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Deviant segments hamper a morphometric approach towards *Halimeda* taxonomy

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Abstract – Traditional taxonomy of the segmented green algal genus *Halimeda* is largely based on descriptive expressions of thallus habit, segment shape and anatomical structures. In the course of the last decade, molecular phylogenetic studies have revealed non-monophyly and cryptic diversity in several species. In an attempt to tackle the taxonomic problems that were raised by these molecular studies, a combined molecular and morphometric method was developed. In this study, a morphometric pilot data set is explored. This resulted in the discovery of segments aberrant in morphology and/or anatomy. These are primarily apical and non-calcified segments, and segments from the basal part of the algal body. To answer the question whether incorporation of comparison of discriminant analyses that included and excluded deviant segments demonstrated the negative influence of such segments on the taxonomic power of the data. Omitting non-calcified and apical segments and segments from the basal thallus region yielded the same results as the exclusion of all deviant segments, irrespective of their location in the algal body. This result permits a simple recommendation towards precluding these types of segments from further studies.

Anatomy / Bryopsidales / Chlorophyta / deviant segments / discriminant analysis / *Halimeda* / morphology / morphometrics / taxonomy

Résumé – **Les segments déviants gênent une approche morphométrique de la taxonomie d'*Halimeda*.** La taxonomie traditionnelle du genre *Halimeda*, une algue verte à segments, est largement basée sur la morphologie générale du thalle, la forme des segments et des caractères anatomiques. Au cours de la dernière décennie, des études moléculaires phylogénétiques ont démontré la non-monophylie et la diversité cryptique de plusieurs espèces appartenant à ce genre. Dans un essai d'élucidation des problèmes taxonomiques qui ont été soulevées par ces études moléculaires, une méthode combinée moléculaire et morphométrique a été développée. Dans cette étude une banque de données morphométriques pilote est explorée. Ceci a résulté en la découverte de segments morphologiquement et anatomiquement aberrants. Ces segments proviennent surtout des parties apicales non calcifiées et des segments de la base du thalle. Malgré cette connaissance, il reste la question si l'incorporation de ces segments aberrants dans la banque de données a un effet négatif sur sa valeur taxonomique. La réponse a été donnée par l'utilisation d'une approche à analyse discriminante, dans laquelle les groupes avaient été déterminés sur la base de méthodes moléculaires et donc indépendants de la morphologie. Le fait que l'assignement de segments à des groupes d'espèces est meilleur lorsque les segments déviants sont omis prouve

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bien l'effet négatif de l'inclusion de ces dernières. L'omission des segments non calcifiés, des segments apicaux et des parties basales du thalle donne les mêmes résultats que l'omission de tous les segments déviants, de n'importe quelle partie du thalle. Ce résultat permet de conclure à la simple recommandation d'exclure ce type de segments aberrants d'études morphométriques futures.

Anatomie / Bryopsidales / Chlorophyta / segments déviants / analyse discriminante / *Halimeda* / morphologie / morphométriques / taxonomie

INTRODUCTION

The chlorophyte genus *Halimeda* is ubiquitous throughout the tropics (Taylor, 1960; Hillis-Colinvaux, 1980; Littler & Littler, 2000, 2003). Its segmented thalli consist of a single giant tubular cell (siphon) that branches and anastomoses to form one of the most architecturally complex algal bodies within the order Bryopsidales (Vroom *et al.*, 1998). The medulla consists of lengthwise siphons that branch off into the cortex, where they form series of short, inflated branches called utricles.

In current *Halimeda* taxonomy, the different sections and species are defined using descriptive expressions of thallus habit, segment shape and anatomical structures (Hillis-Colinvaux, 1980; Verbruggen & Kooistra, 2004). In addition, measurements of segment size and a limited number of anatomical structures are usually specified, and aid to distinguish between certain species. Although the major evolutionary lineages within the genus can be recognized with relative ease (Verbruggen & Kooistra, 2004), species within these lineages are often difficult to identify with existing identification keys and morphological insights. Additionally, molecular phylogenetic studies have shown that several species are nonmonophyletic or incorporate hidden diversity (Kooistra *et al.*, 1999; 2002; Kooistra & Verbruggen, 2005). It is clear that classical approaches do not provide systematicians with the acumen needed to come to an evolutionarily correct species-level taxonomy. This appears not to be the case for *Halimeda* alone; more and more cases of cryptic diversity and erroneous species boundaries within all three major marine macroalgal groups have been demonstrated (e.g. Siemer *et al.*, 1998; van der Strate *et al.*, 2002; Zuccarello *et al.*, 2002; Zuccarello & West, 2003; Saunders & Lehmkuhl, 2003). If we strive for a correct morphological interpretation of species boundaries, new methods of examining and interpreting the morphology are needed.

In this and a parallel paper (Verbruggen *et al.*, 2005), we introduce a combined molecular and morphometric approach towards *Halimeda* taxonomy. While Verbruggen *et al.* (2005) concentrate on the taxonomic utility of the different methods and variables; the focus of this study is on exploration of the morphometric data and on the study of deviant segments within the thallus. More specifically, we identify and characterize deviant segments. Thereafter, their influence on the taxonomic power of the morphometric data is assessed and suggestions towards the exclusion of certain groups of segments from further studies are put forward.

