. The results of Uchimura et al. (2008) based on ITS sequences suggest that *H. gaudichaudii*, *H. okinawensis,* and *H. nipponica* may be conspecific. *Halophila ovalis* and *H. major* are distinct species. Moreover, Waycott et al. (2002) indicated that the taxonomic status of *H. hawaiiana* and *H. johnsonii* needs clarification, as molecular data could not distinguish them from *H. ovalis.* Based on detailed analysis of leaf morphology and ITS analysis, Short et al. (2010) demonstrated that *H. ovalis* and *H. johnsonii* are synonyms.

Also in this work, ITS sequence analysis revealed higher species resolution than single plastid *rbc*L/*mat*K or the concatenated sequences of the two plastid markers.

Interestingly, the result described in chapter 3 indicated that specimens collected in Nha Trang Bay cluster to *H. major* based on ITS analysis. All results obtained based on analysis of clustering, nucleotide differences, evolutionary divergence and morphological data support the conclusion that materials collected in Nha Trang Bay formerly identified as *H. ovalis* is indeed *H. major*. Short et al. (2011) stated that this species should be accepted if there were supporting genetic data for sameness. Hence, results in chapter 3 support the conclusion by Kuo et al. (2006) that *H. ovalis* and *H. major* are distinct species. In the same way, results described in chapter 5 showed that some materials collected in Malaysia and Myanmar formerly identified as *H. ovalis* need to be classified as *H. major* based on ITS markers and in parallel based on careful microscopical analysis of morphology and subsequent statistical treatment. Interestingly, *H. major* is new record for Vietnam, Malaysia and Myanmar. Therefore, the species resolution of genetic markers can be ranged in the following way: ITS > *mat*K+*rbc*L > *mat*K > *rbc*L.

However, our results indicated that boundaries between *H. ovalis* and *H. ovata* based on plastid and nuclear sequences could not be resolved. Despres et al. (2003) stated that AFLP fingerprints were very useful in resolving phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. Therefore, in this study we used the AFLP marker system to resolve the genetic relationship of *H. ovalis* and *H. ovata* which were differentiated from each other by the number of cross-veins (3 to 9 cross vein pairs for *H. ovata* and 10 to 25 cross vein pairs for *H. ovalis*) (Den Hartog 1970). Results of the similarity index, cluster analysis, PCoA (band-based approach) and pair wise genetic differentiation, genetic distance (allele frequency-based approach) among *H. ovalis*, *H. ovalis* subsp. *ramamurthiana* and *H. ovata* (chapter 4) indicated that *H. ovata* and *H. ovalis* are indeed distinct species with high significance in all methods applied. Moreover, populations growing in lagoon and open sea also showed genetic differences. It is the first report on genetic differences between *H. ovata* and *H. ovalis* based on DNA fingerprinting. It is supporting the conclusion of the previous study on morphological characters (Kuo 2000), namely that they should be classified as two distinct species.

Clearly, DNA fingerprinting is another option which can be successfully applied to resolve very close taxa. Papenbrock (2012) indicated that the *H. ovalis* complex has

little genetic variation but wide morphological plasticity. For seagrass, tree- and character-based approaches demonstrated that the *rbc*L sequence fragment is capable of resolving up to family and genus level (Lucas et al. 2012; Papenbrock 2012). In the *Halophila* genus, the result from *rbc*L analysis (chapter 2) indicated that only *H. beccarii* was clearly separated from other species of the *Halophila* genus. The marker sequence *mat*K showed higher species resolution when *H. beccarii* and *H. decipiens* were resolved. Moreover, ITS resolved almost members of *Halophila* except *H. ovata* (chapter 3). AFLP analysis was used to resolve genetic distance among *H. ovalis*, *H. ovata* and *H. ovalis* subsp. *ramamurthiana*. In general, it can be inferred from the present investigation that the AFLP technique is a useful tool for the analysis of genetic diversity among seagrass populations, and can be used as the tool to resolve complex taxonomic issues of seagrasses at species and subspecies level. Hence, marker selection depends on the hypothesis as well as aims of each study.

