Reproductive features of Hawaiian *Halimeda velasquezii* (Bryopsidales, Chlorophyta), and an evolutionary assessment of reproductive characters in *Halimeda*

Peter S. VROOM[a]* & Celia M. SMITH[b]

[a]Coral Reef Ecosystem Division, NOAA Fisheries Honolulu Laboratory, Kewalo Basin Research Facility, 1125B Ala Moana Bvld., Honolulu, HI 96814, USA

[b]Department of Botany, University of Hawaii at Manoa, 3190 Maile Way, Honolulu, Hawaii 96822, USA

Abstract — Reproductive features form the basis of many taxonomic and phylogenetic classification schemes, yet their relative obscurity among many green algae has led researchers to form evolutionary hypotheses based primarily on vegetative features. Here we describe reproductive characters of *Halimeda velasquezii* for the first time and assemble descriptive information for 19 other species of *Halimeda* to assess the importance of reproductive structures for delineation of species and phylogenetic hypotheses within the genus. Multivariate analyses reveal reproductive structures of sand-dwellings species to form a group distinct from at least one other evolutionary lineage within the genus, suggesting that reproductive characters may be evolutionarily informative. Gametophore length appears to be the most phylogenetically informative character, accounting for over 75 % of differences between lineages. Most *Halimeda* species with multiple descriptions of reproductive characters exhibit widely divergent, nonoverlapping size characteristics between geographic locations. This may be explained by possible nonmonophyly of historical species from distant areas or specimens having been collected at different developmental stages.

Evolution / *Halimeda velasquezii* / Hawaii / phylogeny / PRIMER / reproduction / taxonomy

Résumé — **Caractéristiques de la reproduction de *Halimeda velasquezii* (Bryopsidales, Chlorophyta) d'Hawaii et évaluation évolutioniste des caractères de la reproduction chez *Halimeda*.** Les caractéristiques de la reproduction forment la base de nombreux schémas de classification taxinomique et phylogénétique, cependant leur relative obscurité chez les algues vertes a conduit les chercheurs à formuler des hypothèses d'évolution basées d'abord sur les formes végétatives. Nous décrivons, ici pour la première fois, les caractères de la reproduction d'*Halimeda velasquezii* et, en réunissant les informations pour 19 autres espèces d'*Halimeda*, nous montrons l'importance des structures de la reproduction dans la délimitation des espèces et dans l'élaboration d'hypothèses phylogénétiques à l'intérieur du genre. Des analyses multivariables des structures de la reproduction révèlent que les espèces du sable forment un groupe distinct des autres lignées du genre, confirmant que les caractères de la reproduction peuvent donner des informations sur l'évolution. La longueur du gamétophore semble être le caractère le plus informatif sur l'évolution, en effet il permet d'expliquer plus de 75 % des différences entre les lignées. Les nombreuses descriptions de caractères de la reproduction de la plupart des espèces d'*Halimeda* montrent de grandes divergences, les mesures des caractères ne se recouvrant pas d'un lieu géographique à l'autre. Ceci peut être expliqué par la para- ou la polyphylie des espèces historiques provenant d'aires éloignées ou par des spécimens ayant été collectés à différents stades de développement.

Evolution / *Halimeda velasquezii* / Hawaii / phylogénie / PRIMER / reproduction / taxinomie

---

* Correspondence and reprints: Peter.Vroom@noaa.gov

INTRODUCTION

In the green algal order Bryopsidales, current species and genus descriptions are based almost entirely on vegetative characters (Gepp & Gepp, 1911; Hillis-Colinvaux, 1984; Littler & Littler, 1990; Vroom *et al.* 1998). However, recent research has documented reproduction in many genera of siphonous green algae, and has found widely divergent reproductive structures and gamete types within and among historical taxa (Clifton & Clifton, 1999). Similarly, molecular data indicate that well-established genera such as *Udotea*, *Penicillus*, and *Chlorodesmis* are polyphyletic (Kooistra, 2002). Clearly, taxonomic groupings within the Bryopsidales are artificial and need re-evaluation. As with the red algae (Schmitz & Hauptfleisch, 1896), reproductive characters appear to be valid phylogenetic indicators at the genus level that can be used to delimit natural assemblages within the siphonous green algae. What, however, is their usefulness in distinguishing relationships within a genus? Can reproductive features be reliable characters that separate species, or do vegetative characters continue to provide the best resolution at the species level?

Cues that trigger reproduction in *Halimeda* are unknown, but in all species where reproductive structures have been observed, gametes form in multiple clusters of gametangia (termed compound reproductive structures). Although similar structures have been reported for other siphonous genera within the Bryopsidales (e.g. *Chlorodesmis* and *Caulerpella*; see Vroom *et al.*, 1998 for review), the unique morphology of structures in *Halimeda* clearly separate this genus from other evolutionary lineages. Reproductive structures are valuable characters useful for taxonomic purposes at a species level within *Halimeda*, but further studies are needed to determine if they are phylogenetically informative or if they delineate species relationships.

Vegetatively, species of the genus *Halimeda* are characterized by series of calcified segments. The genus appears monophyletic based on molecular and morphological characters (Hillis *et al.*, 1998; Kooistra *et al.*, 1999, 2002). Although reproductive structures are described for 21 of the 33 species, no previous phylogenetic studies have included these structures in their analyses (Hillis *et al.*, 1998; Kooistra *et al.*, 2002). In all cases where reproductive structures have been found, noncalcified, external, compound gametangia develop rapidly along the margins or through the surface of calcified segments. A central stalk, termed a gametophore, bears clusters of globose gametangia (Hillis-Colinvaux, 1980). Specialized discharge tubes (papillae) quickly expel gametes once reproductive cues have been perceived (Drew & Abel, 1988). Where observed, gametangia generally form within a 24-hour period, gametes are released in a synchronized manner across entire populations often slightly before dawn (Drew & Abel, 1988), and adult plants break down rapidly and disappear after reproduction (Clifton, 1997).

Halimeda velasquezii Taylor, a species that has been excluded from previous phylogenetic studies of the genus (Hillis *et al.*, 1998; Kooistra *et al.*, 1999, 2002), is among the most common algae found in the Northwestern Hawaiian Islands (NWHI; PSV, personal observation). Because this species has never been described from a reproductive standpoint, the goals of this study were to 1) scientifically describe reproductive structures of *Halimeda velasquezii* for the first time, 2) assemble descriptions of reproductive structures in *Halimeda* from primary literature in order to compare those of *H. velasquezii* to other species in the genus, and 3) assess the phylogenetic importance of reproductive structures within the genus.