MATERIALS AND METHODS

The procedures for segment dissection and the morphometric methods employed are explained in more detail in Verbruggen *et al.* (2005). We will here restrict ourselves to a short overview of the molecular and morphometric dataset and expand on the statistical analyses used to address this paper's questions. For the tables listing the morphometric variables, we refer to Verbruggen *et al.* (2005: tables 3 & 4). Notations of the type *s*# or *a*# refer to variable numbers in these tables (in this notation # are numbers, while *s* and *a* stand for segment morphological and anatomical variables, respectively)

Species sampling, sequence analysis and morphometrics

Twenty-one specimens from nine species spanning the phylogenetic range of the genus were examined (Table 1). All specimens were deposited in the Ghent University Herbarium, Belgium (GENT). Specimens were assigned to species-level groups by sequencing the nuclear ribosomal DNA (SSU, ITS1, 5.8S, ITS2 and partial LSU) and determining the species-level clade to which they belong in a phylogenetic tree (Verbruggen *et al.*, 2005 and references therein).

Table 1. Specimens in the morphometric study. Given are their accession number in the GENT herbarium and geographic origin.

section	*species*	*GENT*	*geographic origin*
Rhipsalis	*H. borneensis*	HV18-1	Zanzibar Island (Tanzania)
		HV183b	Tahiti, French Polynesia
	H. macroloba	HV38	Zanzibar Island (Tanzania)
		HV45	Mnazi Bay, Tanzania
		HV206	Tahiti, French Polynesia
Micronesicae	*H. micronesica*	H.0014-1	Great Barrier Reef, Australia
		WLS184-02	Wallis Island (France)
		WLS420-02	Wallis Island (France)
Halimeda	*H. lacunalis*	HV306-1	Rangiroa, French Polynesia
		HV308-1	Rangiroa, French Polynesia
	H. taenicola	HV285-1	Rangiroa, French Polynesia
		HV285-2	Rangiroa, French Polynesia
		HV306-3	Rangiroa, French Polynesia
	H. tuna	H.0113-1	Naples, Italy
		HV319	Rosas, Spain
Pseudo-Opuntia	*H. gracilis*	HV312-1	Rangiroa, French Polynesia
		HV317-1	Rangiroa, French Polynesia
Opuntia	*H. goreauii*	H.0257	Bocas del Toro, Panama
		H.0258-1	Galeta, Panama
	H. opuntia	HV46b	Mnazi Bay, Tanzania
		HV61	Moorea, French Polynesia

The morphometric study involved gathering segment morphological and anatomical variables. For analyses of segment morphology, small plants (< 100 segments) were studied in their entirety, whereas series of segments spanning the entire thallus length were studied for larger specimens (totalling between 48 and 89 segments per specimen).

The position of the segments within the thallus was determined as the distance of the segment to the basal segment (number of intermediate nodes; variable *s1*). Segments were classified in three groups according to their location along the thallus axis: the lower-most 25% (*s2* = 1), the central 50% (*s2* = 2), and the apical-most 25% (*s2* = 3). The binary variable apical (*s3*) was set to 1 for apical segments or 0 for non-apical segments. It was also noted whether the segments were calcified or not (*s4*). The local branching pattern was characterized by counting the number of sister segments (*s5*) and the number of daughter segments (*s6*).

All segments were digitally photographed, numbered, and aligned with their base pointing downwards. Six qualitative characters used in traditional taxonomy were gathered for a subset of the specimens (Verbruggen *et al.*, 2005: table 3: *s7–s12*). These variables are referred to as *categorical shape variables*. Using landmarks superimposed on the digital segment pictures, a series of size properties of the segments were calculated (Verbruggen *et al.*, 2005: figure 2a, 2b and table 3: *s13–s17*). These variables are called *conventional measurements*; they showed a log-normal distribution and were transformed using the natural logarithm when necessary. The *ratio shape variables* were calculated from the conventional measurements; they are ratios of different couples of measurements (Verbruggen *et al.*, 2005: table 3: *s18–s22*). A geometric landmark analysis (Bookstein, 1989, 1991; Rohlf & Marcus, 1993) was carried out on the landmarks under the conditions specified in Verbruggen *et al.* (2005). The *partial warp scores* were extracted and saved to variables *s23–s28*. An elliptic Fourier analysis (Kuhl & Giardina, 1982) was carried out on the digitized segment outlines in Morpheus *et al.* (Slice, 2000) with the parameters set as in Verbruggen *et al.* (2005). The ten extracted harmonics yielded a total of 40 *Fourier coefficients* (Verbruggen *et al.*, 2005: table 3: *s29–s68*).

From each specimen, between five and eight segments spanning the thallus from the basal to the apical region were dissected. In total, data was gathered for 30 variables (Verbruggen *et al.*, 2005: table 4: *a1–a30*). Ten replicate measurements were made for all structures. The different measurements are visualized in Verbruggen *et al.* (2005: figures 2d & 2e) Measurements were taken from the medullar filaments throughout the segment (*a1–a8*) and at the node (*a9–a11*). Utricular properties were recorded from the outer three layers (*a12–a16*, *a17–a23* and *a24–a30* for the peripheral, secondary and tertiary utricles, respectively).

