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Genetic Analysis of the Seagrass *Halodule* in Central Visayas, Philippines

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Abstract - Genetic variation of seagrass species *H. uninervis* and *H. pinifolia* with varying leaf width, from different sites, density and exposure in the Central Visayas were assessed using plastid *rbc*L marker specific for *Halodule*. The genetic structures of the *rbc*L sequences analyzed from 83 specimens of *Halodule uninervis* and *H.pinifolia* were 671 base-pairs (bp) long (with a difference of 2 base pairs (bp) substitutes of A in *uninervis* to C and G *in pinifolia*). All specimens of *Halodule* from three different sites in Central Visayas were clustered only into clades: *H. pinifolia* and *H. uninervis*, maintaining that despite significant morphological variation in leaf width due to different sites, density and exposure, the two are still separate species.

Keywords - seagrass, DNA, *rbc*L marker

INTRODUCTION

Seagrasses represent difficult ecological subject in marine ecosystems. The biology of seagrasses is constrained by the environment they inhabit leading to convergence in morphologies. For instance the majority of seagrass species possess flexible strap-like leaves although they do not represent a single evolutionary lineage (den Hartog 1970; den Hartog & Kuo, 2006; and Les et al., 1997). This leaf form allows the plant to thrive in fluid tide and wave-driven environment. The evolutionary trends that have led to these and other convergent characteristics are difficult to interpret without sufficient insight into their origin in both ecological and evolutionary context. Significantly, these suites of adaptations represent dramatic evidence of their adaptive capacity of to evolve and survive in extreme environments and such warrants careful study (Waycott et al., 2006).

Today, the understanding of seagrass ecology, physiology and adaptation has been constrained by the ability to develop a clear understanding of many biological features of these seagrass such as their ability to pollinate in seawater. On the contrary, due to the now widespread invention of new technologies, the details of seagrasses photosynthetic mechanisms as inferred through the applications of Pulse-amplitude modulation fluorometry, water temperature and light intensity using Hobo pendant loggers during different phases of their daily, weekly, monthly growth variation are rapidly being unraveled. However, some of the most basic questions of biology such as evolutionary relatedness, plant age and growth, selection for adaptive traits, breeding systems, and disturbance response strategies, are poorly studied. To date it is now possible to investigate those questions using readily available DNA markers (Reusch 2001; Waycott et al., 2006)

According to Waycott and Les (2000), a number of seagrass species are currently undergoing reassessment employing the genetic methodologies now available to supplement morphological and anatomical studies. The genus *Halodule* has been a source of disagreement and confusion due to its overlapping morphological descriptors; the contribution of genetic analysis of phylogenetic relationships of species and biogeography should aid in resolving this

debate (Short et al., 2007).

With the use of genetic markers in studying the seagrass evolution and ecology (begun 1980-1990s), studies revealed that seagrass were remarkable in their genetic uniformity which indicates that the application of allozyme data to study population processes would not be successful (Les, 1988; McMillan, 1981; Triest, 1991). But the latter studies showed that greater sampling efforts could better detect polymorphism which made way to the improvement of the utilization of allozyme markers (Laushman, 1993; Ruckelshaus, 1996, Waycott, 1995, Williams & David, 1996; Waycott et al., 1997). The introduction of readily applied DNA markers provided significantly greater sensitivity (Alberte et al., 1994; Procacinni et al., 1996; Reusch et al., 1999).

Halodule has been well studied using nuclear Internal Transcribed Spacer (ITS) and chloroplast DNA (cpDNA) such as *rbc*L and *trn*L markers, nonetheless their relationship remains complex. All markers examined so far reveal the genus to be monophyletic. Within the genus, nuclear ITS sequence data analysis yielded one monophyletic clade comprising the New World of *H.wrightii* and another containing the Old World *H. uninervis* and *H. pinifolia*. Population of the latter two species overlap and are not clearly differentiated by ITS data (Les et al., 1997; and Waycott et al., 2006).