MATERIALS AND METHODS

Reproductive *Halimeda velasquezii* were observed and collected by scuba divers as part of rapid ecological assessment (REA) field surveys during 2000 and 2002 research expeditions to the NWHI. Material was frozen at –20°C and brought to the marine macrophytes laboratory at the University of Hawai`i at Manoa in Honolulu. Species identification was confirmed by decalcifying segments and examining nodal regions, utricles, and segment cross-sections microscopically following Hillis-Colinvaux (1980). Microscope preparations of reproductive structures were stained with 1% aniline blue and mounted on a slide with 50 % Karo® syrup: 50 % water. Slide preparations were examined under an Olympus BX-41 compound microscope and photographed with an Olympus DP-11 digital camera. Photographs were printed on a Hewlett Packard 4500-N color laser jet printer and used for enumeration and measurement of reproductive and vegetative features. The remainder of each plant was pressed following standard protocols (see *http://www.nmnh.si.edu/botany/projects/algae/Alg-CoPr.htm*; accessed 10 July, 2003). Voucher specimens of reproductive individuals (IAA27396, IAA27448, IAA27520, PSV20007) are currently housed in the phycology laboratory at the NOAA Fisheries' Coral Reef Ecosystem Division, and are slated for eventual deposition in the Bishop Museum, Honolulu, Hawai`i.

One gametophore (with associated gametangia) from each of 9 specimens was haphazardly selected for analysis (Fig. 1). To determine gametangial and gametophore widths, measurements were made at the widest section of each structure. Gametophore length was measured from where the gametophore attached at the secondary utricle to the tip of the most distal discharge papilla. Because of the branched nature of the compound gametangia, this sometimes required adding the length of two or more branches together (Fig. 1). Descriptive statistics were calculated using Minitab for Windows, ver. 12.1.

Reproductive characters of *Halimeda* species were collected from published and unpublished sources. Many older manuscripts contained drawings and photographs of reproductive material, but these were poorly described in the text. To compare these studies in a meaningful way, drawings were measured to determine approximate sizes of gametangia and gametophores. Size ranges and averages were determined when multiple structures were pictured, but standard errors were not calculated because of the secondary nature of measurements and lack of replication. Species in Tab. 1 were arranged in the same order as presented in Fig. 1 of Kooistra *et al.* (2002) to indicate possible phylogenetic relationships.

To determine if reproductive structures could be used to define evolutionary lineages within *Halimeda*, species where complete descriptions of reproductive characters were found were grouped into molecular-based phylogenetic lineages (Kooistra *et al.*, 2002) for multivariate analysis using Primer software (Clarke & Warwick, 2001). Ordination of lineages was determined by multidimensional scaling (MDS; number of restarts = 30) and differences among lineages compared through analysis of similarities (ANOSIM; maximum permutations = 5000). Similarity percentages (SIMPER) were calculated to determine the importance of individual characters in separating lineages. Additionally, mean sizes of reproductive characters (or median size if only a range is known) were averaged for each record within molecular-based phylogenetic lineages for gross lineage comparison. To determine if gamete size was of phylogenetic importance among lineages, species where gametes had been reported were grouped into molecular-based phylogenetic lineages (Kooistra *et al.*, 2002) for the same types of multivariate analysis described above.
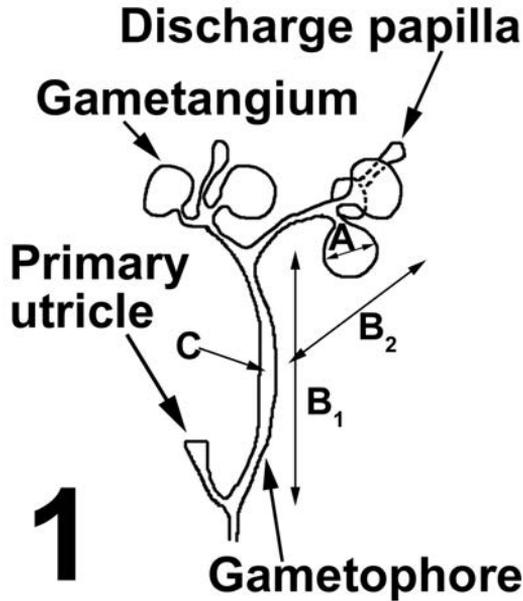
Fig. 1. Schematic diagram of *Halimeda velasquezii* gametophore indicating location of measurements. A. Widest section of gametangium; B_1 & B_2. Lengths added together to attain total length of gametophore; C. Widest section of gametophore.

RESULTS

Halimeda velasquezii

Recent research expeditions to the NWHI have taken place in early- to mid- autumn and have found low numbers of reproductive *H. velasquezii* occurring on a continual basis from September through October. Gametophores bearing deeply pigmented gametangia tended to occur on bleached segments located on the upper half of the plant (Fig. 2). Although the majority of gametangia appeared clustered on terminal margins of segments, gametangia were also commonly found on segment surfaces (Fig. 2). Gametophores arose from secondary utricles, taking the place of a primary utricle (Fig. 3). Six to 18 globose gametangia that ranged in size from 102 μm to 248 μm in diameter (Tab. 1) were produced on gametophores that branched dichotomously between 0-3 times (Figs 4, 5). Gametophores ranged from 526 μm to 1137 μm in length, 37 μm to 57 μm in width, and contained between 1 and 4 discharge papillae (Tab. 1, Figs 3-5).

When reproductive characters of *H. velasquezii* were compared to related species (*H. opuntia*, *H. goreauii*, *H. minima*, *H. renschii*, *H. copiosa*, and *H. distorta*; see Hillis-Colinvaux (1980) and Kooistra *et al.* (2002)), average gametangial diameters and gametophore lengths fall within the mid-range of those reported for the lineage (Tabs 1 & 2). The major difference between *H. velasquezii* and related species is the number of gametangia that commonly occur per gametophore, with an average 1.4 times higher than that found in the lineage as a whole (Tabs 1, 2).

Reproductive characters of *Halimeda*

Scientific descriptions or illustrations of gametangia from 20 species of *Halimeda* (33 records) were collected (Tab. 1). Of these records, twenty-eight

Table 1. Reproductive characteristics of *Halimeda* species. Numerical data = *range*; **average ± SE or SD**†(n). Asterisks indicate that measurements or observations were calculated from published photographs or drawings. G/C = number of gametangia/gametophore, P/C = number of discharge papillae/gametophore, G/P = gametangia:papilla ratio, degree of branching = the number of dichotomies/gametophore. Lineages refer to the hypothesized lineages reported by Kooistra et al. (2002) based on molecular rDNA data. ‡Indicates species not included in the Kooistra (2002) molecular study, lineage alignment hypothesized based on Hillis-Colinvaux (1980). Gepp (1904) has illustrated reproductive structures from *H. gracilis* (a member of lineage 4), but data is not included here.