Exploratory analyses

A number of correlation-matrix based principal component analyses (PCA) were performed on a subset of segments and variables. The first analysis was carried out on the *conventional measurements*, *ratio shape variables* and *partial warp scores* (*s13–s28*). All segments were included in this analysis. The second PCA included only anatomical variables. It concerns medullar and nodal characters *a1–a6*, *a9* and *a10*, the variables associated with the peripheral utricles except *a16*, and some associated with the secondary utricles (*a17–a20*). Variables *a11* and *a16* were omitted because they were not applicable throughout the genus. The tertiary utricles were left out for the same reason. Variables *a7* and *a8* were left out

because they are dependent on *a6* (*a6* + *a7* + *a8* = 1). In this analysis, only dissected segments were included. The third ordination is a combined analysis of segment morphological and anatomical data (*s13–s28*, *a1–a6*, *a9*, *a10*, *a17–a20*). Only dissected segments were included. A fourth ordination was carried out on the combined data set plus thallus structure variables (*s1–s6*). In this analysis too, only dissected segments were included. All ordinations were carried out with the PCA&CA module of Statistica 6.0 (Statsoft Inc., Tulsa, OK).

Calculation of segment deviation and identification of deviant segments

The deviation of a segment within the specimen to which it belonged was estimated as the Euclidian distance between the segment and its specimen mean in the space spanned by the axes of ordinations of different subsets of variables.
Segment morphology – Deviation in *segment size* was calculated for each segment as the Euclidian distance from the segment in question to the specimen mean in the multivariate space spanned by all four principal components resulting from a PCA of the log-transformed *s13–s15* and *s17*. To calculate deviation in *segment shape*, the same procedure was used in the multivariate space spanned by the 23 principal components resulting from a PCA of ratio shape variables, partial warp scores, and fourier coefficients (first three harmonics only). A single measure of deviation of *segment morphology* was calculated by assessing the multivariate distances between the segments and the specimen mean in the space spanned by the four principal components from the *segment size* PCA above and the first four principal components from the *segment shape* PCA above. The three measures of *segment morphology* deviation explained above were calculated for all 1346 segments in the study.
Anatomy – The Euclidian distance between the location of the specimen mean and the segment in the multivariate space spanned by all seven principal components resulting from a PCA of variables *a12–a15* and *a17–a19* was calculated as a measure of deviation in *cortical structure*. The deviation in *medullar structures* was based on the multivariate distances in the space spanned by the axes of the PCA of *a1–a5* and *a9–a10*. The general measure of *anatomical deviation* was calculated for the space spanned by the first four principal components of both anatomy-based PCAs described above. The three measures of *anatomical deviation* were calculated for all 104 dissected segments.
For all six types of deviation, the segments with the 10% highest Euclidean distances were considered seriously deviant.

Typification of the seriously deviant segments

In order to typify the seriously deviant segments, their frequency patterns against thallus part (*s2*), apicality (*s3*), and calcification state (*s4*) were analysed. We used chi-square tests to test for significant deviations from the expected frequencies. Since for some cells in some tables the expected frequency exceeded the absolute value of the difference between the observed and expected frequency, log-likelihood ratio tests were used in addition to the chi-square tests. The G-values listed in Table 2 are equal to the double of the sum of all log-likelihood ratios in the table. For contingency tables containing cells with counts less than five, the chi-square and G-statistics were computed using Yates' correction. To complete the picture, ANOVA was used to test whether or not the deviation measures differed significantly between segments with different states of thallus part (*s2*), apicality (*s3*) and calcification state (*s4*). The effect of thallus part was tested using single-factor ANOVAs and differences were pointed out using Tukey HSD post-

hoc comparisons. The effects of apicality and presence of calcification were tested together in main-effects ANOVAs (no interactions). Segments from the basal thallus region were excluded from this analysis since apical and non-calcified segments usually don't occur in this region.