OBJECTIVES OF THE STUDY

The purpose of this study was to conduct DNA analyses using the *rbc*L marker specific for *Halodule* on specimens *H. uninervis* and *H. pinifolia* with varying leaf width, from different sites, density and exposure in the Central Visayas to assess their genetic variation

MATERIALS AND METHODS

Sampling

Halodule uninervis and *H. pinifolia* (Figs. 4 and 5) were collected from both multispecific and monospecific population in Bantayan Beach, Banilad Beach, and Siquijor from March to December 2010. Shoots were collected from 10 quadrats (1x1 m) in the intertidal and

subtidal area with the high and low density beds. Although whole plants were taken, DNA was extracted only from photosynthetic tissue. Plants were cleaned carefully with filtered seawater. Epiphytes on the leaves were removed by gentle scraping with the hand. The cleaned leaf fragment was immediately placed into labeled plastic bags containing silica gel desiccant or placed in plastic containers and stored in the freezer at -20^{o}C prior to the extraction.

DNA Extraction

Total genomic DNA extractions were performed using a modified CTAB extraction procedure following Zucarello et al. (2006). Dried or frozen were placed in a microtube containing 500 µL CTAB extraction buffer (2% CTAB, 0.1 Tris-HCL, 1.4 M NaCL 20 mM EDTA, 1 % PVP) plus 50 µg RNAse and 10 µL proteinase K, the homogenized. Samples were then incubated at 60^{o}C for 30 minutes with occasional mixing. Two extractions using an equal volume of chloroform: isoamyl alcohols (24:1) were performed, mixing and spinning for 10 minutes at 12,000 x g. DNA was precipitated with an equal volume of 100% isopropanol. The tubes were inverted and incubated at room temperature for 30 minutes spinned at 12,000 g and decanted, then washed with 70 % ethanol to remove the salts, air dried for 30 minutes and 50 µL of 0.1 X TE buffer is added. DNA checked was done on a horizontal electrophoresis using TBE agarose gels. The DNA extractions were then stored in the freezer for subsequent analysis.

DNA Amplification and Sequencing

Amplification of the DNA *rbc*L marker followed procedures of Zucarello et al. (1999). A set of r*bc*L markers specific for genus *Halodule* (provided by 1st BASE) were used with the sequences: forward primer 5'–GAAGCAGGGGCCGCAGTAGC- 3 and reverse primer 5-TGCATGCATTGCGCGGTGA- 3. Amplification was done in 30 µL volumes using the following concentration: 1 X PCR buffer (promega ™) 120 nmol dNTPs, 2 mM of MgCl2, 2.5 pmol of each primer 0.25 % bovine serum albumin (BSA; Sigma), 0.5 U of Tag polymerase (promega ™) an 1 µL of template DNA. Optimal amplification condition was as

follows: initial denaturation was by 95 ºC for 100 second, followed by 95 ºC for 20 second, 55 ºC for 35 second, and 68 º C for 45 second. Finally, the second steps to the fourth step were repeated 29 times. Amplified products were electrophoresed in 2% agarose. Positive PCR were sent to the First BASE Laboratories Sdn. Bhd, Malaysia for sequencing. Nucleic acid sequences were subjected to BLAST/N (Altschul et al., 1990) searches at the National Center for Biotechnology Information (NCBI). Sequencing was then aligned and edited by using MEGA 5.

Phylogenic Analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura, Peterson, Stecher, Nei, & Kumarm , 2011). Unweighted Pair group Method with Arithmetic Mean (UPGMA), Neighbor Joining (NJ) and Minimum Evolution (ME) implemented in Mega 5 version were used to construct a Phylogram from the data set using sequences of *H. pinifolia* and *H. uninervis* sequences from Ito and Tanaka (2010) for rooting. Support for individual branches was determined by bootstrap analysis; 500 bootstrap data sets were generated from resample data. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. To test the interspecific variation of both species, absolute and uncorrected-*p* pairwise distance matrices were constructed (Table 1).

