Molecular Phylogenetic Evidence for a Reversible Morphogenetic Switch Controlling the Gross Morphology of Two Common Genera of Green Seaweeds, *Ulva* and *Enteromorpha*

Ian H. Tan, Jaanika Blomster,† Gayle Hansen,‡ Elina Leskinen,§ Christine A. Maggs,† David G. Mann,* Hans J. Sluiman,* and Michael J. Stanhope†*

*Royal Botanic Garden Edinburgh, Edinburgh, U.K.; †School of Biology and Biochemistry, The Queen's University of Belfast, Belfast, U.K.; ‡Hatfield Marine Science Center, Oregon State University, Newport, Oregon; and §Department of Genetics, Uppsala University, Uppsala, Sweden

Ulva and *Enteromorpha* are two of the most common, ubiquitous, and environmentally important genera of green seaweeds. They are widely regarded as easily distinguishable because of their dramatically different morphologies: *Ulva* species are flat, lettucelike blades two cell layers thick, and *Enteromorpha* species form hollow liquid- or gas-filled tubes one cell thick, which may also be highly branched. We present molecular phylogenetic analyses of nuclear ribosomal RNA ITS sequences from 39 samples representing 21 purported species within these two genera. The results clearly indicate that the two genera are not respectively monophyletic and that the characteristic *Ulva* and *Enteromorpha* morphologies have arisen independently several times throughout the evolutionary diversification of the group. The analyses demonstrate that this radical change in gross morphology can also happen within clades exhibiting sequence divergence typical of conspecific assemblages of this group. We suggest that this morphological flexibility is the result of some form of developmental switch that results in either blades or tubes, but that this putative switch must be activated relatively infrequently, since there is evidence that some lineages have retained their form for significant periods. This discovery suggests a possible new model system for study of the molecular mechanisms involved in the interplay between environmental stimuli and plant development.

Introduction

Ulva (the sea lettuce) and *Enteromorpha* (the gut weed) are two of the best known marine green algal genera. They are responsible for widespread ''green tides'' and marine fouling, both major global environmental problems (Fletcher 1996; Callow et al. 1997). These ubiquitous seaweeds are common throughout the world in marine and estuarine habitats, where they are highly tolerant of variable salinity, temperature, and water quality and grow rapidly in nutrient-rich habitats. They are widely used as model organisms for experimental studies of marine biofilms and spore adhesion (Callow et al. 1997), plant physiology (e.g., Grobe and Murphy 1997), as bioindicators of organic and inorganic pollution (Fletcher 1996; Leal et al. 1997). They also have applications in aquaculture for polyculture systems and as biofilters (Brzeski and Newkirk 1997; Troell et al. 1997) and in medicine as anticarcinogenic, antimutagenic, and antiviral agents (Ivanova et al. 1994; Okai et al. 1994).

Linnaeus (1753) recognized only the genus *Ulva*, but its representatives were subsequently separated into two genera (see Silva 1952) on the basis of their gross morphologies, which differ very significantly: *Ulva* species are flat, lettucelike plants two cell layers thick, and *Enteromorpha* Link (1820) species form hollow liquid- or gas-filled tubes one cell thick, which may also exhibit a profusion of fine branches (fig. 1). *Ulva* and *Enteromorpha* are widely regarded as easily recognizable seaweed genera (e.g., Nybakken 1997). However, despite their obvious differences in habit, they share many cellular, ultrastructural, physiological, and developmental characters, including having the same type of highly tolerant and fast-adhering spores that contribute to their success as significant marine fouling organisms.

Here, we present a molecular phylogenetic analysis of the multicellular green algal genera *Ulva* and *Enteromorpha* which demonstrates that these taxa are not distinct evolutionary entities. In other words, the dramatic differences in gross morphology of these two purported taxa bear no relationship to their evolutionary history. Indeed, this flexibility in gross morphology can be identified even within well-supported clades exhibiting very little sequence divergence, and we therefore suggest that it is the result of a morphogenetic switch activated during early development.