Lineage	Species	Location	Origin of Gametophores	Position of Gametangia	Diameter of Gametangia	G/C	P/C	G/P	Length – Gametophore	Width – Gametophore	Degree of branching	Reference
Lineage 1	*H. incrassata*	JPN	inner utricles, medulla	surface of segments	*266-391**; **327*** (35)	*14-20**; **16*** (3)	*2-4**,**3*** (3)	**5.3*** (3)	*1300-2900*	*150-360*; **243**	*1-2x,* (3)	Kamura (1966)
	(as *H. tridens*)	PR	primary utricles, inner utricles	surface of segments, margins	*156.3-250.0**; **205.0*** (25)	*7-31**; **19*** (3)	*2-5**,**3.3*** (3)	**5.8*** (3)	*1500-2000**; **1777.8*** (3)	*62.5*; **62.5*** (3)	*1-5x*	Howe (1907)
	H. monile		primary utricles, medulla		*100-225**; **163.8*** (10)	*5-10**; **7.5*** (2)	*1-3**,**2*** (2)	**3.75*** (2)	*1025-1700**; **1362.5*** (2)	*62.5*; 100*; **81.3*** (2)	*0-2x** (2)	Hillis (1959); Hillis-Colinvaux (1980)
	H. melanesica	GBR			**234.1 ± 46.2** † (33)	**6.6** (33)	**2** (33)	**3.3*** (33)	**1052.8 ± 263.8** † (33)	**50.0 ± 4.1** † (33)	*2-3x** (1)	Drew & Abel (1988)
	H. macroloba	GUA	medulla	surface of segments, margins	*250-400*	**6**					*0-3x** (3)	Kanda (1940); Merten (1971)
Lineage 2		CHA	utricle*		*135.7-200.0**; **177.0*** (9)	*9** (1)	*2** (1)	**4.5*** (1)	*1900** (1)	**57.1*** (1)	*1-2x** (1)	Meiling & Tseng (1980)
	H. favulosa †		primary utricles, medulla		*100-175**; **133.3*** (6)	*3.7*,**5*** (2)	*1*,**1*** (2)	*5*; **5*** (2)	*900-1100**; **1000*** (3)	*50*; **50*** (3)	*0** (3)	Hillis (1959); Hillis-Colinvaux (1980)
	H. micronesica	GBR			**123.5 ± 21.9** † (29)	**14.5** (29)	**3.5** (29)	**4.1** (29)	**964.0** (29)	**60.0** (29)	*3x** (1)	Drew & Abel (1988)
	H. cryptica	CAR	inner utricles, medulla	under surface of segments	**137** (50)	*5.0-10.0* (100)	*1** (6)	*5.0-10.0**	**600**	*50*; **50*** (3)	*0** (6)	Graham (1975)
Lineage 2	*H. fragilis*	GBR			*106.7-126.7**; **114.4*** (6)	*7** (1)	*2** (1)	**3.5*** (1)	*620** (1)	**53.3*** (1)	*1x** (1)	Drew & Abel (1988)

Table 1. (*suite*)

Lineage 3

Species	Location	Origin of Gametophores	Position of Gametangia	Diameter of Gametangia	G/C	P/C	G/P	Length – Gametophore	Width – Gametophore	Degree of branching	Reference
H. tuna	GBR			**138.2 ± 19.1** (87)	**3.6** (87)	**1.1** (87)	**3.4** (87)	**595 ± 112.2**† (87)	**36.3 ± 4.8**† (87)	0* (1)	Drew & Abel (1988)
	PR	medulla	surface of segments, margins	166.7-260.4*, **215.4*** (18)	3-7*, **6.25*** (5)...						

Table 1. (*suite*)

Lineage 3

Species	Location	Origin of Gametophores	Position of Gametangia	Diameter of Gametangia	G/C	P/C	G/P	Length – Gametophore	Width – Gametophore	Degree of branching	Reference
H. tuna	GBR			**138.2 ± 19.1** (87)	**3.6** (87)	**1.1** (87)	**3.4** (87)	**595 ± 112.2**† (87)	**36.3 ± 4.8**† (87)	0* (1)	Drew & Abel (1988)
	PR	medulla	surface of segments, margins	166.7-260.4*, **215.4*** (18)	3-7*, **4.6*** (5)	1-2*, **1.2*** (5)	**3.8*** (5)	1125-1833.3*, **1388.9*** (3)	41.7-83.3*, **60.4*** (5)	0-1x	Howe (1907)
	FLA	inner utricles	surface of segments, margins	260-280	5.0-6.0			1230-1700	47-63	1	CM Smith (unpublished data)
H. discoidea	GBR			**104.6 ± 15.9**† (149)	**11.5** (149)	**2.9** (149)	**4** (149)	**1055.4 ± 175.7**† (149)	**40.4 ± 8.1**† (149)	2-3x* (1)	Drew & Abel (1988)
	MHI	primary utricles		32-59*, **46.8*** (16)	19* (1)	4* (1)	4.75* (1)	404.5* (1)	18.2* (1)	2x* (1)	Egerod (1952)
		primary utricles, medulla		87.5-112.5*, **103.1*** (12)	3-7*, **6.25*** (4)	2*, **2*** (4)	**3.2*** (4)	350-700*, **575*** (5)	50-62.5*, **55*** (5)	0-2x* (4)	Hillis (1959); Hillis-Colinvaux (1980)
H. macrophysa	GBR			93.3-106.7*, **100*** (3)	3* (1)	1* (1)	3* (1)	373.3* (1)	33.3* (1)	0* (1)	Drew & Abel (1988)
H. gigas	GBR			160-173.3*, **170.1*** (4)	4* (1)	1* (1)	4* (1)	693.3* (1)	26.7* (1)	0* (1)	Drew & Abel (1988)
H. taenicola	GBR			112-126.7*, **118.1*** (4)	5* (1)	1* (1)	5* (1)	480* (1)	40* (1)	0* (1)	Drew & Abel (1988)
H. cuneata	JPN	primary utricles	surface of segments	150-180; **181.8*** (12)	5-8*, **6.3*** (3)	1*, **1*** (3)	**6.3*** (3)	594-1050*, **823*** (3)	46.9-56.3*, **51.1*** (3)	0* (3)	Chihara (1956)
H. scabra‡		medulla		100-175*, **130.4*** (14)	5-10*, **8*** (3)	1-3*, **2.3*** (3)	**3.5*** (3)	1100-1150*, **1133*** (3)	50-62.5*, **58.3*** (3)	1-2x* (3)	Hillis (1959); Hillis-Colinvaux (1980)
		FLA		160-300*, **221.3*** (30)	7-24*, **13*** (4)	1-3*, **2*** (4)	**6.5*** (4)	1625* (1)	53-83*, **72.5*** (4)	0-2x* (4)	Howe (1905)