Influence of deviant segments on taxonomic power

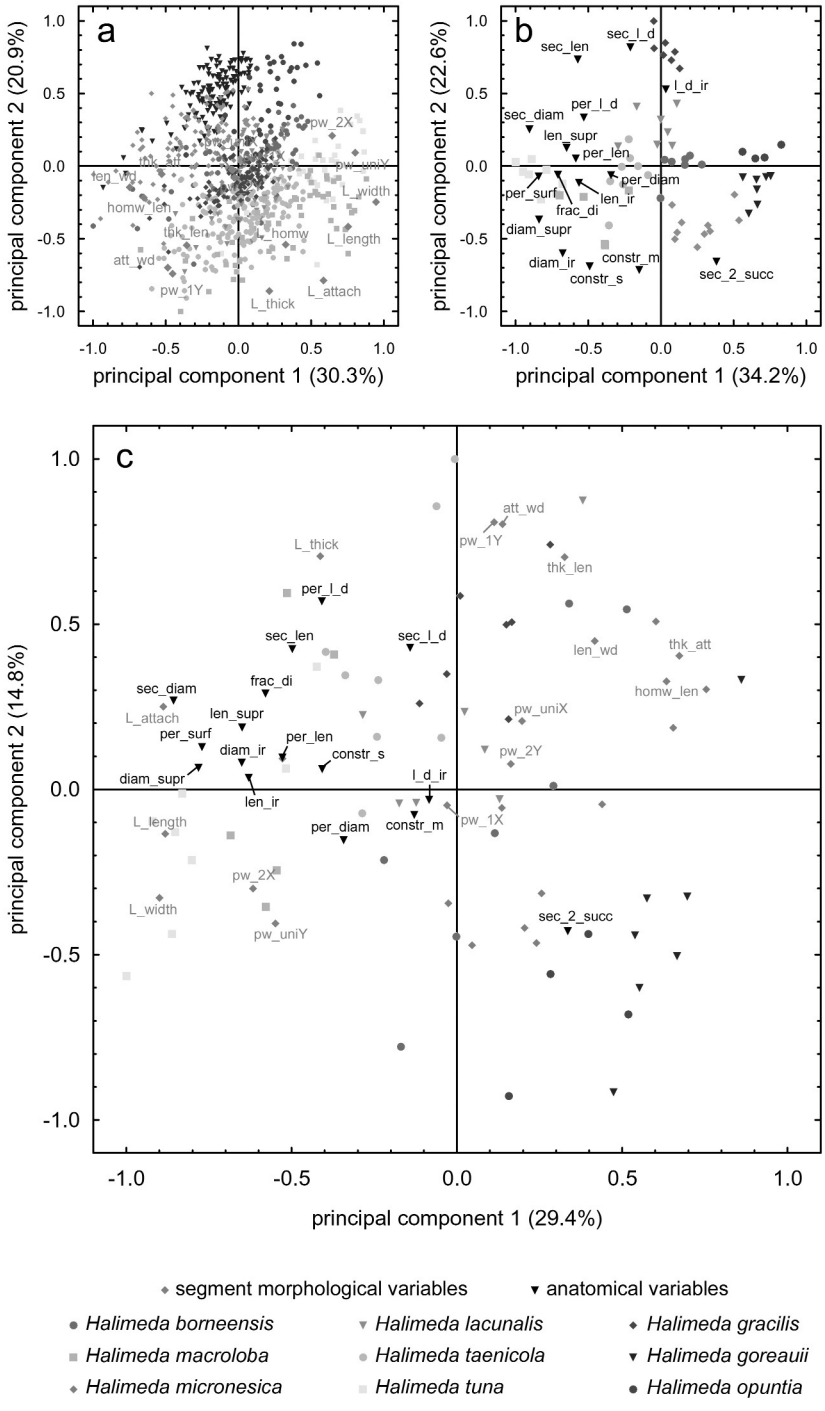
Taxonomic power of the morphometric data was estimated by comparing the adequacy of group membership prediction of segments in discriminant analysis (DA). Species-level clades from the molecular phylogeny were used as the *a priori* groups for the DA. To assess the influence of the deviant segments, three different DA models for discriminating between the nine species in the study were built from a selection of segment morphological variables (*s13*, *s17*, *s21–s28*). The three models differed in the segments that they included. The first model included all segments; the second model included only segments that were not seriously deviant; and the third model excluded segments from the basal thallus region, apical segments and non-calcified segments. Henceforth, these latter three segment types will be abbreviated as B-segments (basal zone), A-segments (apical) and N-segments (non-calcified), and the union of these segment types as BAN-segments. All effects were entered at once, prior probabilities were set to equal and cross-validation was set so that 50% randomly chosen segments were used to build the model and the remaining segments were used to evaluate it. This procedure was repeated 20 times with different randomisations of which segments to use for model building and which for testing.

For the anatomy, a similar approach was used. The same three DA models were built using variables *a1–a5*, *a9*, *a10*, *a12–a15*, *a17–a19*. Again, we performed DA in a non-stepwise manner with equal prior probabilities. Owing to the relatively low number of segments after the removal of BAN-segments, we chose not to use cross-validation to test the anatomy-based model. So in all three cases, all available segments (except those excluded by the model) were used to build and test the model.

Although most assumptions for DA were met, heteroscedasticity (non-constant variance) was an issue. Because DA is robust against violation of the heteroscedasticity assumption (Lachenbruch, 1975; Klecka, 1980), we further disregarded this assumption (see also Verbruggen *et al.*, 2005). Owing to the meticulous selection of variables for our models, multicollinearity was not an issue (see also Verbruggen *et al.*, 2005). All DAs were performed using the GDA module of Statistica 6.0 (Statsoft Inc., Tulsa, OK).

The degrees of classification success of the three discriminant models based on morphological data were compared using a one-way ANOVA and a Tukey HSD post-hoc test. Both these analyses were carried out in the ANOVA module of Statistica 6.0 (Statsoft Inc., Tulsa, OK).