However, the methodology of AFLP experiment and post-run data analysis are complex and time consuming compared with other markers. Also AFLP analysis requires very high DNA quality to avoid poor quality profiles with low reproducibility (Meudt et al. 2007). When plant material has to be collected in tropical areas with high temperature and humidity it is not always possible to conserve the DNA. The same applies to herbarium samples. Hence, in this study another approach was used to overcome the disadvantages of AFLP. Microsatellite analysis (SSRs) shows some advantages genetic markers over the AFLP technique including locus-specificity, a high degree of polymorphism, and therefore reliable results also with partially degraded DNA (Kimberly and Toonen 2006). Thus, SSRs were used to investigate the genetic differences of seagrass samples collected within and among the Western Pacific and Eastern Indian Ocean which is isolated by the Thai-Malay peninsula.

## Genetic variation and barriers

Geographic isolation refers to a situation where a species, or a population of a species, becomes separated by some kind of barrier, allowing each group to diverge along separate evolutionary paths (Braillet et al. 2002). The effect of geographic isolation is that the two populations are subjected to different selection pressures, since the conditions in the two areas will be different (Roy et al. 2006). So different alleles will be selected for, and genetic differences will gradually accumulate between the populations.

The present study, which is the first report on *H. ovalis* in this area, showed a genetic differencences across the Thai-Malay peninsula in the AFLP approach. Cluster analysis based on materials collected in the Gulf of Thailand and the Andaman Sea showed two distinct clades with 100% bootstrap value in the band-based approach. Nei's genetic distance between the Gulf of Thailand population and the Andaman population was higher than within populations in the Andaman Sea. The value of genetic differentiation between the Andaman Sea populations and the Gulf of Thailand populations was higher than 0.25. It indicated that very low gene flow occurred between the Andaman Sea populations and the Gulf of Thailand populations. Moreover, the AMOVA also indicated that there were significant differences ($p < 0.01$) between these two areas. Almost all data of AFLP analysis suggested the great genetic differentiation between *H. ovalis* population in the Gulf of Thailand and the Andaman Sea, and the Thai-Malay peninsula which blocks the free floating connection between the Gulf of Thailand and the Andaman Sea was considered as geographic barrier.

Beside geographic barriers, ecological barriers also play an important role in genetic differentiation of *H. ovalis* as was shown in a case study in India. In the present study, AFLP analysis (chapter 4) showed that *H. ovalis* populations in two different habitats including open sea and lagoon – estuary with a lower salinity were genetically different. For the allele frequency-based approach, the value of genetic differentiation (FST) gained from AFLP (chapter 4) indicated that populations occurred in the lower salinity (estuary, lagoon) had lower FST when compared to populations occurred in the open sea (higher salinity) and vice versa. Likewise, cluster analysis of the band-based approach also indicated that *H. ovalis* collected along the Tamil Nadu Coast,

India, including lagoon, estuary and open sea distributed in two clades: estuary, lagoon vs open sea. Nei' genetic distance (Nei 1978) as well as AMOVA also supported the conclusion that *H. ovalis* found in different habitats (low and high salinity) are genetically different.

Geographic and ecological barriers might be the result of the specific hydrophilous way of reproduction. *Halophila ovalis* pollen floats on the water surface because the pollen itself is hydrophobic (Cox and Knox 1988). However, the propagation of *H. ovalis* is mainly vegetative and may also form homogeneous colonies with clones (Les 1988). Kendrick et al. (2012) revealed that pair wise genetic distance significantly increased with geographical distance. How about the relationship between genetic and geographic distance? The answer was presented in the chapter 5 based on SSRs analysis the materials collected in the Western Pacific and the Eastern Indian Ocean.