Table 1. *(suite)*

Lineage 5

Species	Location	Origin of Gametophores	Position of Gametangia	Diameter of Gametangia	G/C	P/C	G/P	Length – Gametophore	Width – Gametophore	Degree of branching	Reference
H. goreaui	FLA	medulla	surface of segments, margins	215	4.5			672	37.8	1	CM Smith (unpublished data)
H copiosa	JAM	cortical, subcortical.	surface of segments.	190-238(-288); 205* (8)	4.0-8.0	1*,1* (6)	4.0-8.0*	980* (1)	50* (1)	0	Goreau & Graham (1967)
	GBR			130.2 ± 25.2† (48)	6.9 (48)	2 (48)	3.4 (48)	824.1 ± 69.2 (48)	41.0 ± 6.5 (48)	0* (1)	Drew & Abel (1988)
(as H. hederacea)	GBR			147.0 ± 26.3† (201)	8.7 (201)	1.8 (201)	4.9 (201)	1128.5 ± 282.7† (201)	36.9 ± 4.9† (201)	3x* (1)	Drew & Abel (1988)
H. opuntia	GBR			211.1 ± 45.8† (268)	7.9 (268)	2.5 (268)	3.2 (268)	999.2 ± 113.2 (268)	45.1 ± 7.8† (268)	4x* (1)	Drew & Abel (1988)
	FLA	utricles, medulla	surface of segments, margins	170	7.2-12.9			890-1190	32-39	1	CM Smith (unpublished data)
f. typica	JPN	primary utricles medulla	surface of segments, margins	139-231*; 198* (28)	4-14*; 9.8* (4)	1-3*; 1.7* (4)	5.4* (4)	700-1750	100-225; 162	0-2x* (4)	Kamura (1966)
f. intermedia	JPN	primary utricles	surface of segments, margins	120-216*; 152* (25)	8-12*; 7.3* (3)	1-2*; 1.7* (3)	4.3* (3)	400-950	90-250; 151	0-1x* (3)	Kamura (1966)
H. distorta	GBR			80-106.7*; 92.8* (7)	7* (1)	1* (1)	7* (1)	373.3* (1)	26.7* (1)	1x* (1)	Drew & Abel (1988)
H. velasquezii‡	NWHI	inner utricles	surface of segments, margins	102.0-248.3; 168.2 ± 4.6 (68)	6-18 (-21); 11.1 ± 1.5 (9)	1-4; 2.1 ± 0.3 (9)	5.3 (9)	526.3-1137.3; 852.8 ± 80.6 (9)	36.8-56.4; 46.3 ± 2.5 (9)	0-3x (9)	this study

CAR = Caribbean, CHA = China, FLA = Florida, USA, GBR = Great Barrier Reef, Australia, JAM = Jamaica, JPN = Japan, MHI = Main Hawaiian Islands, NWHI = Northwest Hawaiian Islands, PAN = Panama, PR = Puerto Rico
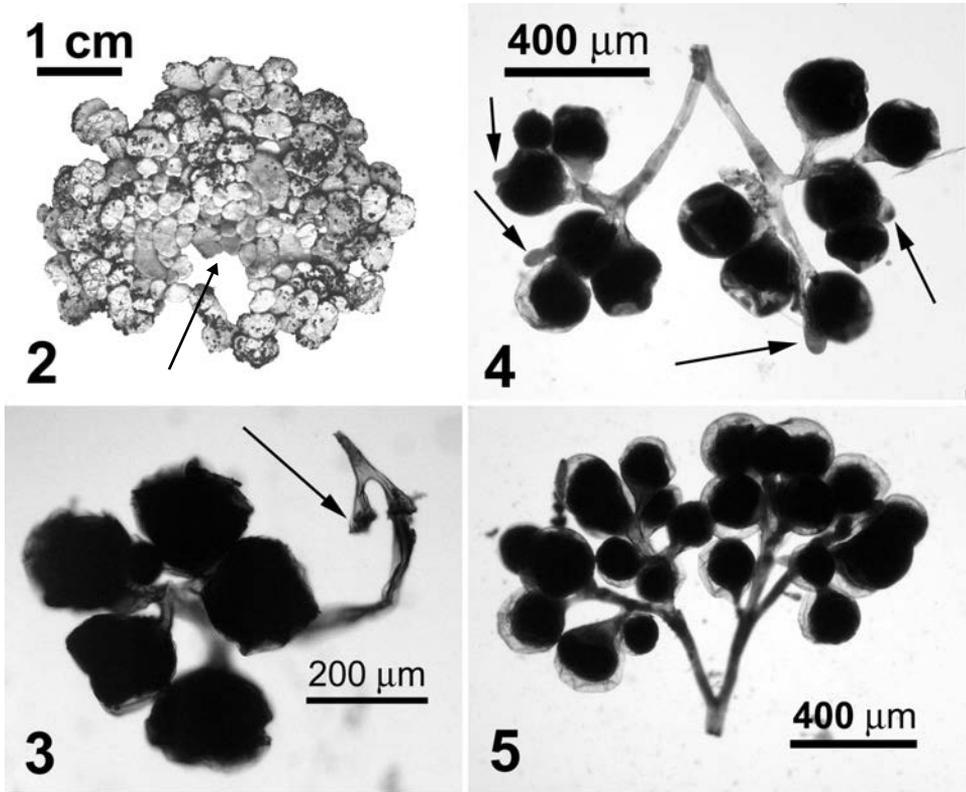
Fig. 2. Reproductive *Halimeda velasquezii* (PSV20007) from French Frigate Shoals. Gametangia most commonly occur on the margins of segments located in the upper half of the plant but can be found on almost all plant surfaces. Plant holdfast indicated with arrow. Fig. 3. Gametophores arise from secondary utricles, replacing a primary utricle (arrow). Fig. 4. Branched gametophore exhibiting 2 dichotomies. Four discharge papillae are indicated by arrows. Fig. 5. Gametophore exhibiting dense cluster of 21 gametangia.

contained complete information for characters analysed in this study. Multiple descriptions were found for 6 species (*H. incrassata*, *H. tuna*, *H. discoidea*, *H. scabra*, *H. copiosa*, and *H. opuntia*). Of these, five reported dissimilar (nonoverlapping) gametangial size ranges and two reported dissimilar gametophore lengths (Tab. 1). Because of these within species differences, two multivariate analyses were conducted: one including all 28 records (with multiple records for some species), and one including only a single record for each species, with the largest (and presumably most mature) reproductive structures represented for each species.

Before analyses, species were labeled according to the evolutionary lineages revealed through molecular studies (Kooistra *et al.*, 2002). In analyses containing all 28 records, MDS plots spatially mapped relationships within and among species from each lineage (Fig. 6) but did not reveal species to cluster into discrete groups. However, when bubble values were added to the MDS plots for each character, it was clear that members of lineage 1 tended to exhibit larger gametangial diameters and longer gametophore lengths than species from other lineages

Table 2. Reproductive characters averaged for species within phylogenetic lineages. Numerical data = *range*; average ± SE (n). G/C = number of gametangia/gametophore, P/C = number of discharge papillae/gametophore. Lineages refer to the hypothesized lineages reported by Kooistra *et al.* (2002) based on molecular rDNA data.

