BULLETIN OF MARINE SCIENCE, 71(3): 1299–1308, 2002

EVOLUTIONARY TRENDS IN THE SEAGRASS GENUS *HALOPHILA* (THOUARS): INSIGHTS FROM MOLECULAR PHYLOGENY

Michelle Waycott, D. Wilson Freshwater, Robert A. York, Ainsley Calladine and W. Judson Kenworthy

ABSTRACT

Relationships among members of the seagrass genus *Halophila* (Hydrocharitaceae) were investigated using phylogenetic analysis of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA. The final aligned ITS sequence data set of 705 base pairs from 36 samples in 11 currently recognised species included 18.7% parsimony informative characters. Phylogenetic analysis yielded two most parsimonious trees with strong support for six groups within the genus. Evolutionary trends in *Halophila* appear to be toward a more reduced simple phyllotaxy. In addition, this study indicates that long distance 'jump' dispersal between major ocean systems may have occurred at least in the globally distributed *H. decipiens*. Results of ITS analyses also indicate that the widespread pacific species *H. ovalis* is paraphyletic and may contain cryptic species. Likewise, the geographically restricted species *H. hawaiiana* and *H. johnsonii* could not be distinguished from *H. ovalis* with these data and warrant further investigation.

Marine angiosperms have evolved to survive a particular set of environmental constraints (Arber, 1920; den Hartog, 1970; Larkum and den Hartog, 1989). A detailed molecular systematic analysis of the subclass Alismatidae which included all seagrass genera, demonstrated, that there have been at least three independent origins of the seagrasses in three major family groups, marine Hydrocharitaceae, Zosteraceae and a group containing the Cymodoceaceae, Posidoniaceae and Ruppiaceae (Les et al., 1997). Among these three groups, the marine members of the family Hydrocharitaceae (*Enhalus*, *Halophila* and *Thalassia*) are a tropical derivation from the other more wide-spread members of this family that are primarily freshwater aquatics. At present, the major seagrass lineages are assumed to have evolved primarily through vicariance and are an old group whose origins are in the Cretaceous (den Hartog, 1970; McCoy and Heck, 1976; Waycott, 2000). Improving our current understanding of seagrass evolutionary trends will benefit from more detailed studies of closely related taxa using a wide array of techniques (Waycott and Les, 2000).

As exemplified by their different pollen morphologies, the marine Hydrocharitaceae differ dramatically from other marine angiosperm lineages. Seagrasses in the Cymodoceaceae complex and Zosteraceae possess filamentous pollen lacking an exine. In contrast, the two hydrophilous marine Hydrocaritaceae genera (*Halophila* and *Thalassia*) have strings of spherical pollen embedded in mucilage (Les et al., 1997). The seagrass genus *Halophila* is distributed world wide, primarily in the tropics but it also occurs in south-eastern Africa and southern Australia (den Hartog, 1970). This genus is also one of the most speciose among seagrasses [13 species at present (Kuo and McComb, 1989)] and represents an exciting challenge to better understand the nature and origins of this diversity. Recent species descriptions have enhanced the sense that there are many undiscovered taxa in this genus (Greenway, 1979; Larkum, 1995). Several *Halophila* species have very wide geographic distributions such as *H. ovalis* (throughout the Indo-Pacific) or *H. decipiens* (global tropical waters), however several species have restricted distribu-

tions such as *H. johnsonii* (Florida, USA) or *H. hawaiiana* (Hawaii) (den Hartog, 1970; Walker and Prince, 1987; Kuo and McComb, 1989; Mukai, 1993). Of particular interest is the question of whether these distributions have arisen recently through 'jump' dispersal (i.e., dispersal between major ocean systems) or through long term historical processes such as vicariant evolutionary diversification.

The morphology of *Halophila* species is unique among seagrasses in having a petiolate leaf lacking a leaf sheath (den Hartog, 1970). The plants are diminutive and lack lignified tissue making them flexible but vulnerable to physical disturbance. Their low stature and flexibility make them ideal sediment surface inhabitants. In addition, many *Halophila* species are primary colonisers, being the first to enter areas of bare sand or disturbed seagrass beds (see den Hartog, 1970). Of particular interest, many *Halophila* species have an incredible tolerance range for temperature, salinity and water depth (den Hartog, 1970; Lee Long et al., 1996). The plants are rapidly growing, sometimes with sand-binding roots or at least many root hairs (den Hartog, 1970; Kuo, 1982; Kuo and McComb, 1989). *Halophila* species also form many tiny seeds which may be as small as 0.2 mm in diameter (Inglis, 2000; Kenworthy, 2000) and are produced throughout the year in tropical waters but more seasonally in temperate climes (den Hartog, 1970; Kuo et al., 1993).