Materials and Methods

Plant material was collected from various locations around the world, and voucher specimens for most of the studied samples were deposited in various herbaria (table 1). Samples were identified on the basis of morphological characters such as habit and details of cell arrangement and organelles (see Blomster, Maggs, and Stanhope 1998), using the identification scheme proposed by Bliding (1963, 1968) and modified by subsequent authors (e.g., Koeman and van den Hoek 1981, 1982*a*, 1982*b*, 1984). Epiphyte-free samples were subjected to a modified total genomic DNA extraction protocol for algal material (Tan and Druehl 1996). The polymerase chain reaction (PCR) was used to amplify the nuclear ribosomal internal transcribed spacers (ITS1 and ITS2) and 5.8S rDNA for 19 *Ulva* samples and 17 *Enteromorpha* samples, representing, in total, 21 cur-

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Address for correspondence and reprints: Michael J. Stanhope, Smith-Kline Beecham, 1250 S. Collegeville Rd., Collegeville, PA 19426. E-mail: michael_j_stanhope@sbphrd.com.

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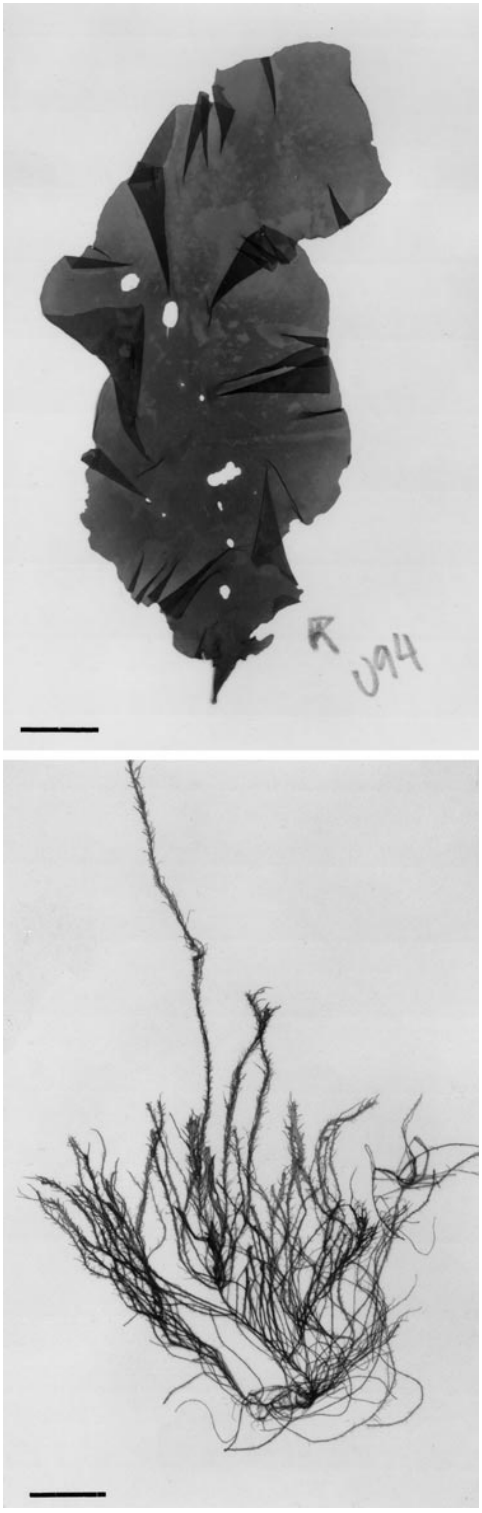

FIG. 1.—Samples of *Ulva* sp. (above) and *Enteromorpha muscoides* (below), both from Ireland, showing contrast in morphology between unbranched blades and highly branched tubes (scale bars represent 1 cm).

rently recognized species from around the world. Primers for the PCR were as follows: ITS5 (5′-GGCAATAACAGGTCTGT-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), situated in the 18S and 26S loci, respectively (White et al. 1990). The resulting fragments were directly sequenced on both strands by dye terminator cycle sequencing reactions (ABI) that were subsequently loaded on an ABI 373 or 377 sequencer. Internal sequencing primers were designed as necessary. Sequences were obtained on both strands.