Lineage	Diameter of gametangia	G/C	P/C	Length - gametophore	Width - gametophore
1	*100.0 – 400.0*; 231.4 ± 33.1 (6)	*3 – 31*; 10.0 ± 2.4 (6)	*1 – 5*; 2.3 ± 0.4 (5)	*900 – 2900*; 1459 ± 212 (5)	*50 – 360*; 97.4 ± 36.9 (5)
2	*106.7 – 137.0*; 125.0 ± 6.6 (3)	*5 – 15*; 9.7 ± 2.4 (3)	*1 – 4*; 2.2 ± 0.7 (3)	*600 – 924*; 728 ± 118 (3)	*50 – 60*; 54.4 ± 2.9 (3)
3	*32.0 – 300.0*; 150.0 ± 18.3 (12)	*3 – 24*; 7.8 ± 1.5 (11)	*1 – 4*; 1.8 ± 0.3 (11)	*350 – 1833.3*; 884 ±126 (12)	*26.7 – 83.3*; 45.6 ± 4.5 (12)
5	*80.0 – 288.0*; 168.9 ± 12.5 (10)	*4 – 18*; 7.9 ± 0.6 (10)	*1 – 4*; 1.7 ± 0.2 (8)	*373.3 – 1750*; 877 ± 80 (10)	*26.7 – 250*; 63.2 ± 15.7 (10)
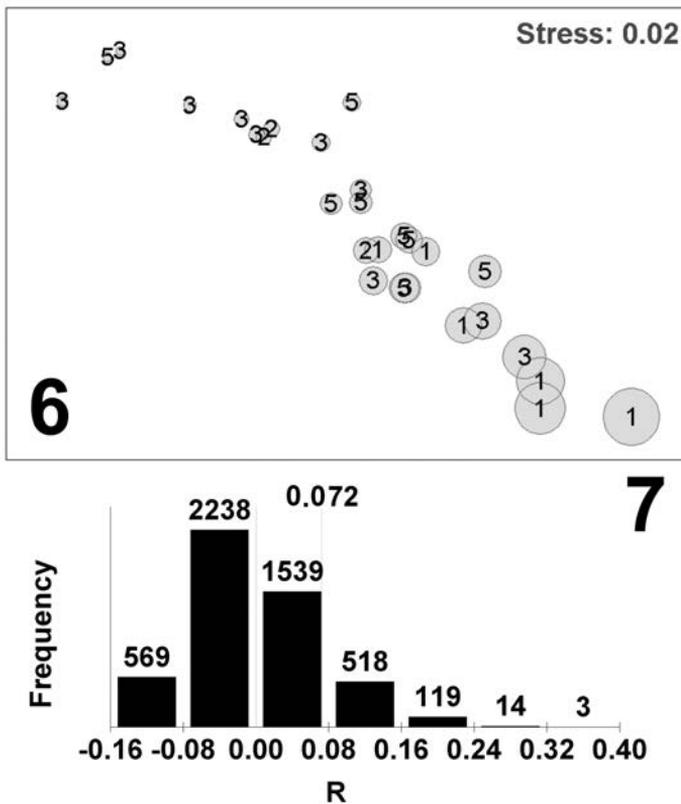
Fig. 6. Comparison of *Halimeda* reproductive structures. MDS bubble plot with average gametophore lengths represented. Numbers correspond to lineages shown in Tab. 2.
Fig. 7. Comparison of *Halimeda* reproductive structures. Histogram of ANOSIM R-statistics.

(Fig. 6). SIMPER analysis confirmed that gametophore length was responsible for explaining over 75 % of differences between lineages, while gametangial diameter was responsible for about 9 % of differences. Other reproductive characters were not informative in determining lineage relationships. Average reproductive features calculated for each phylogenetic group show lineage 1 (Kooistra *et al.*, 2002; roughly corresponds to *Halimeda* section *Rhipsalis* in Hillis-Colinvaux, 1980) to exhibit gametangial diameters at least 1.4 times greater and gametophore lengths at least 1.7 times longer than the other 3 lineages tested (Tab. 2).

As suggested by the MDS plot, low R-statistics from ANOSIM analyses with all 28 records reveal much overlap in morphology among reproductive characters from most lineages (global R = 0.072, significance level = 15.9 %; Fig. 7). Pairwise tests show only lineages 1 and 2 to be distinct (R = 0.549, significance level = 2.4 %; Tab. 3). The R-statistic for pairwise comparisons between all other groups ranged from -0.233 to 0.213, indicating that reproductive structures alone cannot be used to sufficiently separate these species into their hypothesized evolutionary lineages. The ANOSIM analyses using only the largest record for each species did not clarify species or lineage relationships (global R = 0.006, significance level = 42.5 %).

Descriptions of gametes from 10 species of *Halimeda* (14 records) were collected (Tab. 4). Of these records, eleven contained information for all characters analysed in this study. A MDS plot of gamete size separated *H. incrassata* and *H. simulans* from the majority of other *Halimeda* species based on the great size disparity exhibited between male and female gametes (Fig. 8). However, because *H. monile* (also a representative of lineage 1) exhibits gamete types similar to the other 3 lineages tested, ANOSIM reveals overlapping lineage comparisons (global R = 0.147, significance level = 12.9 %, Fig. 9). When *H. incrassata* and *H. simulans* were removed from MDS (not shown) and ANOSIM analyses, the remaining species remained clustered together without any lineage resolution (global R = – 0.357, significance level = 99.3 %).

DISCUSSION

This study provides the first descriptive information of reproductive structures for *Halimeda velasquezii*, and offers the first comprehensive multivariate comparison of reproductive structures for the genus. Analyses presented

Table 3. ANOSIM pairwise test results comparing reproductive structures between lineages within *Halimeda*.

Lineages	R-statistic	Significance level %	Possible permutations	Actual permutations	Number ≥ observed
1, 2	0.549	2.4	84	84	2
1, 3	0.213	4.6	12376	5000	229
1, 5	0.222	3.5	3003	3003	104
2, 3	– 0.233	99.7	364	364	363
2, 5	0.032	38.2	165	165	63
3, 5	– 0.032	55.4	75582	5000	2769

Tab. 4. Gamete characteristics of *Halimeda* species. Numerical data = *range*; **average ± SE or SD**†(n). Megagametes are indicated with a ♀, microgametes are indicated with a ♂. Lineages refer to the hypothesized lineages reported by Kooistra *et al.* (2002) based on molecular rDNA data.