The use of molecular phylogeny in the study of systematics and evolutionary biology has contributed significant insights into evolutionary processes and a deeper understanding of relationships among taxa at all levels of the biological hierarchy (Soltis and Soltis, 2000). Large DNA sequence data sets for conservative regions of both the nuclear and chloroplast genomes have been utilised in phylogenetic analyses to better develop our understanding of the major groups of angiosperms (Chase et al., 1993; Soltis et al., 2000). These large data sets have demonstrated that the Alismatales, (which includes all the seagrasses) are sister to the remaining monocotyledons. The position of the Alismatales at the base of the monocotyledons supports the notion that this is an old group. More rapidly evolving DNA sequences have been shown to be very useful at unravelling relationships among more closely related taxa. Among these the internal transcribed spacers (ITS) region of nuclear ribosomal DNA (nrDNA) has been utilised extensively in phylogenetic studies in many angiosperm families and has been particularly useful in improving our understanding of species relationships in these families (Baldwin et al., 1995).

In this study, we have utilized DNA sequences of the ITS region of nrDNA to infer relationships among species and biogeographic trends in the seagrass genus *Halophila*.

Methods

Collections and Selection of Taxa.—Vegetative material, whole leaves and/or shoot meristems, of eleven of the currently described *Halophila* species (Table 1) were placed and stored in self-indicating silica gel desiccant (Sigma-Aldrich Cat# 85344) immediately after field collection. Dried shoots were stored in the desiccant at room temperature or in the refrigerator until use. Collections were obtained from as wide a geographic distribution as possible and identification of taxonomic status was made using the taxonomic keys of (den Hartog, 1970; Phillips and Meñez, 1988; Kuo and McComb, 1989) and local keys where available. Where possible voucher herbarium samples have been collected and will be lodged at recognised herbaria (contact the authors for details).

DNA Extractions.— DNA extractions were done using the Qiagen Dneasy Plant Mini Kit or as described here. A small amount of leaf tissue was ground in a microfuge tube with acid-washed sand and 500 μL SDS grinding buffer similar to Huff et al. (1993) but with 1% SDS, 1% PVP-40 and 1% PVP-360 and 5M potassium acetate was added to make up 10% of the buffer volume and

mixed well. The mixture was incubated at 65°C for 45 min and then placed on ice for 10 min. Cell debris was removed by microcentrifugation (3 min at 11,500 g) and the clear, aqueous supernatant was placed into another tube. DNA was extracted in one volume of isoamyl alcohol:chloroform (1:24). After mixing well and centrifuging at 11,500 g for 3 min, the top, aqueous layer was separated into a clean tube. DNA was precipitated from the supernatant by adding an equal volume of isopropanol and placing at -20°C for at least 1 h or overnight. The DNA was pelleted by microcentrifugation (3 min at 11,500 g), washed once in 500 ml 70% ethanol (the tube was shaken, the pellet dislodged from the bottom of the tube and then centrifuged to re-pellet) and then alcohol was poured off and excess alcohol removed from the pellet by air-drying. Following resuspension of the DNA in 100 μl of sterile TE (100 mM Tris, 5 mM Na_2EDTA), the DNA was further purified using a glassmilk purification protocol (MoBio™ UltraClean-15 DNA purification kit), the final elution being made at 65°C for 15 min. DNA quality and approximate concentration were assessed by agarose gel electrophoresis.

In one instance, *Halophila ovalis* from Shoalwater Bay, Australia, DNA was extracted from a herbarium specimen. A clean, healthy looking specimen was chosen and one apical meristem removed. DNA was extracted as above to the first precipitation step. At this stage, the DNA (which appeared brown, unusual for *Halophila* DNA extractions which are most often bright yellow) was run on a low melting agarose gel and the high molecular weight DNA band was excised before the glassmilk purification protocol as above. This was to recover high quality DNA from the large proportion of degraded DNA in this sample due to the slowness of drying and storage in less than optimal environments.