RESULTS AND DISCUSSION

The sequences analyzed from 83 specimens of *Halodule uninervis* and *H.pinifolia* yielded 671 base-pairs (bp) long. All specimens of *H. uninervis* from three different sites in Central Visayas were very similar to *H. uninervis* (AB 571205) and the specimens of *H. pinifolia* were similar to *H. pinifolia* (AB 571205) described by Ito and Tanaka (2011). In terms of their *rbc*L sequences there was a difference of 2 base pairs (bp) substitutes of A in *uninervis* to C and G *in pinifolia* (Table 1). The phylogenetic trees obtained from analysis of the *rbc*L data are using tree clustering method presented in Figs. 1-3.

Table 1. Pairwise divergence in rbcL sequences between specimens of *Halodule uninervis* and *H. pinifolia* used in this study

	[1]	[2]	[3]	[4]	[5]	[6]
[1] *H. uninervis*, Banilad (15)	_	0	0	0.003	0.003	0.003
[2] *H. uninervis*, Siquijor (2)	0	_	0	0.003	0.003	0.003
[3] *H. uninervis*, Bantayan (1)	0	0	_	0.003	0.003	0.003
[4] *H. pinifolia*, Banilad (4)	2	2	2	_	0	0
[5] *H. pinifolia*, Bantayan (5)	2	2	2	0	_	0
[6] *H. pinifolia*, Siquijor (11)	2	2	2	0	0	_

Number in parenthesis represents number of samples. Each number indicates absolute distances (below diagonal) and uncorrected p-distances (above diagonal).

The result of the previous research of this study documented the variation in morphology of leaf width of *Halodule* among three sites in the Central Visayas. Samples collected in this study were identified by leaf morphology according to the taxonomic criteria of den Hartog (1970). Examination of all data across the sites indicates that the leaf widths of both *H. uninervis* and *H. pinifolia* were much wider within Banilad site relative to the Siquijor and Bantayan sites. Moreover the mean leaf width of *H. uninervis* was found to vary highly between intertidal and subtidal areas, with larger leaf width observed in subtidal area. Leaf width of *H. pinifolia* on the other hand, was found to be much larger in Banilad than those in Bantayan and Siquijor. Even though the two specimens, *H. pinifolia* and *H. uninervis*, exhibited significant morphological variation in leaf width due to different sites, density and exposure; the genetic analysis revealed that all specimens collected be clustered into clade H. *pinifolia* or clade *H. uninervis*.

The DNA analyses confirmed the seagrass taxonomic identity. The present phylogenetic analyses of the *rbc*L data further support the occurrence of the taxonomic maintenance of the genus *Halodule*. With nuclear (ITS) sequence data, Less et al., 1997 and Waycott yielded two clade whereas the first clade containing *H. wrightii* and the second clade comprising the overlapping of *H. pinifolia* and *H. uninervis*. This study however, does not support this analysis. The *rbc*L analysis data yielded the two species of *Halodule* in a different clade (1 or 2) with bootstrap

support 75 % of *H. pinifolia* and 87 % of *H. uninervis* in UPGMA, 87 - 86 % in NJ, and in 87-87 % in ME Phylogram tree analysis whereas, H. *wrightii* and *H. beaudettei* (from Western tropical Atlantic), as the out group species forming their own specific clade (3 and 4). Evidence by their *rbc*L 0.3 % divergence in a 671-base pair sequence revealed that *H. pinifolia* and *H. uninervis* are different species.