	Species	Location	Length - ♀ gametes	Width - ♀ gametes	- Length ♂ gametes	Width ♂ gametes	♀ gametes: ♂ gametes	Reference
Lineage 1	*H. incrassata*	JPN	*12.4-22.5*	*7.0-13.5*	*3.5-11.0*	*2.5-6.0*		Kamura (1966)
		PAN	*13.5-19.5;* **15.5 ± 0.2** (80)	*7.0-10.3;* **8.7 ± 0.1** (80)	*5.3-6.3;* **5.5 ± 0.1** (80)	*2.3-3.0;* **2.5 ± 0.1** (80)	34.1	Clifton & Clifton (1999)
	H. simulans	PAN	*16.5-21.0;* **19.7 ± 0.7** (100)	*5.3-9.0;* **8.9 ± 0.1** (100)	*5.0-6.8;* **5.5 ± 0.1** (100)	*2.3-3.8;* **2.5 ± 0.1** (100)	45.4	Clifton & Clifton (1999)
	H. monile	PAN	*6.0-9.0;* **7.7 ± 0.1** (80)	*2.3-3.0;* **2.9 ± 0.1** (80)	*5.0-6.8;* **5.6 ± 0.1** (80)	*1.5-3.0;* **2.4 ± 0.1** (80)	2	Clifton & Clifton (1999)
	H. macroloba	GUA	*6.0-10.0*	**3**	*1.5-2.1*			Kanda (1940); Merten (1971)
Lineage 2	*H. cryptica*	CAR	*6.0-9.0*					Graham (1975)
Lineage 3	*H. tuna*	PAN	*6.8-9.0;* **7.5 ± 0.1** (80)	*2.0-4.5;* **3.1 ± 0.1** (80)	*5.3-6.8;* **5.4 ± 0.1** (80)	*2.3-3.0;* **2.6 ± 0.1** (80)	2	Clifton & Clifton (1999)
	f. *platydisca*	MED	*7.0-8.0*	*3.0-4.0*	*5.0-6.0*			Feldmann (1951)
	H. discoidea	PAN	*6.0-9.0;* **7.7 ± 0.1** (80)	*2.3-3.5;* **2.9 ± 0.1** (80)	*4.5-6.8;* **5.6 ± 0.1** (80)	*2.3-3.0;* **2.4 ± 0.1** (80)	2	Clifton & Clifton (1999)
	H. cuneata	JPN	*6.3-7.5*	*2.5-3.4*	*5.0-6.3*	*2.0-2.9*		Chihara (1956)
Lineage 5	H. goreaui	PAN	*6.8-9.0;* **7.8 ± 0.1** (80)	*2.0-4.3;* **3.1 ± 0.1** (80)	*4.5-6.8;* **5.7 ± 0.1** (80)	*2.3-3.8;* **2.5 ± 0.1** (80)	2.1	Clifton & Clifton (1999)
	H. opuntia	PAN	*5.3-9.0;* 7.5 ± 0.1	*2.3-4.5;* 3.0 ± 0.1	*5.0-6.3;* 5.4 ± 0.1	*2.3-3.0;* 2.5 ± 0.1	2	Clifton & Clifton (1999)
	f. *typica*	JPN	*5.0-7.5*	*2.2-3.5*	*4.8-7.0*	*2.0-3.0*		Kamura (1966)
	f. *intermedia*	JPN	*6.8-8.4*	*2.6-3.3*	*4.8-6.0*	*2.5-3.7*		Kamura (1966)

here reveal that the sand dwelling species in lineage 1 of Kooistra *et al.* (2002) have reproductive structures that are statistically distinct from species found in lineage 2 (Tab. 3, Fig. 7), thus supporting the concept that reproductive structures may be diagnostic features capable of supporting some evolutionary-based taxonomic groupings.

In her monograph of *Halimeda*, Hillis-Colinvaux (1980) hypothesized that gametophore length, extent of branching, and the size, shape, and number of
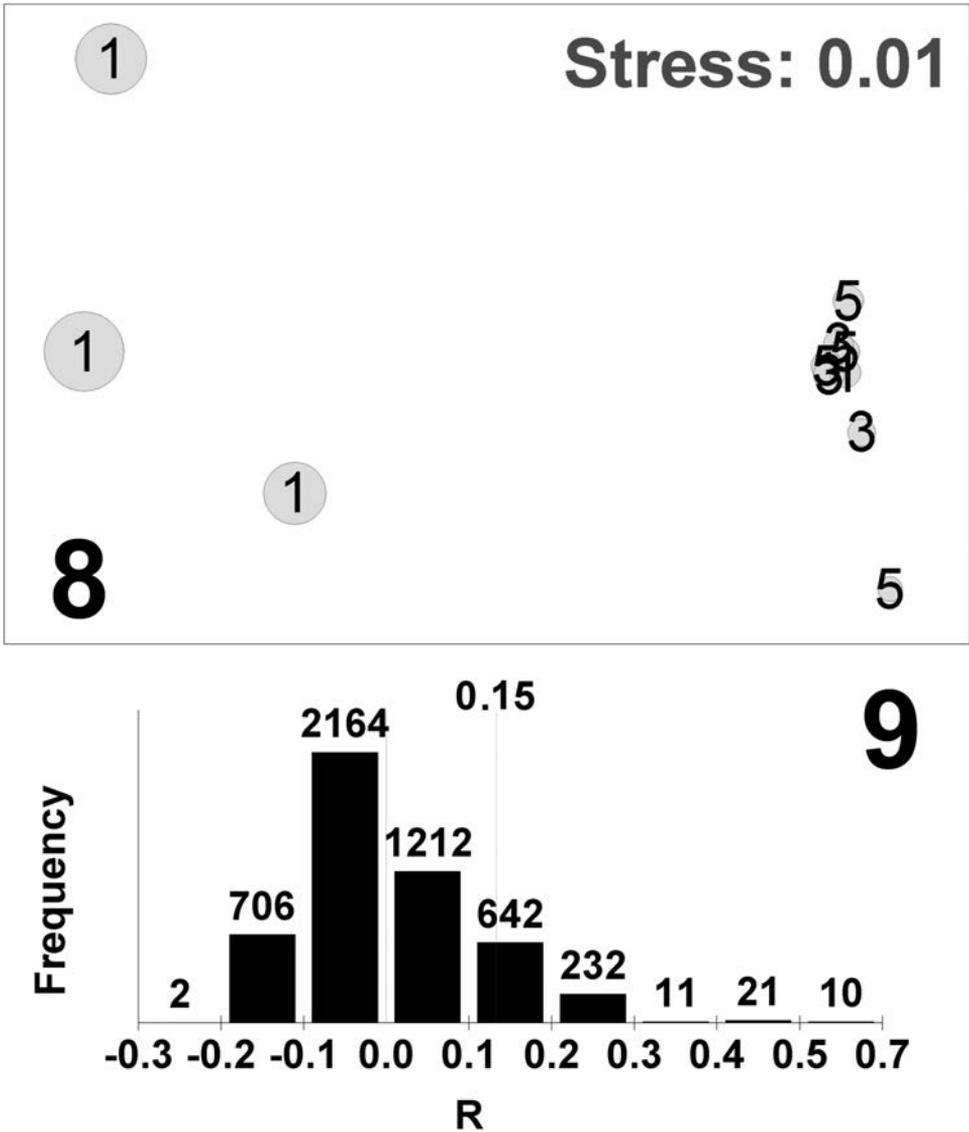
Fig. 8. Comparison of *Halimeda* gametes. MDS bubble plot with average gamete diameters represented. Numbers correspond to lineages shown in Tab. 2. Fig. 9. Comparison of *Halimeda* gametes. Histogram of ANOSIM R-statistics.