The three sequences from France and the sequences for *Enteromorpha compressa* (Ireland) and *Enteromorpha procera* (Sweden) came from earlier publications (Leskinen and Pamilo 1997; Blomster, Maggs, and Stanhope 1998; Coat et al. 1998). The *Gloeotilopsis planctonica* sequence is from GenBank, and the remaining 35 sequences are new to this study and have been deposited in GenBank (table 1). We used CLUSTAL X (Thompson et al. 1997) to align the sequences initially, and SeqPup (Gilbert 1996) to ''fine-tune'' the alignment by eye. The final alignment was arrived at using a conservative approach in which we excluded all positions in which there was any possible ambiguity in the sequence alignment. The resulting data set comprised 504 aligned nucleotide positions for 39 operational taxonomic units (OTUs). The sequence alignment is available from the EMBL file server under accession number ds38913.

Phylogenetic analyses, including neighbor joining, maximum parsimony and maximum likelihood, were performed using PHYLIP 3.5c (Felsenstein 1993) and PAUP 4.0 (Swofford 1998). Two representatives of a different genus from the same order as *Ulva* and *Enteromorpha* (*Blidingia chadefaudii* and *Blidingia minima*; order Ulvales), as well as a filamentous green alga (*Gloeotilopsis planctonica*) from a different order (Ulotrichales), served as outgroup taxa in all phylogenetic reconstructions. All trees were rooted at *Gloeotilopsis planctonica.* Clade strength was assessed by bootstrap, with 1,000 replications for neighbor-joining and maximum-parsimony analyses and 100 replications for maximum-likelihood analysis. Neighbor-joining trees were estimated by maximum-likelihood distances. Parsimony analyses employed 50 random input orders. Maximum-likelihood analyses (DNAML) used empirical base frequencies, a transition : transversion ratio of 2:1, and assumed equal rates between sites. In several instances, statistical tests were conducted to assess the likelihood of constrained topologies relative to the most parsimonious trees. These included winning-sites Prager and Wilson (1988), Templeton (1983), and Kishino-Hasegawa (Kishino and Hasegawa 1989) tests and were implemented using PAUP 4.0. Kishino-Hasegawa tests were also used to evaluate the likelihood of constrained topologies relative to the maximum-likelihood trees.

Results and Discussion

All phylogenetic analyses resulted in a monophyletic *Ulva*/*Enteromorpha* assemblage with 100% bootstrap support, but the respective genera were not monophyletic (fig. 2). Sequence divergence within the *Ulva*/*Enteromorpha* clade ranged from 0% to 21%; however, all divergences in excess of 17% involved pairwise comparisons with one particular OTU, *Ulva olivascens*, which occupied a strongly supported sister group posi-

Table 1
Samples of *Enteromorpha, Ulva,* and *Blidingia* Sequenced, Along with Collection Locality, Herbarium Accession Number, and EMBL Accession Number