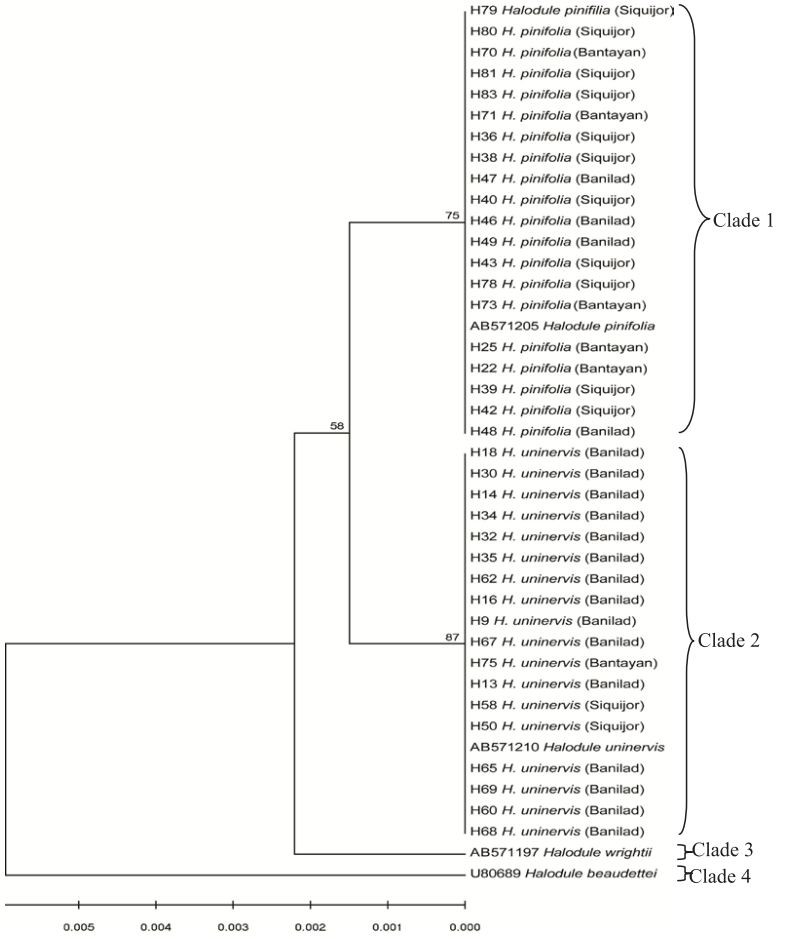

Fig. 1. UPGMA (Unweighted Pair Group Method) tree from *rbc*L spacer (543 characters) using Mega 5.01 Number on branch = bootstrap support (500 replicates).