Fig. 1. Ordinations of morphometric data. **a**. PCA biplot of segment morphological data. **b**. PCA biplot of anatomical data. **c**. Biplot of the PCA based on segment morphological and anatomical data. Case coordinates were divided by the maximum absolute value to fit within the [-1,1] range.


a
principal component 2 (20.9%)
principal component 1 (30.3%)
pw_2X
pw_uniX
L_width
ten_wd
homw_len
thk_len
L_homw
L_length
att_wd
pw_1Y
L_thick
L_attach
b
principal component 2 (22.6%)
principal component 1 (34.2%)
sec_l_d
sec_len
l_d_ir
per_l_d
sec_diam
len_supr
per_len
per_diam
per_surf
len_ir
frac_di
diam_supr
diam_ir
constr_m
constr_s
sec_2_succ
c
principal component 2 (14.8%)
principal component 1 (29.4%)
att_wd
pw_1Y
L_thick
thk_len
per_l_d
sec_len
sec_l_d
len_wd
thk_att
homw_len
sec_diam
frac_di
L_attach
len_supr
pw_uniX
per_surf
per_len
diam_ir
constr_s
pw_2Y
diam_supr
l_d_ir
len_ir
pw_1X
L_length
per_diam
constr_m
pw_2X
L_width
sec_2_succ
pw_uniY
segment morphological variables
anatomical variables
Halimeda borneensis
Halimeda lacunalis
Halimeda gracilis
Halimeda macroloba
Halimeda taenicola
Halimeda goreauii
Halimeda micronesica
Halimeda tuna
Halimeda opuntia

RESULTS

Molecular phylogeny

Figure 3 from Verbruggen *et al.* (2005) shows the placement of specimens used in our morphometric analyses within a phylogeny of the genus. All our specimens clustered within or as the closest sister to existing species clades.

Basic statistics

The dataset consisted of 21 specimens (Table 1). From a total of 1346 segments, data for 62 variables relating to the morphology of the segment and local thallus structure were gathered (6 thallus structure variables, 5 size variables, 5 ratio variables, 6 partial warp scores, 40 Fourier coefficients). In addition, six categorical variables were coded for a subset of 536 segments. Within the 104 segments that were dissected, a total of 2193 utricles were measured. Of these, 1008 were peripheral. From the slides with the medullar siphons, data was gathered from 827 nodal structures and 982 medullar siphon branches.

Ordinations

Fig. 1 shows the biplots of the PCAs performed on the segment morphological (Fig. 1a), anatomical (Fig. 1b), and combined (Fig. 1c) data sets. In all three PCAs the first two principal components accounted for about 50% of the total variance. The biplot of the segment morphological data (Fig. 1a) showed no separation between species. Although species clusters were clearly present, they showed serious overlap, both between closely and distantly related taxa. As for the variables, the distinction between the size descriptors (fourth quadrant; e.g. L_length, L_attach) and the shape descriptors (first and third quadrant; e.g. att_wd, pw_2X) was clear. Nonetheless, certain shape aspects were clearly correlated with size (e.g. pw_uniY and L_width).

In the PCA biplot of anatomical data (Fig. 1b) species clusters showed substantially less overlap than was the case for the segment morphological data. In general, closely related species clustered together. *Halimeda gracilis* fell out of the bunch because of its high loadings on the second principal component.

The third graph (Fig. 1c) was based on segment morphological and anatomical data. The segment size and shape variables were located primarily in the first and third quadrants whereas the anatomical variables concentrated in the second quadrant. The first axis was strongly correlated with variables measuring segment sizes and anatomical structures. Many segment shape descriptors (ratio variables, partial warp scores) had intermediate loadings on the first axis while shape characters of anatomical structures had low loadings. The highest loadings on the second principal component came from a subset of the segment shape variables. Owing to the limited number of cases, species clusters could hardly be recognized.

A fourth ordination (not shown) including the location of the segment along the thallus axis and the number of sister and daughter segments made clear that some of the morphometric variables correlated with these properties. For example, segments further away from the base were smaller and their medullar siphons were narrower. Also, the number of daughter segments was positively correlated with the width of the mother segment.