PCR of ITS Region and Analysis.—The region of the genome being analysed in this study is the nuclear ribosomal DNA internal transcribed spacers (ITS) which comprises the region between the 18S rDNA and the 26S rDNA including ITS-1, 5.8S nrDNA and ITS-2 (White et al., 1990). PCR reactions were conducted using PCR primers that amplify from the 3′ end of the 18S rDNA [primer 18S of Baldwin (1992) or ITS-1 of White et al. (1990)] and the 5′ end of the 26S rDNA [ITS-4 of White et al. (1990)] in 25 μl total volume using 1 unit QIAGEN Taq DNA polymerase per reaction, 2.5 μl 10X PCR buffer (including $MgCl_2$), 1 μl 50 mM $MgCl_2$, 0.4 μl 10 mM dNTPs. PCR amplification was performed using the following profile: 2 min at 96°C; 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C; with a final extension time of 10 min at 72°C. PCR products were separated by agarose gel electrophoresis using low melting temperature agarose, the bands excised and purified using a QIAQuick Gel Purification kit and PCR product concentration was determined by visual comparison with known amounts of DNA on an agarose gel. DNA sequencing reactions were carried out using ABI BigDye or dRhodamine Dye Terminator cycle sequencing reactions in 10 μl total volume. DNA sequences were run on ABI Prism 377 DNA autosequencer, chromatograms were manually edited. Sequences were readily manually aligned using Se-Al (Rambut, 1995). Final edited sequences have been submitted to Genbank (http://www.ncbi.nlm.nih.gov/), accession numbers are presented in Table 1.

Phylogenetic analyses of DNA sequence data under parsimony and maximum likelihood criteria were conducted using PAUP (v. 4.0b4a, Swofford, 2000). Sites where gaps were required to maintain the alignment of sequences were excluded from analyses. Minimal parsimony trees were found using a heuristic search protocol with simple taxon addition, tree-bisection-reconnection (TBR) branch swapping, and saving all most parsimonious trees (MULPARS). Support for nodes on the resulting parsimony trees was determined by bootstrap analysis consisting of 500 replications of heuristic searches with simple taxon addition, TBR and MULPARS. Likelihood analyses were done by initially determining the transition:transversion ratio (tn:tv) that maximized the log-likelihood value by plotting a range of tn:tv against the corresponding inferred log-likelihoods. The determined value was 1.7 and this was used in all subsequent maximum likelihood analyses. The maximum likelihood analysis of the ITS data was replicated 10 times using empirical base frequencies, random orders of sequence addition, and TBR branch swapping. The maximum likelihood tree was bootstrapped using 100 resamplings of a single random addition of taxa and TBR branch swapping. Trees were rooted using *Halophila engelmanii* and *H. beccarii* as outgroups based on

preliminary analyses of conserved, readily alignable ITS regions only, that included *Thalassia* as the outgroup (data not shown). Trees resulting from rbcL sequence analyses including a limited sampling of *Halophila* species and multiple outgroup taxa showed the same polarity as that presented here (Freshwater, unpubl. data).

Results

PCR products were approximately 750 base pairs in length, which included short end fragments of the 18S and 26S nrDNA. The final aligned ITS sequence data set was of 705 base pairs from 36 samples in 11 currently recognised species (Table 1). After excluding 133 positions where indels were coded, 164 of the remaining positions were variable (28.6%) and 107 of these were parsimony informative (18.7%). Phylogenetic analysis yielded two most parsimonious trees (Fig. 1), of 265 steps (CI excluding uninformative characters = 0.710, retention index (RI) = 0.886, rescaled consistency index (RC) = 0.685). These two trees from this analysis were virtually identical in topology and differed only in the within *H. ovalis* group branching. Alternate analysis conducted using the data set with gaps included and treated as missing data yielded 36 most parsimonious trees of virtually identical topology, but requiring more steps (L = 345 steps). Bootstrap analysis indicated high support for all major clades within the phylogram (Fig. 1).

The 10 replicate maximum likelihood analyses all found the same tree (LnLi = -2215.89505), which was identical to one of the two most parsimonious trees. Maximum likelihood bootstrap values were congruent to those found in the parsimony analyses except that the clade containing *H. engelmanii* and *H. beccarii* was only poorly supported (66% maximum likelihood, 96% parsimony). The maximum likelihood bootstrap analysis also indicated good support for a monophyletic *H. spinulosa* within the *H. spinulosa*/*H. tricostata* clade.