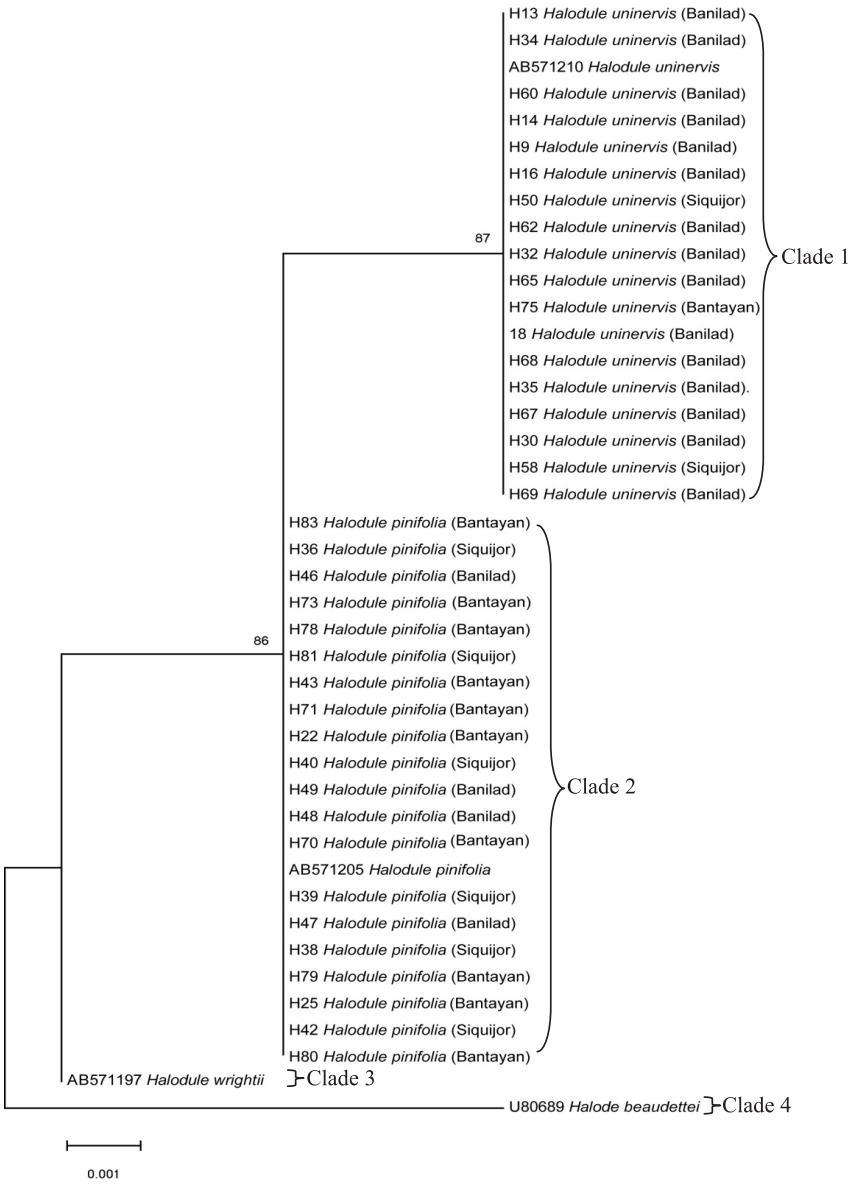

Fig. 2. Neighbor Joining (NJ) tree from *rbc*L spacer (543 characters) using Mega 5.01 Number on branch = bootstrap support (500 replicates).

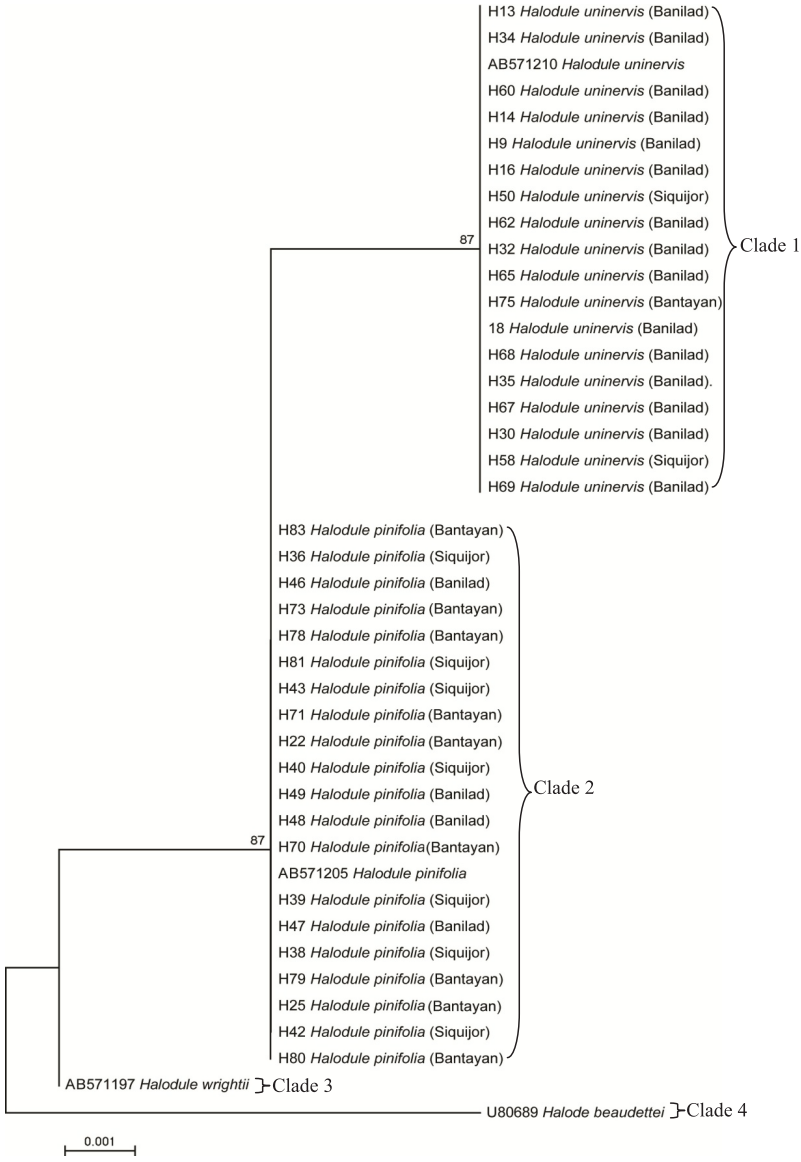

Fig. 3. Minimum Evolution (ME) tree from *rbc*L spacer (543 characters) using Mega 5.01 Number on branch = bootstrap support (500 replicates).