gametangia found in species would be valuable taxonomic characters. Using these characters, this study revealed that only gametophore length and gametangial diameter were informative markers for separating lineages. When species of *Halimeda* were mapped on a MDS plot (Fig. 6) and compared with ANOSIM (Fig. 7), lineages 1 and 2 slightly overlapped, but were clearly different based on a pairwise R-statistic of > 0.50. Pairwise R-statistics of < 0.25 between all other

lineages suggest that they do not differ greatly from each other (Tab. 3, Fig. 6). However, because the R-statistics generated from comparisons between lineages 1 to 3 and 5 were close to 0.25, and because significance values were below 5 %, it seems likely that inclusion of additional samples or species may distinctly separate lineage 1 from all other lineages. Lineages 2, 3, and 5 overlapped, indicating that our present knowledge of reproductive characters within the genus cannot be used to adequately describe these sections within *Halimeda*.

One of the larger concerns revealed by this study involves the disparate reports of reproductive structures within single species that were found during our literature search (Tab. 1). Fast hypotheses may explain these ambiguities: 1) specimens were collected at different developmental stages (Drew & Abel, 1988); 2) historical species represent para- or polyphyletic entities that have converged on similar vegetative morphologies, but reproductive structures remain distinct (van Oppen *et al.*, 1996, Kooistra *et al.* 2002); 3) reproductive characters may be environmentally plastic, and ambient conditions may affect their morphology; or 4) previously published data does not represent adequate species descriptions. The relevance of each of these hypotheses will be discussed below.

As first discussed by Drew and Abel (1988), gametangial diameter may be correlated to developmental stage, and unless descriptions of each species were collected at exactly the same stage, size measurements may not be valid for taxonomic purposes. Dramatic size changes over short time periods have already been documented in the related genus, *Rhipidosiphon*, where gametangia expanded in length from ~800 μm to over 5000 μm during a single day (Vroom *et al.*, 2001). If mature gametangia were collected for each species of *Halimeda*, it is hypothesized that this character would prove more informative in resolving lineage relationships. Despite the fact that no information exists on the developmental stage of gametangia presented in Tab. 1, MDS and SIMPER analyses presented here show gametangial diameters to be useful in explaining some inter-lineage differences.

Kooistra *et al*. (2002) show that at least 2 lineages of *Halimeda* separate into distinct Atlantic and Indo-Pacific clades, and it is hypothesized that species in these lineages represent genetically distinct cognate pairs. This is supported by some reproductive data collected in Tab. 1. Two representatives of *H. copiosa* from the Great Barrier Reef exhibit statistically similar gametangial diameters, while a Caribbean representative exhibits a larger gametangial diameter outside the range found in Australia. *Halimeda incrassata* and *H. tuna* from the Atlantic and Pacific oceans also exhibit non-overlapping gametangial diameters. Additionally, molecular data found Atlantic and Pacific members of *H. opuntia* to group together in one clade (Kooistra *et al.*, 2002), suggesting recent dispersal. This finding is also supported by reproductive data (Tab. 1) that shows overlapping gametangial diameters in representatives of *H. opuntia* from Japan, Australia, and the Caribbean.

Environmental influences on gametangial morphology in *Halimeda* has never been examined, however it is hypothesized that internal regulatory factors such as turgor pressure will have a greater influence on gametangial morphology than external factors (Vroom & Smith, 2003). What is more of a concern is that data presented here were derived from published illustrations and may not indicate true size values, or are too small to be considered fair assessments of population or species limits. Additionally, the reproductive characters illustrated may not represent average specimens because of observer bias. However, because the data presented in Tab. 1 support geographic, within species differences suggested by molecular data (Kooistra *et al.*, 2002), it is assumed that the rough size estimates of reproductive characters collected here are representative of the species they represent.

Confusion remains about the usefulness of gametes to infer phylogenetic relationships, yet some data collected by Clifton & Clifton (1999) supports the close relationship of Caribbean *H. incrassata* and *H. simulans* suggested by molecular studies (Kooistra *et al.*, 2002). Both these species were found to produce unusual megagametes considerably larger and morphologically distinct from other species of *Halimeda* (Tab. 4). Fig. 1 in Kooistra *et al.* (2002) shows a clade with 63 % bootstrap support containing Caribbean *H. incrassata*, *H. simulans*, and *H. monile* (a species that does not produce giant megagametes), but the relationship of the 3 species in the clade is clouded by less than 50 % bootstrap support. From the results of Clifton & Clifton (1999), it can be hypothesized that a phylogeny slightly different than presented in Kooistra *et al.* (2002) might be likely: *H. monile* is sister to a clade containing the other 2 species.

From Kooistra (2002) and Famà *et al.* (2002), it is clear that compound reproductive structures have arisen more than once among Bryopsidalean taxa. *Chlorodesmis*, *Caulerpella*, and *Halimeda* do not appear to share a close relative. Kooistra (2002) presents a molecular-based phylogeny for the Bryopsidales containing 8 historical genera that shows *Rhipiliopsis* to be a close relative to *Halimeda*. Because reproductive features for *Rhipiliopsis* have been reported for only Australian *R. peltata*, the range of reproductive characters extant in the genus remains unknown. However, the solitary, stalked gametangia of *R. peltata* (Womersley, 1984) may likely be precursors to the compound gametangia found in all *Halimeda* species. Vroom *et al.* (2001) suggest that *Tydemania* may represent a pivotal genus between a flabellate ancestor similar to *Rhipiliopsis* and morphologically complex species of *Halimeda*. Once reproductive features become known for *Tydemania*, a relationship between these 3 genera may become clear.

In conclusion, this study found reproductive structures of *Halimeda velasquezii* fell within the range of other members of its evolutionary lineage (Tab. 2). Based on MDS and SIMPER analyses, gametophore length and gametangial diameter were the most informative reproductive characters for species and lineage comparison (Fig. 6). Multivariate analyses found compound gametangia may be useful in separating the lineage containing sand dwelling species of *Halimeda* from other species in the genus, however most species concepts and relationships must remain based on vegetative characters at present.