Two major goups are identified in this analysis (Fig. 1). *Halophila engelmanii, H. beccarii, H. tricostata* and *H. spinulosa*, are resolved within two lineages of species with complex phyllotaxy that are basal to the clade containing *H. decipiens*, *H. stipulacea*, *H. ovalis*, *H. minor*, *H. johnsonii* and *H. hawaiiana*, all species with simple phyllotaxy.

The *H. decipiens* clade, seven samples from the Caribbean and Indo-Pacific, was not resolved into different geographically distinct groups. Only 5 differences were found among these sequences: 2 transitions and a 2 base deletion in samples from Malaysia and Gladstone compared to the others; a transversion between the Caribbean and Dunk Island samples and others, and a transversion unique to the Curaçao sample.

H. stipulacea was resolved at the base of the *H. minor*, *H. australis*, *H. ovalis*, *H. johnsonii* and *H. hawaiiana* complex (the *H. ovalis* 'complex' of den Hartog, 1970). The large *H. ovalis* 'complex' includes three, well supported, clades. One clade contains samples of *H. minor* from Guam and the Philippines that differed by only 2 transitions. A second clade contains *H. australis*, samples of *H. ovalis* from the Philippines and one other *H. ovalis* with unusual leaf form from Shoalwater Bay on the southern Queensland coast (R. Coles, pers. comm.). Among these 4 samples there were 12 transitions and 3 transversions. The third clade contains the majority of *H. ovalis* samples plus the geographically restricted *H. hawaiiana* and *H. johnsonii*. Sequence differences in this clade consisted of 5 transitions and 15 transversion, but most of these differences (1 transition, 13 transversions) were unique to the sample from Gia Luan in Vietnam. *H. johnsonii* was indistinguishable from the remainder of the Australian/Malaysian/Japan *H. ovalis* group. *H. hawaiiana* had

Table 1. Species in the seagrass genus *Halophila*, their distribution and localities of samples utilised in this study (data collated from den Hartog, 1970; Phillips and Meñez, 1988; Kuo and McComb, 1989). Genbank accession numbers in parentheses.

Species	General distribution	Localities of collections (Genbank accession numbers of ITS sequences)
Section *Halophila*		
H. australis Doty & Stone	Southern Australia	Two Peoples Bay, southwestern Australia (AF366414)
H. capricorni Larkum	Tropical north-eastern Australia, Coral Sea	Not obtained for this study
H. decipiens Ostenfeld	Tropics worldwide	Caribbean Indian River, Florida, USA (AF366407); southeastern Costa Rica (AF366409); Bocas del Toro, Panama (AF366408); Curacao (AF366413) Indo-Pacific Dunk Island (AF366411), Gladstone Harbour (AF366410), Australia; Kuala Setiu, Malaysia (AF366412)
H. ovalis (R. Br.) Hook. f	Tropics, Indo-Pacific, temperate Australia and Africa	Australia Silvers Beach (AF366423), Towra (AF366422), Narrabeen Lake (AF366428)-Sydney Moreton Bay (AF366418), Gladstone Harbour (AF366424), Shoalwater Bay (AF366415) -S Queensland Pioneer Bay (AF366434), Dugong Inlet (AF366430), Dingo Beach (AF366431)-Whitsundays Queensland Upstart Bay (AF366435), Cape Cleveland (AF366429), Picnic Bay (AF366427 and AF366433), Herald West-Rattlesnake Island (AF366432)-N Queensland Central & North Indo-Pacific Makadong (AF366417), Lucerno (AF366419), Hilutangan (AF366416)-Philippines; Kuala Setiu, Malaysia (AF366420); Gia Luan, Vietnam (AF366437); Okinawa, Japan (AF366421).
H. hawaiiana Doty & Stone	Hawaii	Oahu, Hawaii (AF366426).
H. johnsonii Eiseman	Florida, USA	Sebastian Inlet, Florida, USA (AF366425).
H. minor (Zollinger) den Hartog	Tropical Indo-Pacific	Guam (AF366405); Zamboanga, Philippines (AF366406).
H. stipulacea (Forsskal) Ascherson	Western Indian Ocean, Mediterranean invasion	Sicily, Italy (AF366436).
Section Microhalophila		
H. beccarii Ascherson	South China Sea, Bay of Bengal	Gia Luan, Vietnam (AF366441)
Section Spinulosae		
H. spinulosa (R. Br.) Ascherson	Tropical northern Australia, Malaysia, New Caledonia	Whitsunday Island, Australia (AF366439); Pulau Perhentian, Malaysia (AF366440).
Section Trichostata		
H. trichostata Greenway	Tropical northeastern Australia	Whitsunday Island, Australia (AF366438).
Section Americanae		
H. englemanii Ascherson	Caribbean	Florida Bay, USA (AF366404).
H. baillonis Ascherson	Caribbean, Brazil	Not obtained for this study.