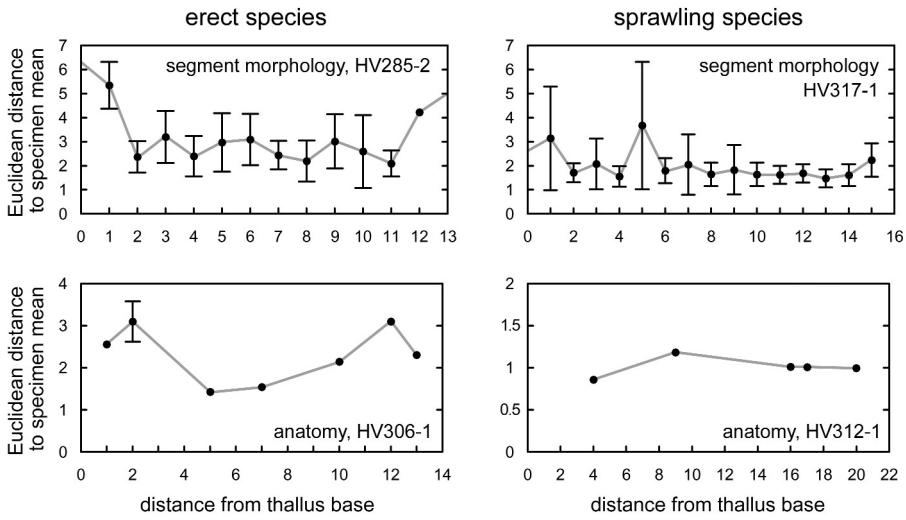


Fig. 2. Deviance of segments as a function of their distance from the thallus base. The graphs depict the course of the Euclidean distance between individual segments and the average segment in multivariate space as a function of the segments' location in the thallus. The upper graphs refer to segment morphological data; the lower graphs concern anatomical data. The graphs on the left hand side concern erect species (HV285-2, *H. taenicola* and HV306-1, *H. lacunalis*); those on the right hand side refer to sprawling species (HV312-1 and HV317-1, both *H. gracilis*). Deviances are given as the average ± 1 S.D.

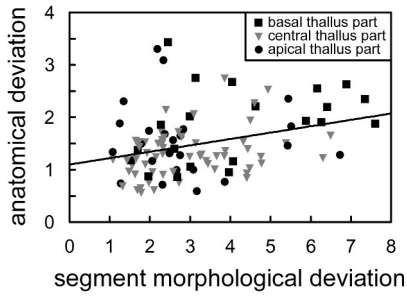


Fig. 3. Scatterplot of deviation in segment morphological versus deviation in anatomical characters.

Deviant segments

Fig. 2 shows representative examples of the patterns that segments' deviations follow along the thallus axis. Patterns tended to change according to the thallus habit of the species. For erect species, segment morphology was strongly deviant in the basal thallus region and less but variably deviant higher up the thallus. Sprawling species, on the other hand, showed no such pattern. Anatomical data, did not show consistent. Nonetheless, the correlation between segment mor-

phological deviation and anatomical deviation was significant (Fig. 3; Spearman rank test: $R = 0.3$, $p = 0.001$).

One-way ANOVA tests showed that Euclidean distances between the segments and their specimen mean were significantly different in the different thallus parts, both for segment morphology ($F = 65.2$, $p < 0.001$) and for anatomy ($F = 4.28$, $p = 0.016$). The patterns however, differed between segment mophological and anatomical deviations. Deviations of segment morphology were significantly higher in the basal thallus region than higher up the thallus (Tukey HSD: $p < 0.001$) while there was no significant difference between the central and the apical thallus regions ($p = 0.889$). Anatomical deviations were high in the basal and apical thallus parts, and low in the central part. The only significant difference was between the deviations of the basal and central parts (Tukey HSD, $p = 0.015$).

The apicality of a segment proved to have a significant effect on its morphological deviation ($F = 4.13$, $p = 0.042$) but not on its anatomical deviation ($F = 0.79$, $p = 0.37$). Calcification, on the other hand, seemed to influence the deviation of the anatomical and not of the segment morphological observations: the deviation of anatomical observations was significantly higher for non-calcified segments ($F = 3.98$, $p = 0.049$).

Frequency tables of seriously deviant segments (Table 2) supported the observation that segment morphology was much more deviant in the basal thallus region. The probability of encountering a seriously deviant segment in the basalmost quarter of the thallus was about 40% and the chi-square and log-likelihood-ratio tests showed that serious segment morphological deviation and thallus part were related (p-values < 0.001 for deviation of size, shape and general morphology). In general, the groups of apical and non-calcified segments did not turn out to contain more deviant segments than could be expected by chance alone.

Whereas the graphs for the anatomical data (Fig. 2, lower two graphs) did not show recurrent patterns and the ANOVAs left doubt for some characters, the contingency tables indicated that deviations of the observed frequencies from the expected frequencies were significant in the majority of cases. Significant deviation was observed for all the examined segment properties (thallus part, apicality and calcification). In all cases, the chi-square and log-likelihood ratio tests were significant for counts of segments seriously deviant in their medullar and general anatomical characters. When only cortical characters were examined, the observed frequencies of seriously deviant segments corresponded to the frequencies that could be expected by chance.

Influence of deviant segments on taxonomic power

Fig. 4 shows the classification success of individual segments in species groups, according to the six DA models built. Models incorporating all segments performed worst. For segment morphology, they achieved an average of 58% successful allocations. Both other models reached classification successes of about 10% higher. One-way ANOVA showed that the differences were significant ($F = 178.15$, $p < 0.001$, for both cases), and a post-hoc comparison found significant differences between the model with all segments (model 1) and both other (2 & 3) models (Tukey HSD: $p < 0.001$ for both cases). The models that excluded all deviant segments (model 2) and BAN-segments (model 3) did not differ significantly.

Models based on anatomical variables yielded very good (> 95%) separation between species. The model built using all segments performed worse than both other models, who achieved very comparable membership predictions (96.1% versus 98.8% and 98.4%).