Sample	Collection Locality and Country	Herbarium Accession No.	EMBL Accession No.
E. compressa I	Ythan Estuary, Aberdeenshire, Scotland	E00068503[a]	AF013981
E. compressa II	Ythan Estuary, Aberdeenshire, Scotland	E00068504[a]	AF013982
E. compressa III	Quarterland Bay, Strangford Lough, Northern Ireland	F11409[b]	AJ234301
E. compressa IV	Portaferry, Strangford Lough, Northern Ireland	F11404[b]	AJ234302
E. flexuosa	Sweden	A00277[c]	AJ234306
E. intestinalis I	Ythan Estuary, Aberdeenshire, Scotland	E00068499[a]	AJ000207
E. intestinalis II	Opinan, Wester Ross, Scotland	E00068500[a]	AJ000212
E. intestinalis III	Gills Bay, Highland, Scotland	E00068501[a]	AJ234299
E. intestinalis IV	Bamfield, British Columbia, Canada	E00068502[a]	AJ234300
E. intestinaloides	Dunbar, East Lothian, Scotland	E00068505[a]	AJ234303
E. linza I	Ythan Estuary, Aberdeenshire, Scotland	E00068506[a]	AJ000203
E. linza II	Dunbar, East Lothian, Scotland	E00068507[a]	AJ000204
E. muscoides	Backstrand, Tramore, Ireland	F11622[b]	AJ234307
E. procera (as *E. ahlneriana*)	Sweden	A00278[c]	AJ012276
E. prolifera I	Ythan Estuary, Aberdeenshire, Scotland	E00068508[a]	AJ234304
E. prolifera II	Ythan Estuary, Aberdeenshire, Scotland	E00068509[a]	AJ234305
Enteromorpha sp.	Ythan Estuary, Aberdeenshire, Scotland	E00068510[a]	AJ234308
U. armoricana	Brittany, France[d]		
U. californica	Otter Crest, Oregon, U.S.A.	E00068515[a]	AJ234315
U. fenestrata	North Boardman St. Park, Oregon, U.S.A.	E00068516[a]	AJ234316
U. lactuca I	Kirkwall pier, Orkney, Scotland	E00068511[a]	AJ234309
U. lactuca II	Ballyhenry Island, Strangford Lough, Northern Ireland	F11621[b]	AJ234310
U. lactuca III	Sweden	A00279[c]	AJ234311
U. olivascens	Carna, County Galway, Ireland	F11623[b]	AJ234322
U. pertusa	Guryongpo, East Coast, Korea	E00068521[a]	AJ234321
U. pseudocurvata I	Ythan Estuary, Aberdeenshire, Scotland	E00068512[a]	AJ234312
U. pseudocurvata II	Ythan Estuary, Aberdeenshire, Scotland	E00068513[a]	AJ234313
U. pseudocurvata III	Ythan Estuary, Aberdeenshire, Scotland	E00068514[a]	AJ234314
U. rigida I	Redpoint, Wester Ross, Scotland	E00068518[a]	AJ000208
U. rigida II	Skara Brae, Orkney, Scotland	E00068519[a]	AJ234319
U. rigida III	Brittany, France[d]		
U. rotundata	Brittany, France[d]		
U. scandinavica I	Langstone Harbour, Portsmouth, England	E00068517[a]	AJ234317
U. scandinavica II	Langstone Harbour, Portsmouth, England	F11676[b]	AJ234318
U. taeniata	Seal Rock, Oregon, U.S.A.	E00068520[a]	AJ234320
Ulva sp.	Stromness Harbour, Orkney, Scotland	E00068522[a]	AJ234323
B. chadefaudii	Carnalea, Belfast Lough, Northern Ireland	F11624[b]	AJ012309
B. minima	Scotland	E00068523[a]	AJ000206
Gloeotilopsis planctonica	Sucdol, former Czechoslovakia		Z28970

[a] Royal Botanic Garden Edinburgh Herbarium.
[b] Ulster Museum Herbarium.
[c] Uppsala University Herbarium.
[d] Coat et al. (1998).

tion to all other *Ulva*/*Enteromorpha* samples (fig. 2). Several strongly supported interspecific and intergeneric clades were evident within which sequence divergence was extremely low (less than 0.5%; see fig. 3 for tree with branch lengths drawn proportional to amount of sequence change). The greatest sequence divergence between any two *Ulva* and *Enteromorpha* species (21%) was less than that between *B. chadefaudii* and *B. minima* (34%), which were not monophyletic in any of our analyses.