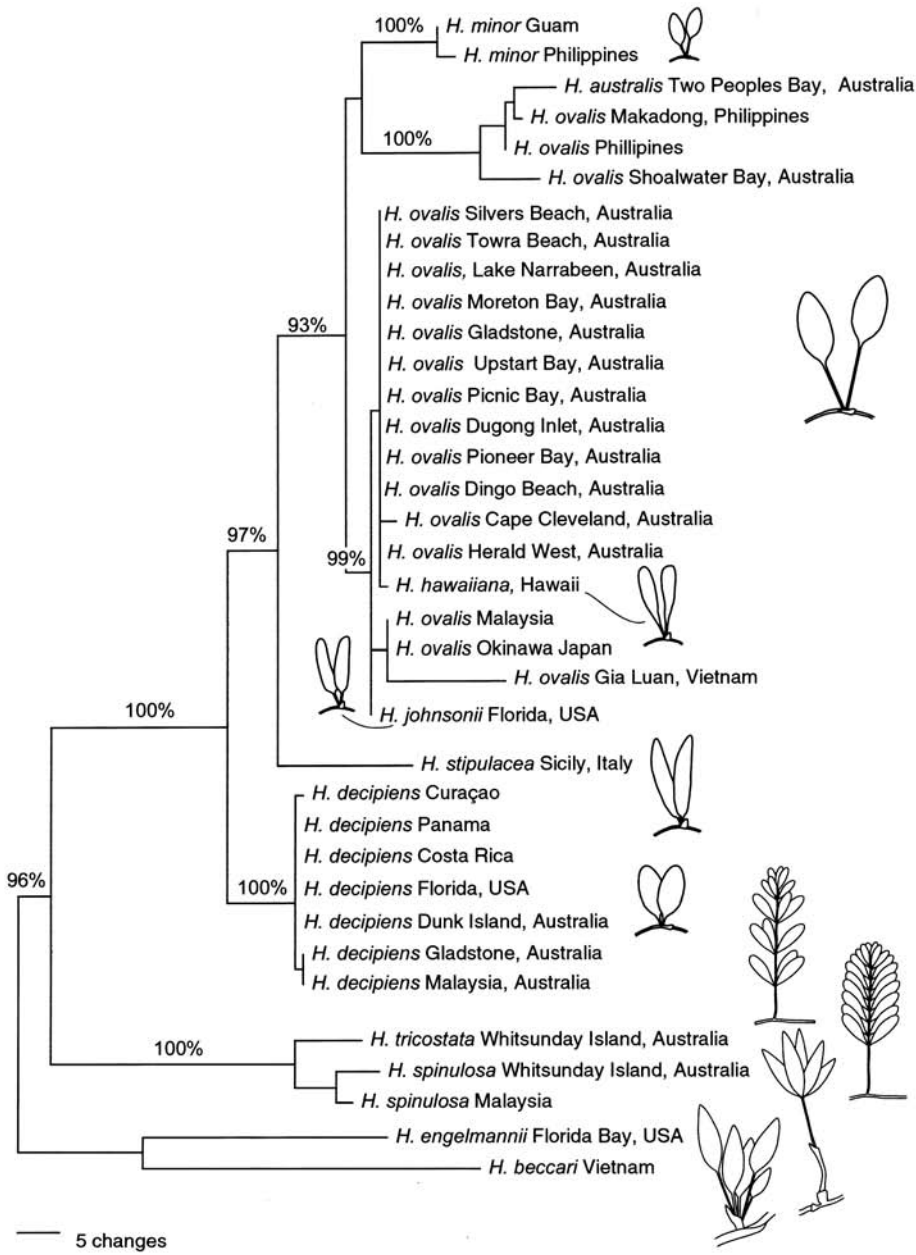

Figure 1. One of two most parsimonious phylograms from a heuristic search of a 705 base pair alignment of nuclear ribosomal internal transcribed spacers (ITS) DNA sequences. Bootstrap support (percentages) over 50% are shown above branches. Taxa with unresolved polytomies are arranged south–north (the order is not representative of relationships). Line drawings represent a generalised morphology of each species or group (based on information in den Hartog, (1970); Phillips and Meñez, (1988); Kuo and McComb, (1989) and pers. observ.).