REFERENCES

ABBOTT I.A., 1999 – *Marine Red Algae of the Hawaiian Islands*. Honolulu, Hawaii, Bishop Museum Press, 477 p.

CHIHARA M., 1956 – Studies on the life-history of the green algae in the warm seas around Japan. IV. On the life-history of *Halimeda cuneata* Hering. *The Journal of Japanese Botany* 31: 102-110.

CLARKE, K.R. & WARWICK, R.M., 2001 – *Change in marine communities: an approach to statistical analysis and interpretation*, 2nd edition. PRIMER-E: Plymouth.

CLIFTON K.E., 1997 – Mass spawning by green algae on coral reefs. *Science* 275: 1116-1118.

CLIFTON K.E. & CLIFTON L.M., 1999 – The phenology of sexual reproduction by green algae (Bryopsidales) on Caribbean coral reefs. *Journal of Phycology* 35: 24-34.

DREW E.A. & ABEL K.M., 1988 – Studies on *Halimeda*. II. Reproduction, particularly the seasonality of gametangia formation, in a number of species from the Great Barrier Reef Province. *Coral Reefs* 6: 207-218.

EGEROD L.E., 1952 – An analysis of the siphonous Chlorophycophyta: with special reference to the Siphonocladales, Siphonales, and Dasycladales of Hawaii. *University of California Publications in Botany* 25: 325-423, pls 29-42.

FAMA P., WYSOR B., KOOISTRA W.H.C.F. & ZUCCARELLO G.C., 2002 – Molecular phylogeny of the genus *Caulerpa* (Caulerpales, Chlorophyta) inferred from chloroplast tufA gene. *Journal of Phycology* 38: 1040-1050.

FELDMANN J., 1951 – Sur la reproduction sexuée de l'*Halimeda tuna* (Ell. *et* Sol.) Lamour. f. *platydisca* (Decaisne) Barton. *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences, Paris* 233: 1309-1310.

GEPP E.S., 1904 – The sporangia of *Halimeda. Journal of Botany, British and Foreign* 42: 193-197.

GEPP A. & GEPP E.S., 1911 – The Codiaceae of the Siboga Expedition. *Monographs of the Siboga Expedition* Vol. 62, 150 p. + XXII plates.

GOREAU T.F. & GRAHAM E.A., 1967 – A new species of *Halimeda* from Jamaica. *Bulletin of Marine Science* 17: 432-441.

GRAHAM E.A., 1975 – Fruiting in *Halimeda* (order Siphonales) 1. *Halimeda cryptica* Colinvaux and Graham. *Bulletin of Marine Science* 25: 130-133.

HILLIS L.W., 1959 – A revision of the genus *Halimeda* (order Siphonales). *Contributions in Marine Science* 6: 321-403.

HILLIS L.W., ENGMAN J.A. & KOOISTRA W.H.C.F., 1998 – Morphological and molecular phylogenies of *Halimeda* (Chlorophyta, Bryopsidales) identify three evolutionary lineages. *Journal of Phycology* 34: 669-681.

HILLIS-COLINVAUX L., 1980 – Ecology and taxonomy of *Halimeda*: primary producer of coral reefs. *Advances in Marine Biology* 17: 1-327.

HILLIS-COLINVAUX L., 1984 – Systematics of the Siphonales. *In*: Irvine D.E.G. & John D.M. (eds), *Systematics of the Green Algae*. Systematics Association, Special Volume 27, London, Academic Press, pp. 271-296.

HOWE M.A., 1905 – Phycological studies – I. New Chlorophyceae from Florida and the Bahamas. *Bulletin of the Torrey Botanical Club* 32: 241-252, pls. 11-15.

HOWE M.A., 1907 – Phycological studies – III. Further notes on *Halimeda* and *Avrainvillea. Bulletin of the Torrey Botanical Club* 34: 491-516, pls. 25-30.

KAMURA S., 1966 – On the sexual reproduction of two species of *Halimeda* (Chlorophyta). *Bulletin of Arts & Science Division, University of the Ryukyus, Mathematics and Natural Sciences* 9: 302-313.

KANDA C., 1940 – [Notes on *Halimeda macroloba* Decne.]. *Kagaku Nanyo* 3: 20-26. [In Japanese].

KOOISTRA W.H.C.F., 2002 – Molecular phylogenies of Udoteaceae (Bryopsidales, Chlorophyta) reveal non-monophyly for *Udotea, Penicillus* and *Chlorodesmis. Phycologia* 41: 453-462.

KOOISTRA W.H.C.F., CALDERON M. & HILLIS L.W., 1999 – Development of the extant diversity in *Halimeda* is linked to vicariant events. *Hydrobiologia* 398/399: 39-45.

KOOISTRA W.H.C.F., COPPEJANS E.G.G. & PAYRI C., 2002 – Molecular systematics, historical ecology, and phylogeography of *Halimeda* (Bryopsidales). *Molecular Phylogenetics and Evolution* 24: 121-138.

LITTLER D.S. & LITTLER M.M., 1990 – Systematics of *Udotea* species (Bryopsidales, Chlorophyta) in the tropical western Atlantic. *Phycologia* 29: 206-252.

MEILING D. & TSENG C.K., 1980 – Studies on some marine green algae from the Xisha Islands, Guangdong province, China. II. *Studia Marina Sinica* 17: 1-9.

MERTEN M.J., 1971 – Ecological observations of *Halimeda macroloba* Decaisne (Chloro-
          phyta) on Guam. *Micronesica* 7: 27-44.
SCHIMTZ F. & HAUPTFLEISCH P., 1896 – Helminthocladiaceae, Chaetangiaceae. *In*:
          Engler A. & Prantl K. (eds), *Die natürlichen Pflanzenfamilien*. T. 1 Abt. 2, Leipzig,
          Engelmenn, pp. 327-339.
VAN OPPEN M.J.H., KLERK H., OLSEN J.L. & STAM W.T., 1996 – Hidden diversity in
          marine algae: some examples of genetic variation below the species level. *Journal
          of the Marine Biological Association of the United Kingdom* 76: 239-242.
VROOM P.S. & SMITH C.M., 2003 – Life Without Cells. *Biologist* 50: 222-226.
VROOM P.S., SMITH C.M. & KEELEY S.C., 1998 – Cladistics of the Bryopsidales: a
          preliminary analysis. *Journal of Phycology* 34: 351-360.
VROOM P.S., SMITH J.E. & SMITH C.M., 2001 – Observations of reproduction in
          Rhipidosiphon javensis (Halimedineae, Bryopsidales) in Hawaii. *Phycologia* 40:
          97-100.
WOMERSLEY H.B.S., 1984 – *The Marine Benthic Flora of Southern Australia*. Part I.
          Adelaide, Government Printer, 329 p.