The phylogenetic analyses showed clearly that the overall morphology of a sample (broad bladelike vs. narrow tubular) was not correlated with its position within the *Ulva*/*Enteromorpha* clade (figs. 2 and 3). All methods of phylogenetic analysis congruently and convincingly supported several mixed clades of *Ulva* and *Enteromorpha* (figs. 2 and 3). Bootstrap support (BP) for mixed clades containing representatives of both genera ranged between 90% and 100%. The type species of both genera, *Ulva lactuca* and *Enteromorpha intestinalis*, were placed in a well-supported clade (BP 90–93) with two other *Enteromorpha* species (*E. compressa* and *E. intestinaloides*) and three other *Ulva* species (*U. pseudocurvata*, *U. californica*, and *U. rotundata*) (fig. 2). All three of these methods of phylogenetic analysis used both transition and transversion substitutions in the phylogenetic reconstructions. Graphical plots indicated that there was no evidence for transition saturation at even the most extreme evolutionary distances within this clade (data not shown), suggesting that the use of both types of substitutions in phylogenetic reconstructions is appropriate. Even so, overly conservative analyses, such

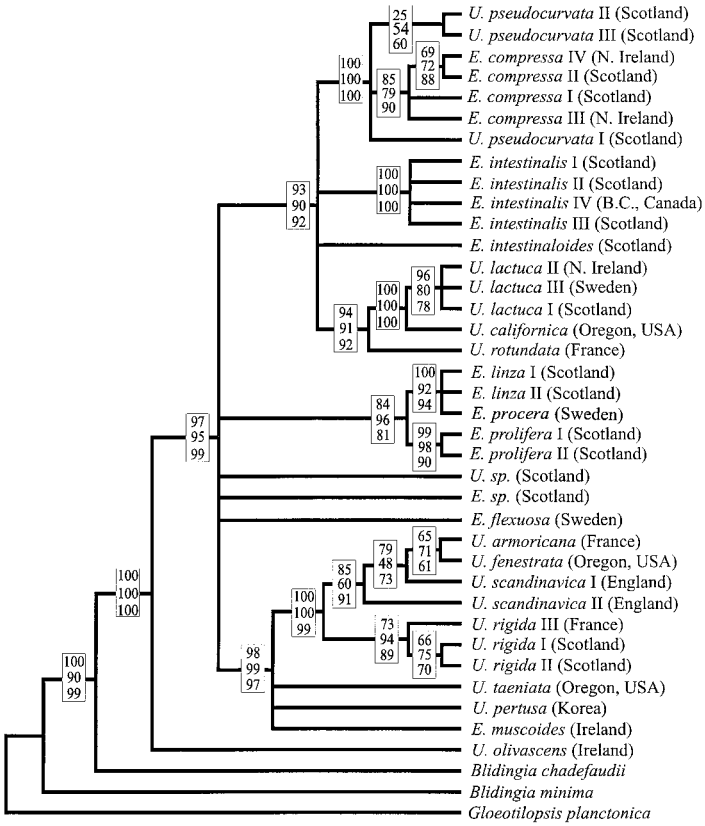

FIG. 2.—Majority consensus bootstrap tree of parsimony (top bootstrap value), neighbor-joining (middle value), and maximum-likelihood (bottom value) analyses of *Ulva, Enteromorpha,* and *Blidingia* internal transcribed spacer sequences.

as transversion parsimony, that discard all the phylogenetic signal contained within the transitions still resulted in mixed generic clades with similarly high levels of bootstrap support (92%–100%).