In the broader collecting sites, materials collected from the Western Pacific to the Eastern Indian Ocean were analyzed by the SSRs approach and showed great genetic differentiation between the Western Pacific and the Eastern Indian Ocean (chapter 5). The unrooted neighbor-joining tree based on Slatkin's genetic distance among 14 populations from both the Western Pacific (ten populations) and the Eastern India Ocean (four populations) showed two main clades: Clade Western Pacific Ocean and clade Eastern Indian Ocean with a 100% bootstrap value. For the Western Pacific Ocean clade, three groups were formed corresponding to the geographic distribution of the populations: Western part, Southern part and Eastern part of the South China Sea. Populations in the Celebes Sea are close to the populations from the Eastern part of the South China Sea. Interestingly, two populations with close geographic distance in Viet Nam showed significant differences. In fact, there are greatly different environmental conditions where the two populations grow: in the lagoon and the open sea. It could be explained by differentiation base on a salinity gradient with high salinity in the open sea and low salinity in the lagoon. This result supports the genetic differentiation of *H. ovalis* in different habitats (low and high salinity) found in India based on AFLP analysis. For the Eastern Indian Ocean, there were two groups

corresponding to the geographic distribution of populations: Bay of Bengal and Andaman Sea. In the Andaman Sea, there was no genetic differentiation within populations in the area while larger genetic differentiation was observed within populations in the Bay of Bengal. Again, SSRs analysis confirmed the results obtained by AFLP when genetical differences between lagoon – estuary and open sea populations were found. Based on the results from the genetic differentiation, genetic distance, especially the unrooted neighbor-joining tree gained from SSRs for two cases in Viet Nam populations and Indian populations, we suggest that the evolution of lagoon – estuary populations may originate from open sea populations. The Mantel test indicated significant correlation between genetic and geographic distance for all populations in the study area.

Hence, all above results presented in chapter 4 and 5 showed that geographic, ecological barriers as well as geographic distance are the main causes to the evolution of *H. ovalis*. For the geographic barrier, Thai-Malay peninsula is typical example which seems to block the gene flow between Andaman Sea and South China Sea. The role of Thai-Malay peninsula to genetic differences was demonstrated in the results of AFLP and SSRs. Another barrier affected to genetic differences was found in this study is ecological barrier, open sea vs lagoon. Finally, geographic distance also effected to genetic distance. The more geographic distance increase, the more genetic distance increase.

## Suggestions for further studies to learn more about species and haplotype diversity and genetic differentiation within species

Although this present study resolved the genetic relationship among members of *Halophila* genus as well as genetic populations of *H. ovalis*, more populations in the Eastern part of the South China Sea (The Philippines) and Northern part of Bay of Bengal (Myanmar or Bangladesh Coast) should be included.

The distribution of halophyte species/clones can be understood not only by geographical but also by latitudinal temperature ranges. Hence, we recommend for the next studies on the genetic variation among *H. ovalis* populations collected at latitudinal temperature ranges (from Japan (40°N) via equator (0°) to Australia (40°S)) to understand the evolution of this species. Un-reported data gained from ITS sequence analysis (chapter 5) indicated that *H. major* also clusters into two subgroups: Pacific and Indian Ocean. The next studies should carry out the AFLP and SSRs analyses to demonstrate again the role of the Thai-Malay peninsula as geographic barrier to *H. major*. Results from this present study showed the importance of SSRs analysis in terms of genetic population study, therefore we would like to introduce SSRs approach should be applied to other species. In the recently years, there are several primer sets of SSRs suggested for seagrass species namely *Zostera nigricaulis* (Smith et al. 2013), *Z. muelleri* (Sherman 2012), *Halodule wrightii* (Larkin 2012), *Enhalus acoroides* (Nakajima et al. 2012), *Thalassia hemprichii* (Matsuki et al. 2013), *Halophila beccarii* (Jiang 2011) and *Syringodium isoetifolium* (Matsuki et al. 2013). However, there are no any detail studies on genetic population of above species. Hence, more studies on genetic diversity as well as evolution of seagrass should be carried out.