only one transition difference as compared to the Australian/Malaysian/Japan *H. ovalis* group. It should be noted that DNA sequences for these two samples were obtained independently (the Australian/Asian samples at JCU in Australia or at STRI in Panama whereas the samples of *H. johnsonii* and *H. hawaiiana* were extracted and sequenced at UNC-Wilmington, USA precluding any chance of contamination). The range of leaf morphologies observed among the *H. ovalis* samples and of *H. hawaiiana* and *H. johnsonii*, was extreme from diminutive (<0.5 cm) to large robust (>5 cm) leaves.

Discussion

This analysis yielded a high proportion of informative sites (excluding indels) and resulted in well resolved major groups of species within the genus. The high level of support for these clades indicates that ITS sequence analysis may be useful for investigations of sub-generic relationships within other seagrass genera. The unresolved relationships within clades, mostly of widespread species, indicate that either resolution among closely related taxa is poor or, alternatively, that current species circumscriptions need revision. The phylogram demonstrates that the basal groups in this genus belong to the more structurally complex species (*H. engelmanii*, *H. beccarii*, *H. trichostata* and *H. spinulosa*, Fig. 1) whose distribution includes both the Indo-Pacific and the Caribbean. Analyses including outgroup taxa are required to determine if species with more complex phyllotaxy are monophyletic. Such analyses would require the use of a more conservative region of DNA, and our work in progress with the chloroplast-encoded *rbc*L should answer this question. The occurrence of species in both the Indo-Pacific and Caribbean represents possible vicariant speciation (see McCoy and Heck, 1976) although empirical data is needed to resolve this question.

The more derived lineage in *Halophila* contains species with only two leaves per shoot (Fig. 1). This increasingly simple phyllotaxy might be seen as a particularly successful evolutionary strategy since these species demonstrate very wide geographic, salinity and depth range distributions. Although they show simple phyllotaxy, morphological diversity among and within these species is high and represents a particular challenge in understanding the link between morphological plasticity, genotypic diversity and speciation. Den Hartog (1970) points out that among the *H. ovalis* 'complex', there is an unusual range of morphological variability that differs across the range of the species. He goes on to emphasise the need for detailed study of this group as a whole to better understand the link between morphological variability, environmental parameters (light, sediment type, nutrients etc.) and the possibility that some of the variation may be genotypically determined. Such an investigation would be of particular value, especially if it included the broadest range of taxa and geographic regions as possible.

Halophila decipiens, is perhaps the only seagrass species with a truly global distribution other than *Zostera marina* (den Hartog, 1970). This distribution must have arisen in recent geological history as there is virtually no difference in the sequences between samples from the Caribbean and from around the Indo-Pacific. The lack of differentiation on such a wide scale suggests recent 'jump' dispersal, the direction of which can not be ascertained in this analysis. Such evidence of 'jump' dispersal is the first indication that seagrasses have the capacity to survive long distance dispersal between ocean systems. The possible method of long distance dispersal remains obscure, perhaps *Halophila* spe-

cies, with their tiny seeds, can become entangled in larger drift mats of alga or other vegetation and relocate as 'hitchhikers'.

At the base of the *H. ovalis* 'complex' clade is the western Indian Ocean species *H. stipulacea*. This species shares the serrated leaf margins and sometimes-hairy leaves with *H. decipiens* but in this analysis appears to be more derived than *H. decipiens* (Figure 1). *H. capricorni*, another species that has serrated leaf margins and leaf hairs, has not been included in this analysis.

The largest group of specimens sampled in this study were in the *H. ovalis* 'complex' (Table 1) and within this group there are three clearly identified clades. *H. minor* appears as one clade, *H. hawaiiana* and *H. johnsonii*, with *H. ovalis* from Australia, Malaysia, Japan and Vietnam form another clade and *H. australis*, *H. ovalis* from the Phillipines and from Shoalwater Bay in Australia form the third. There were few sequence differences between the two *H. minor* specimens analysed but many differences to the remainder of the *H. ovalis* group supporting the notion that they are a separate species. An unusual grouping is the *H. australis* clade which combines samples from Australia and the Philippines. This group demonstrates paraphyly in specimens identified as *H. ovalis* and that cryptic species occur. Among the main group of *H. ovalis* (including *H. hawaiiana* and *H. johnsonii*) the lack of DNA sequence variation, despite the morphological variation, supports the notion that members of this group are either very closely related or the same taxon.