Constrained trees that supported the respective monophylies of *Ulva* and *Enteromorpha* added 134 substitutions to the most parsimonious tree, representing an increase of approximately 17% (score of most parsimonious tree = 792). All three parsimony-based tests, as well as the single maximum-likelihood-based statistical test, rejected the concept of these two genera as distinct entities at $P < 0.0001$ (highest likelihood tree: −LnL = 3,623.03; best tree supporting separate monophyly of *Ulva* and *Enteromorpha*: −LnL = 3,892.67; SD = 37.32). This was true with or without the inclusion of the clearly distinct and outlying sequence for *Ulva olivascens* (without *U. olivascens,* number of steps for most parsimonious tree = 723; number of steps for best tree supporting *Ulva* and *Enteromorpha* monophyly = 849; highest likelihood tree: −LnL 3,375.49; best tree supporting *Ulva* and *Enteromorpha* monophyly: −LnL = 3,640.29; SD = 38.22).

Our data show that both bladelike and tubular morphologies can occur even within groups exhibiting very little sequence divergence. *Ulva pseudocurvata* samples had the typical *Ulva* morphology of simple distromatic blades without cavities, yet formed a strongly supported clade with samples of the tubular, usually highly branched, *E. compressa* (fig. 2). Sequence divergence within this clade ranged from only 0.42% to 1.7% (fig. 3), comparable with levels of sequence divergence within other clearly monospecific groupings (e.g., *E. intestinalis,* 0.00%–2.0%). Another example of dramatic difference in form between very closely related plants involves the *Enteromorpha linza*/*Enteromorpha procera* clade. *Enteromorpha linza* has an almost *Ulva*-like morphology, and *E. procera* is highly branched, yet bootstrap support for this clade is in excess of 92%, and sequence divergence between the members is less than 0.5%.

This flexibility of form among genetically homogeneous plants corroborates results of earlier culture studies that showed the development of both tubular and bladelike thalli from the same zoospore populations of several *Ulva* species (Gayral 1967; Bonneau 1977; Provasoli and Pintner 1980). Gayral (1967) reported the occurrence of tubular thalli from both zoospore and gamete (parthenogenetic) cultures. The majority of both zoospores and parthenogenetic gametes developed into leafy thalli, whereas some developed into tubular ones. Bonneau's (1977) culture study showed progeny with a combination of leafy and cylindrical morphologies; some of the thalli were distromatic in some parts and tubular in others. On the basis of these experiments, Bonneau (1977) questioned the validity of maintaining *Ulva* and *Enteromorpha* as two separate genera. Provasoli and Pintner (1980) showed that *Ulva* cultures can form uniseriate filaments when axenic or *Enteromorpha*-

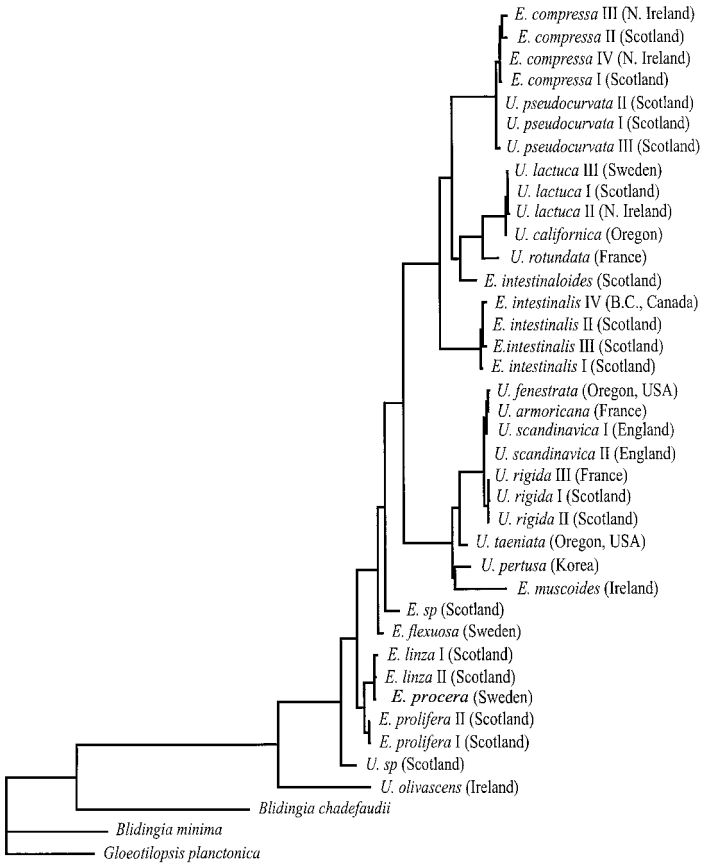

FIG. 3.—Maximum-likelihood tree of *Ulva, Enteromorpha,* and *Blidingia* ITS sequences with branches drawn proportional to amount of sequence change.