Table 2. Frequency tables showing patterns of deviance. The observed frequencies of non-deviant and deviant segments are given in each first and second data column, respectively. Each third column gives the probability of deviance for the segment property in question. When larger than 0.25, this value is in boldface. Frequency tables are given for each of six variable groups (columns) and three segment characteristics (rows). The results of the chi-square and log-likelihood ratio tests for deviances from the expected frequencies are also listed: the chi-square and G-values are followed by the level of significance (*: 0.05 > p > 0.01; **: 0.01 > p > 0.001; ***: 0.001 > p). Subscripts c denote that Yates' correction was used.

		segment size			*segment shape*			*segment morphology*		
		no	*yes*		*no*	*yes*		*no*	*yes*	
thallus part	base	85	59	**0.41**	89	55	**0.38**	90	54	**0.38**
	center	763	47	0.06	758	52	0.06	755	55	0.07
	apex	361	31	0.08	362	30	0.08	364	28	0.07
		χ^2 = 169 (***)			χ^2 = 139 (***)			χ^2 = 132 (***)		
		G = 115 (***)			G = 96.1 (***)			G = 91.3 (***)		
apical	no	886	92	0.09	870	108	0.11	875	103	0.11
	yes	323	45	0.12	339	29	0.08	334	34	0.09
		χ^2 = 2.33			χ^2 = 2.93			χ^2 = 0.49		
		G = 2.25			G = 3.07			G = 0.50		
calcified	no	177	29	0.14	187	19	0.09	191	15	0.07
	yes	1032	108	0.09	1022	118	0.1	1018	122	0.11
		χ^2 = 4.05 (*)			χ^2 = 0.24			χ^2 = 2.23		
		G = 3.73			G = 0.25			G = 2.41		

		medulla			*cortex*			*anatomy*		
		no	*yes*		*no*	*yes*		*no*	*yes*	
thallus part	base	14	7	**0.33**	15	6	**0.29**	15	6	**0.29**
	center	55	3	0.05	53	5	0.09	55	3	0.05
	apex	21	3	0.13	22	2	0.08	20	4	0.17
		χ^2_c = 10 (**)			χ^2_c = 5.8			χ^2_c = 7.5 (**)		
		G_c = 7.7 (**)			G_c = 3.5			G_c = 5.6 (*)		
apical	no	78	8	0.09	74	12	0.14	78	8	0.09
	yes	12	5	**0.29**	16	1	0.06	12	5	**0.29**
		χ^2 = 5.2 (*)			χ^2_c = 0.27			χ^2 = 5.2 (*)		
		G = 4.3 (*)			G_c = 0.29			G = 4.3 (*)		
calcified	no	9	5	**0.36**	13	1	0.07	9	5	**0.36**
	yes	81	8	0.09	77	12	0.13	81	8	0.09
		χ^2 = 7.8 (**)			χ^2_c = 0.05			χ^2 = 7.8 (**)		
		G = 6.1 (*)			G_c = 0.06			G = 6.1 (*)		

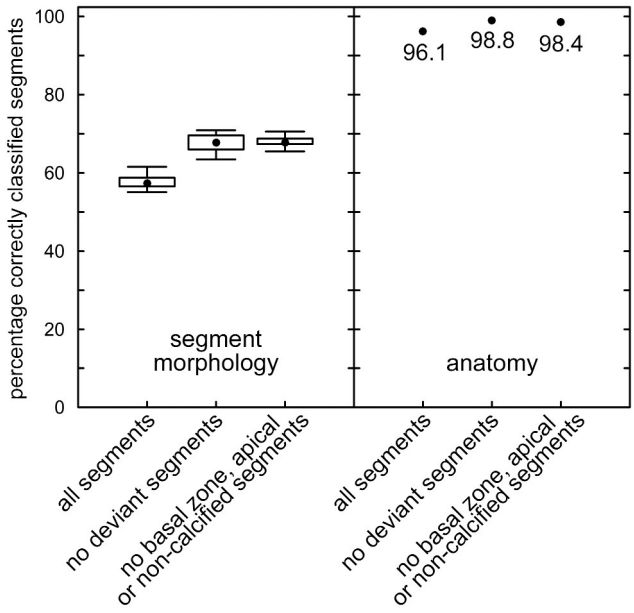


Fig. 4. Adequacy of species group membership prediction of different groups of segments. The left-hand side of the graph depicts classification success of segment morphology-based DA models; the right-hand side is based on DA models built using anatomical variables. The segments that were included/excluded from the analyses are indicated along the horizontal axis. Boxes indicate the 25–75 percentile range and whiskers depict the non-outlier range.

DISCUSSION

Qualitative characters and measurements of a very limited number of structures have dominated *Halimeda* taxonomy for many years. Over the last few decades, however, it has become apparent that quite a few taxa are non-monophyletic, embody considerable cryptic diversity, or are, perhaps as a consequence of regional bias, ill-defined (Noble, 1987; South, 1992; Dargent, 1997; Kooistra *et al.*, 1999, 2002; Verbruggen & Kooistra, 2004). This and a parallel study (Verbruggen *et al.*, 2005) aim to lay the foundation for a new approach towards taxonomy that encompasses molecular and morphometric information.

In the course of explorative analyses of our data, segments showing considerable morphometric deviation from the remainder of the segments in the specimen were detected. Deviations in both anatomical features and segment morphology were observed. The revelation that segments towards the base of the algal body showed larger deviation in erect species while they did not in sprawling species does not require much explanation. The basal segments of erect species are adapted for attachment to the substratum. Sprawling species, on the other hand, depend on secondary holdfasts for attachment. Their primary base is often difficult to track down or becomes lost altogether when the plants spread out and,

with time and incidents, lose connection with their base. In our specimens of *H. gracilis* and *H. opuntia*, no primary holdfasts were present and the basal reference segment was arbitrarily chosen.