Knowledge from literatures and results of this study, we suggested the morphological traits to identify the three closely related species: *Halophila ovalis, H. ovata* and *H. major*. *H. major* is classified from *H. ovalis* based on the ratio of the distance between the intra-marginal vein and the lamina margin. Samples should be treated as *H. major* when this ratio is 1:20-25. In contrast, materials are *H. ovalis* if the ratio is 1: 12-16. In the case of *H. ovata*, the number of cross veins is main criteria when this number is

less than 9 which is lower than number of cross veins of *H. ovalis* and *H. major* (more than 10) In the case, this ratio is between *H. major* and *H. ovalis,* the ITS analysis should be added. In the case ITS cannot resolve the very closely related species, the DNA fingerprinting such as AFLP should be include. Hence, traditional classification of leaf morphology and modern approach: molecular are the best choice.

Because seagrass has decreased and degraded worldwide, the strategies of conservation of seagrass in particular and marine ecosystem in general have been implemented in last decades (Short et al. 2011). Genetic diversity is the main criteria to evaluate the health of habitat/ecosystem (Smith et al. 2013). Genetic diversity should be done on other seagrass species. Hence, the future studies of genetic diversity should be linked to the conservation genetics resource, especially in the Tropical Indo-Pacific. In the present study, genetic diversity including haplotype diversity, allele richness of *Halophila ovalis* in Western Pacific and Indian Ocean were showed and genetic diversity were different in from population to population. Conservation of genetic diversity is essential to the long-term survival of any species, particularly in light of changing environmental conditions. Reduced genetic diversity may negatively impact the adaptive potential for a species. In addition, low genetic diversity leads to an increased risk of inbreeding effects, through the uncovering of deleterious recessive alleles (Nakajima et al. 2012). Consequently, management of genetic diversity is an important component of recovery strategies for seagrass (Matsuki et al. 2013). Monitoring of seagrass not only conducted in biological parameter such as biomass, coverage, shoot density, leaf growing rate, but also genetic diversity, haplotype diversity are considered. Clearly, for the Tropical Asian area, evaluation of genetic diversity based on SSRs for all species should be carried out to point out the trend of degradation/increasing of seagrass under view of molecular. It is main criteria for strategies of conservation (Short et al. 2007).

## Conclusion

The aims of the present study were achieved. There are no genetic differences of sequence of two plastid genes although variation of leaf morphology of *H. ovalis* collected in Viet Nam was detected. DNA barcoding suggested for seagrass did not resolve the genetic distance between *H. ovalis* and *H. major*. Nuclear sequence (ITS) showed higher species resolution when *H. major* was resolved from *H. ovalis* complex. The morphological and ITS data revealed new record of *H. major* in Viet Nam, Malaysia and Myanmar. *H. ovata, H. ovalis* and *H. ovalis* subsp. *ramamurthiana* are genetically differences based on AFLP analysis. AFLP and SSRs analysis also indicated that geographic and ecological barrier cause genetic differences of *H. ovalis* populations from the Western Pacific to Eastern Indian Ocean.

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01.2003 – 08.2004 Graduate studies at the Asian Institute of Technology (AIT), Thailand; Topic of the thesis: „Integrated management of seagrass beds in Krabi estuary, Thailand: A GIS and remote sensing approach"

10.1997 – 12.2002 Worked at Institute of Oceanography, Vietnam Academy of Science and Technology; Subject: Taxonomy and ecology of marine plants.

10.1993 – 6.1997 Graduate studies at Dalat University, Viet Nam; Field of study: Biology; Topic of the thesis: „Study of effect of environmental factor to growing of marine algae – *Chlorella* sp"

09.1990 – 09.1993 Studied at Nguyen Trai High school, Khanh Hoa province, Viet Nam

# List of publications:

## PUBLICATIONS IN JOURNALS

**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

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## POSTER PRESENTATIONS

**Nguyen** XV, Thangaradjou T, Papenbrock J. 2013: Genetic variation among *Halophila ovalis* (Hydrocharitaceae) and closely related seagrass species from the coast of Tamil Nadu, India – An AFLP fingerprint approach. The Evolutionary Biology Meeting, Marseille, France, September 2013.