like tubes if grown with particular bacteria. They concurred with Bonneau's (1977) conclusion that there were no valid criteria for the two genera, but their caution was virtually ignored, as *Ulva* and *Enteromorpha* are still currently regarded as separate autonomous genera (see, e.g., Burrows 1991; Nybakken 1997). Nakanishi et al. (1996) found that live bacteria are required for normal morphogenesis of *Ulva pertusa* in culture. These experiments were conducted on a range of *Ulva* and *Enteromorpha* species but only under laboratory conditions. Our results indicate that the switch between blade and tube morphology happens in wild populations under natural conditions, e.g., in *E. compressa* in the Ythan Estuary, Scotland, and has occurred at various times throughout the evolutionary diversification of the *Ulva*/*Enteromorpha* group.

Our molecular phylogenetic results help place these earlier experimental results in their proper evolutionary context. *Ulva* and *Enteromorpha* are not distinct evolutionary entities, and encoded within the genomes of the members of this clade is some form of developmental switch (or switches) that can result in a plant with either a blade or a tube morphology. It should be noted, however, that it is possible that the final gross morphology of these organisms may not arise through the same developmental pathway (i.e., single reversible switch), but instead several nonhomologous developmental pathways (i.e., multiple nonreversible switches) may be utilized to achieve this same end. Our data do not allow us to clearly differentiate between these two possibilities. The data do suggest, however, that in the sea at least, this switch(es) is activated infrequently. Otherwise, we would expect our phylogenetic tree to have even more mixed clades of *Ulva* and *Enteromorpha.* Instead, we have several nodes that support the monophyly of geographically distinct isolates clearly recognizable as *Ulva,* with a similar scenario for certain groups of *Enteromorpha.* It would seem, therefore, that the switch may stay locked in one position for a significant period. From this, we suggest that certain relatively rare environmental circumstances may act as the stimulus for the morphogenetic switch. This, of course, raises the conundrum of why in culture studies the switching appears to be much more frequent, and, unfortunately, for this we have little clear explanation. Perhaps the conditions of culture create a type of stress-induced switching that is less common in the wild. At the same time, it should be pointed out that we have sampled only a portion of the putative species of *Enteromorpha* and *Ulva.* Although there are no published estimates of the number of species worldwide, in the European Atlantic alone, there are at least 15 putative species recorded for *Ulva* and at least 28 putative species for *Enteromorpha* (Gallardo et al. 1993; Guiry 1997). The two genera are also found in many geographic areas that we have not

sampled. Therefore, it is quite likely that more samples would reveal many more such switching events.

Salinity variation might be one of several factors causing the change between leafy and cylindrical morphologies in *E. compressa*/*U. pseudocurvata.* The three *U. pseudocurvata* samples and the two Scottish *E. compressa* samples were collected from the same 4-km-long estuary in Aberdeenshire, Scotland. Both of the *E. compressa* samples were collected from sites by the mouth of the estuary opening up to the North Sea, while the three *U. pseudocurvata* samples were collected from the top of the estuary closest to the freshwater source from the Ythan River. However, brackish water alone does not promote flat distromatic morphology, for *Enteromorpha* species with distinctive hollow cylindrical morphologies are commonly found in freshwater habitats (Canter-Lund and Lund 1995). We suggest that various forms of environmental stress, including the absence or presence of certain bacteria, may play important roles in activating the morphogenetic switch.