The observation that deviations at the base exceeded those of other thallus parts was confirmed statistically in subsequent analyses. It was also demonstrated that apical and non-calcified segments had a tendency towards aberrance. Although the latter had been previously indicated by the ANOVAs, the contingency tables provided conclusive evidence for the divergent proportions of seriously deviant segments in these segment types. Notwithstanding the fact that chi-square analyses prohibit drawing conclusions in terms of cause and consequence, in combination with the high probabilities of encountering seriously deviant segments in the non-calcified and apical segment classes and the results of the ANOVAs, little doubt remains about the anomaly of these segments. Here again, the explanation is obvious from the biology of *Halimeda* thalli. Branches grow one segment at a time (Hillis-Colinvaux *et al.*, 1965, 1980; Drew & Abel, 1988; Hay *et al.*, 1988). New segments develop at night (Hay *et al.*, 1988) and, during the following 2–3 days, grow to their full size and calcify (Hillis-Colinvaux *et al.*, 1965, 1980; Hay *et al.*, 1988). This probably explains why segment size is more often deviant in non-calcified segments. The number of segments with seriously deviant shape, on the other hand, did not appear to be related to the degree of calcification of the segment, indicating that segments take on a shape similar to that of adult segments before completion of the calcification process. This is also suggested in fig. 1b from Hay *et al.* (1988).

From an anatomical perspective, segment formation starts from uncorticated regions on the distal rim of the mother segment (Hillis-Colinvaux, 1980). From these regions, a tuft of filaments grows outwards. This tuft becomes increasingly organized and starts showing differentiation between medulla and cortex between day 1 and 2. Utricles attach to one another near the end of this period. After this, substantial growth of filaments and utricles still has to occur to attain the final segment size around day 2–3. This explains why the non-calcified segments in our study were often seriously deviant in anatomical characters. These non-calcified segments typically had attached utricles and had already taken the final segment shape, but had not yet attained the final segment size. As a consequence, utricles and medullary filaments were smaller than those of adult segments.

The question remains: does the incorporation of deviant segments into a morphometric dataset hamper taxonomic studies based on this dataset? Using the discriminant analysis methods introduced in Verbruggen *et al.* (2005), the effect of exclusion of certain segment types on the taxonomic power of the morphometric data was verified. The statistical experiment was designed to allow three comparisons. Firstly, the effect of introducing deviant segments to the analysis was verified. Secondly, the effect of introducing BAN-segments was examined. Lastly, the taxonomic power of models with deviant segments was compared to that of models with BAN-segments.

The juxtaposition of the results with and without deviant segments leaves no doubt about the significant deterioration of discriminating power caused by the deviant segments (Fig. 4, left panel). For the anatomical data, results cannot be compared statistically, but a rise of taxonomic power was observed when deviant segments were omitted (Fig. 4, right panel). Similarly, excluding BAN-segments yielded results comparable to analyses that excluded deviant segments. This result, in combination with the observation that deviant segments are primarily found in

BAN-regions, suggests that BAN-segments are primarily responsible for the lowered taxonomic power in our analyses that included all segments.

In earlier taxonomic works, the use of mature segments from the central thallus region for anatomical investigation was proposed (Taylor, 1950; Hillis-Colinvaux, 1980), however without any rationale being given. In more recent literature it was shown that aberrant basal segments can actually aid taxonomy. Noble (1986) was the first to illustrate the difference in anatomy of segments from the base and center of thalli and used this information as supplementary evidence to erect *H. magnidisca*. Dragastan *et al.* (2002) investigated the anatomy of segments throughout the thalli of recent species and compared these to the anatomy of fossil segments. They judged that several fossil segments were mistakenly described as new species while they actually concerned aberrant (basal) segments of recent species.

Deviant segments from the basal thallus region have thus on the one hand generated substantial taxonomic confusion in the past, but on the other hand integration of their morphological properties has apparently led to a firmer taxonomy. Our data clearly confirm the first statement: we demonstrated that basal segments blur group boundaries. Unfortunately, in its current form and extent, our dataset prohibits testing the second statement of increased taxonomic adeptness by incorporating information of basal segments as a set of additional characters.

The present study explored a combined molecular and morphometric approach towards *Halimeda* taxonomy. Our aim with this combined approach was to tackle taxonomic issues within complexes of closely related or morphologically similar species. With the present sampling and analysis methods, deviant segments significantly lowered the taxonomic power of the data. Exclusion of all seriously deviant segments or only the BAN-segments yielded nearly-identical results. Therefore, we suggest the exclusion of BAN segments from future studies. However, if the present morphometric method fails in certain cases, the answer could lie in the incorporation of deviant segments. In this case, data from the basal segments must not be merged with that of segments from the central region but must be coded in separate variables. In other words, they must be treated as a supplementary source of data that does not compete with the data from non-deviant segments.

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