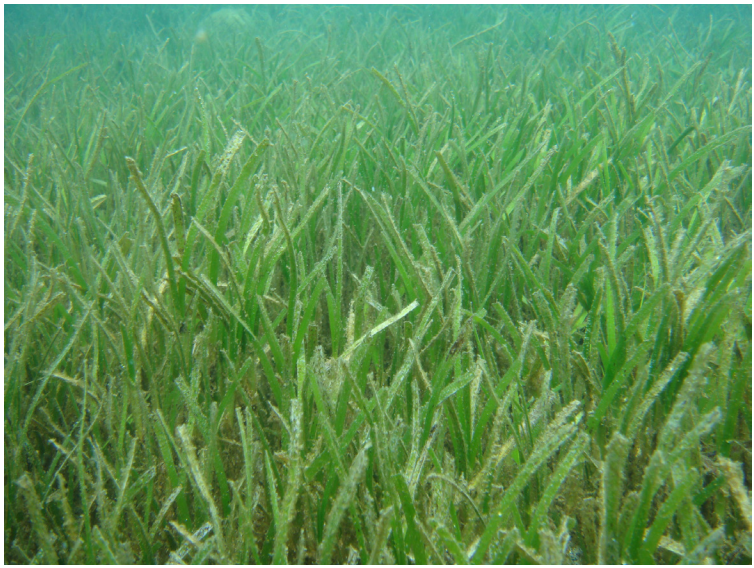

Fig. 4. *Halodule uninervis* in Bantayan beach Dumaguete City

Fig. 5. *Halodule pinifolia* in Bantayan beach Dumaguete City

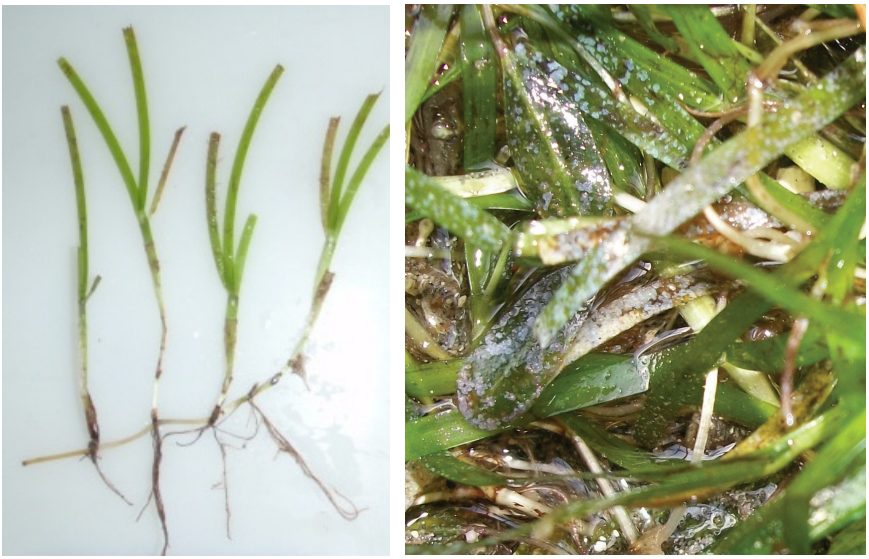

Fig. 6. *Halodule pinifolia* (Miki) den Hartog

Fig. 7. *Halodule uninervis* (Forsskål) Ascherson

CONCLUSION

The *rbc*L sequences showed that there was a difference between *H. uninervis* and *H. pinifolia* with a difference of two base pairs (bp) due to substitutes of A in *uninervis* to C and G *in pinifolia*. This 0.3 % divergence in a 671-base pair sequence of an *rbc*L marker confirms that *H. pinifolia* and *H. uninervis* are different species despite significant morphological variation in leaf width due to different sites and exposure.