Ulva and *Enteromorpha* are simple algae with a limited number of morphological characters, many of which are quantitative in nature. A virtually exhaustive list of morphological characters, over and above blade morphology, that might be useful in distinguishing taxa would include the following: (1) number of pyrenoids per cell, (2) cell organization (short rows, long rows, unorganized), (3) cell size, (4) chloroplast position in the cell, and (5) chloroplast motility. However, a thorough examination of these characters from representatives in figures 2 and 3 does not allow us to identify any unique morphological synapomorphies for clades in this tree. Thus, there are no currently recognized morphological features that uniquely distinguish any systematic level within this group (species, section, or genus); however, some species, at least, can be identified by a suite or combination of characters. For example, *E. compressa* can generally be distinguished from *E. intestinalis* by the presence of branching (Blomster, Maggs, and Stanhope 1998). Although new morphological characters can be sought, we are not hopeful that they will be found, because of the overall simplicity of these algae. There are numerous modern-day examples of multicellular organisms wherein morphology results in phylogenetic trees that are in disagreement with molecular data (e.g., Gatesy et al. 1996; Bena et al. 1998; Dubuisson, Hébant-Mauri, and Galtier 1998; Feller and Hedges 1998; Stanhope et al. 1998). This is particularly true of phylogenies dealing with higher systematic levels at which there are fewer characteristics identifiable as synapomorphies (e.g., Gatesy et al. 1996; Feller and Hedges 1998; Stanhope et al. 1998). However, it is extremely rare in multicellular organisms for all of the recognizable morphological characteristics of a genus to bear no relationship whatsoever to the documentation of common ancestry of, or within, that group. This is, of course, more likely the simpler the organism, and it may be additionally confounded within this group because of the possibility that some of the above five characteristics are mere allometric consequences of the radical change in form from blade to tube or vice versa.

Because our data indicate that there is no evolutionary distinction to be made between *Ulva* and *Enteromorpha,* we propose collapsing *Enteromorpha* back into *Ulva* as originally circumscribed by Linnaeus (1753). We are not, however, suggesting that this means there is only a single species. On the contrary, with sequence divergence of up to 21% within the *Ulva*/*Enteromorpha* clade, and with several strongly supported clades within this larger grouping, there must be several species. We also know that members of different well-supported clades within *Ulva*/*Enteromorpha* do not form viable hybrids (e.g., *E. compressa* and *E. intestinalis*; see Blomster, Maggs, and Stanhope 1998). Within what we now propose to be a single genus, we believe that some species, at least, will continue to be identifiable using a suite of morphological characters in concert with particular environmental features.

Within this single monophyletic *Ulva*/*Enteromorpha* grouping, there are several cryptic clades that could not be detected without molecular data. In addition to the *pseudocurvata*/*compressa* grouping, other such cryptic clades include the association of *U. lactuca* with *U. californica* and the grouping of *U. armoricana, U. fenestrata, U. scandinavica,* and *U. rigida.* Sequence divergence figures within both of these clades are less than 0.5%. Such low sequence divergence suggests a more detailed examination of hypotheses proposing these entities to be distinct taxa is certainly warranted.

These results will be of interest not only to marine ecologists, but also to those concerned with plant developmental biology. Marine biologists concerned with the control of green tides and marine fouling will need to be cognizant of the fact that they cannot with any assurance discriminate an *Ulva* taxon from an *Enteromorpha* taxon. This means, for example, that control programs could well be faced with different marine fouling populations that possess tremendous genetic diversity. We also feel that our findings could provide the basis for the study of heterochronic genes in a very different model organism. Most of the studies pertaining to such developmental timing genes in plants are on slime molds and corn (Slack and Ruvkun 1997). The *Ulva*/*Enteromorpha* algal assemblage could provide an important intermediate group in the comparative study of the mechanisms controlling developmental timing, although this would need to be accompanied/preceded by further work on the basic biology of these green algae.

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