This study indicates that the ITS region of nrDNA is useful at differentiating major groups within *Halophila* and will be informative for investigating relationships within other seagrass genera. These data show that evolutionary trends in *Halophila* are towards a more reduced, simple phyllotaxy and that long distance 'jump' dispersal has occurred at least in *H. decipiens*. In addition, this data set provides evidence for the presences of cryptic species in *H. ovalis*, but no support for the differentiation of *H. hawaiiana* and *H. johnsonii* from the *H. ovalis* complex. Sequence data from a less conserved DNA region is presently being generated to better resolve the close relationships among these taxa. Data for *H. capricorni* and *H. baillonis* are also needed to attain a complete picture of evolution in this genus.

Acknowledgments

The authors would like to thank the following people for contributing samples: E. Serràò, G. Inglis, H. Marsh, W. H. C. F. Kooistra, S.-M. Phang and D. W. Ginsburg. Also G. Procaccini for the ITS sequence of *H. stipulacea* from Italy. M.W. was supported by a James Cook University Postdoctoral Fellowship, a Mellon Foundation Postdoctoral Fellowship at the Smithsonian Tropical Research Institute, an ARC Large Grant and an ARC Postdoctoral Fellowship. D. W. F. and R. A. Y. were supported by a grant from NOAA-CIFO and NSF DEB-9726170 with REU supplement. W. J. K. received funding from NOAA.

Literature Cited

Arber, A. 1920. Water plants: A study of aquatic angiosperms. London, Cambridge University Press.

Baldwin, B. G. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. Mol. Phyl. Evol. 1: 3–16.

_____, M. J. Sanderson, J. M. Porter, M. F. Wojciechowski and M. J. Donoghue. 1995. The ITS region of nuclear ribosomal DNA–a valuable source of evidence on angiosperm phylogeny. Ann. Mo. Bot Gard. 82: 247–277.

Chase, M. W., Soltis, D. E., Olmstead, R. G., Morgan, D., Les, D. H., Mishler, B. D., Duvall, M. R., Price, R. A., Hills, H. G., Qiu, Y. L., Kron, K. A., Rettig, J. H., Conti, E., Palmer, J. D., Manhart, J. R., Sytsma, K. J., Michaels, H. J., Kress, W. J., Karol., K. G., Clark, W. D., Hedren, M., Gaut, B. S., Jansen, R. K., Kim, K. J., Wimpee, C. F., Smith, J. F., Furnier, G. R., Strauss, S. H., Xiang, Q-Y., Plunkett, G. M., Soltis, P. S., Swensen, S. M., Williams, S. E., Gadek, P. A., Quinn, C. J., Eguiarte, L. E., Golenberg, E., Learn Jr., G. H., Graham, S. W., Barrett, S. C. H., Dayanandan, S. and Albert, V. A. 1993. Phylogenetics of seed plants - an analysis of nucleotide sequences from the plastid gene rbcL. Ann. Mo. Bot Gard. 80: 528–580.

den Hartog, C. 1970. The sea-grasses of the world. Amsterdam, North-Holland Publishing Company.

Greenway, M. 1979. *Halophila tricostata* (Hydrocharitaceae), a new species of seagrass from the Great Barrier Reef region. Aquat. Bot. 7: 67–70.

Inglis, G. J. 2000. Variation in the recruitment behaviour of seagrass seeds: implications for population dynamics and resource management. Pacif. Cons. Biol. 5: 251–259.

Kenworthy, W. J. 2000. The role of sexual reproduction in maintaining populations of *Halophila decipiens*: implications for the biodiversity and conservation of tropical seagrass ecosystems. Pacif. Cons. Biol. 5: 260–268.

Kuo, J. 1982. Notes on the biology of Australian seagrasses. Proc. Linn. Soc. N.S.W. 106: 225-245.

_____, W. Lee Long and R. G. Coles. 1993. Occurrence and fruit and seed biology of *Halophila tricostata* Greenway (Hydrocharitaceae). Aust. J. Mar. Freshw. Res. 44: 43–57.