LITERATURE CITED

Alberte, R.S., G.K. Suba, G. Procaccini, R.C. Zimmerman & S.R. Fain
1994 Assessment of genetic diversity of seagrass population using DNA fingerprinting: Implications for population stability and management. Proceedings National Academic Science USA 91: 1049-1053.

Altschul, D.T., W. Gish, W.E.W. Miller & D.J Lipman
1990 Basic local alignment search tool. *Journal of Molecular Biology* 215: 403-410.

den Hartog, C.
1970 The Seagrasses of the World. North Holland Publishing, Amsterdam pp. 275.

den Hartog, C. & J. Kuo
2006 Taxonomy and biogeography of seagrass. *In:* Seagrass Biology, Ecology and Conservation. A.W.D. Larkum, R.J.Orth and C.M. Duarte (eds.) Springer. Nederlands pp. 1-23.

Ito, Y. & N. Tanaka
2011 Hybridization of a tropical seagrass genus *Halodule* (Cymodocea) inferred from plastid and nuclear DNA phylogenies. Teleopela 13 (1-2): 219-231.

Laushman, R.H.

1993 Population genetics of hydrophilous angiosperm. Aquatic Botany 44: 147-158.

Les, D.H.
1988 Breeding system, population structure and evolution in hydrophylous angiosperms, Annual molecular Botanical Garden 75: 819-835.

Les, D. H. M.A. Cleland & M. Waycott
1997 Phylogenitic studies in Alismatidae, II-evolution of marine angiosperms (seagrasses) Hydrophily. *Systematic Botany* 22: 443-463.

McMillan, C.
1981 Morphologycal variation and isozymes under loboratory conditions in *Cymodocea serrulata*. Aquatic Botany 10: 356-370.

Reusch, T.B.H.,
2001 New Markers-old questions: Population genetics of seagrasses. Marine Ecology Progress Series 211: 261-274.

Reusch. T. B.H., W.T. Stam & J. L. Olsen
1999 Size and estimated age of genets in eelgrass *Zostera marina* L. assessed with microsatellite markers. *Marine Biology* 133: 519–525.

Ruckelshaus, M.H.
1996 Estimation of genetic neighborhood parameters from pollen and seed dispersal in the marine angiosperm *Zoostera marina* L. Evolution 50: 856-864.

Short, F.T., T.J. Carruthers, W.C. Dennison & M. Waycott.
2007 Global seagrass distribution and diversity: A bioregional model. www.co2science.org/articles/V14/N1/B2.php

Tamura, K., D. Peterson, N. Peterson, G .Stecher, M. Nei & S. Kumar
2011 MEGA 5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution (submitted). Available on line: http://www.kumarlab.net/publications

Triest, L.
1991 Enzyme polymorphism and its relationship to biological features in aquatic plants. *In*: Tries, L. (ed.). Isozymes in Water Plans. National Botanical Garden of Belgium, Belgium pp. 201-241.

Waycott, M.
1995 Assessment of genetic variation and clonality in the seagrass *Posidonia australis* using RAPD and allozyme analysis. *Marine Ecology Progress Series* 116: 289-295.

Waycott, M. & D.H. Les.
2000 Current perspectives on marine angiosperm evolution. *Biology of Marine Mediterranean* 7: 160–163.

Waycott, M., S.H. James & D.I. Walker
1997 Genetic variation within and between populations of *Posidonia australis*, a hydrophilous, clonal seagrass. *Heredity* 79: 408-412.

Waycott M., G. Procaccini, D.H. Les & T.B.H. Reusch
2006 Seagrass evolution, ecology and conservation: a genetic perspective. *In:* A.W.D. Larkum, R.J.Orth & C.M. Duarte (eds.) Springer. Seagrass Biology, Ecology and Conservation. Nederlands pp. 26-45.

Williams, S.L. & ,C.A. Davis.
1996 Population genetic analyses of transplanted eelgrass (*Zoostera marina*) beds reveal reduced genetic diversity in Southern California. *Restoration Ecology* 4: 63-180.

Zucarello, G.C., G. Burger, J.A. West & R.J. King
1999 A mitochondrial marker for red algal intraspecific relationships. *Molecular Ecology* 8: 1443-1447.