_____ and A. J. McComb. 1989. Seagrass taxonomy, structure and development. Pages 6–73 *in* A. W. D. Larkum, A. J. McComb and S. A. Shepherd, eds. Biology of the seagrasses: A treatise on the biology of seagrasses with special reference to the Australian region. Elsevier, Amsterdam.

Larkum, A. W. D. 1995. *Halophila capricorni* (Hydrocharitaceae): a new species of seagrass from the Coral Sea. Aquat. Bot. 51: 319–328.

_____ and C. den Hartog. 1989. Evolution and biogeography of seagrasses. Pages 112–156 *in* A. W. D. Larkum, A. J. McComb and S. A. Shepherd, eds. Biology of the seagrasses: A treatise on the biology of seagrasses with special reference to the Australian region. Elsevier, Amsterdam.

Lee Long, W. J., R. G. Coles and L. J. McKenzie. 1996. Deepwater seagrasses in northeastern Australia-how deep how meaningful? Pages 41–50 *in* J. Kuo, R. C. Phillips, D. I. Walker and H. Kirkman, eds. Seagrass biology: Proc. Int'l workshop, Rottnest Island, Western Australia, 25–29 January 1996. Faculty of Sciences, The University of Western, Perth, Australia.

Les, D. H., M. A. Cleland and M. Waycott. 1997. Phylogenetic studies in the monocot subclass Alismatidae II: evolution of marine angiosperms ("seagrasses") and hydrophily. Syst. Bot. 22: 443–463.

McCoy, E. D. and K. L. Heck. 1976. Biogeography of corals, seagrasses and mangroves: an alternative to the centre of origin concept. Syst. Zool. 25: 201–210.

Mukai, H. 1993. Biogeography of the tropical seagrasses in the Western Pacific. Aust. J. Mar. Freshw. Res. 44: 1–17.

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

Rambut, A. 1995. Se-Al, Sequence Alignment Program. v. 1.d1.

Soltis, D. E., P. S. Soltis, M. W. M. E. Chase, Mort, D. C. Albach, M. Zanis, V. Savolainen, W. H. Hahn, S. B. Hoot, M. F. Fay, M. Axtell, S. M. Swensen, L. M. Prince, W. J. Kress, K. C. Nixon, and J. S. Farris. 2000. Angiosperm phylogeny inferred from 18S rDNA, *rbc*L, and *atp*B sequences. Bot. J. Linn. Soc. 133: 381–461.

_____ and P. S. Soltis. 2000. Contributions of plant molecular systematics to studies of molecular evolution. Pl. Mol. Biol. 42: 45-75.

Swofford, D. L. 2000. PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, Massachusetts, Sinauer Associates.

Walker, D. I. and R. I. T. Prince. 1987. Distribution and biogeography of seagrass species on the northwest coast of Australia. Aquat. Bot. 29: 19–32.

Waycott, M. 2000. Mating systems and population genetics of marine angiosperms (seagrasses). Pages 277–285 *in* K. L. Wilson and D. Morrison, eds. Monocots: Systematics and Evolution. Sydney, CSIRO Publishing. Vol 1.

_____ and D. H. Les. 2000. Current perspectives on marine angiosperm evolution. Biol. Mar. Med. 7: 160–163.

White, T. J. B. T., S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315–324 *in* M. A. Innes, D. H. Gelfand and T. J. White, eds. PCR Protocols: A guide to methods and applications, Academic Press.

ADDRESSES: (M.W.,A.C.) *School of Tropical Environment Studies, Geography and Tropical Biology, James Cook University, Townsville 4811 Australia.* (M.W.) *Smithsonian Tropical Research Institute, Apartado 2072, Balboa Ancon, Republic of Panama*. (D.W.F.,R.A.Y.) *Center for Marine Science, University of North Carolina at Wilmington, 5600 Marvin Moss Lane, Wilmington, North Carolina 28409.* (W.J.K.) *Center for Coastal Fisheries and Habitat Research, NOAA, 101 Pivers Island Road, Beaufort, North Carolina 28516.* CORRESPONDING AUTHOR: (M.W.) *School of Tropical Biology, James Cook University, Townsville 4811 Australia. Tel. + 617 4781 5246, Fax + 617 4725 1570. E-mail: <michelle.waycott@jcu.edu.au>*.