The use of medullary unit patterns of intergenicula and genicula in the taxonomy of *Amphiroa* (Corallinaceae, Rhodophyta)

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Branch structure and growth in *Amphiroa* generated by primarily dividing apical medullary cells was documented by light and scanning electron microscopy. Specific numbers of long- and short-celled tiers are formed when intergenicular and genicular medullary cells periodically divide, leading to the unit pattern concept. Zonations on the intergenicular surface, grooves on the decalcified intergenicular surface and spaces in longitudinal section of intergenicula underlie units. Three unit pattern types are recognized: angled medullary tiers arranged in a straight horizontal row with lateral initials cut off abruptly from peripheral medullary cells in *A. annulata* and *A. anastomosans* (type A), medullary tiers whose length gradually reduces as they curve into the cortex in *A. fragilissima* var. *debilis*, *A. rigida* var. *antillana*, *A. spina* and *A. verruculosa* (type B), and rounded medullary tiers arranged in slightly irregular horizontal rows with lateral initials or cortical cells surrounding circularly peripheral medullary cells that are cut off from the medulla in *A. cuspidata* (type C). These patterns were statistically analysed in four Bermudan species: *A. annulata*, *A. cuspidata*, *A. fragilissima* var. *debilis* and *A. rigida* var. *antillana*. Unit patterns can usefully distinguish species. The association of intergenicula and genicula at the branch apex during the initial stage of branch formation is explained for the following four species: *A. annobonensis*, *A. crustiformis*, *A. fragilissima* var. *debilis*, and *A. itonoi*.

Key words: *Amphiroa*, anatomy, Corallinaceae, genicula, intergenicula, morphology

Introduction

Amphiroa Lamouroux (Fig. 1) is a common tropical and subtropical genus of articulated corallines involved in coral reef formation around the world (Goreau, 1963). The genus is primarily characterized by the anatomy of the branching fronds, which are made up of groups of filaments derived from the meristematic tissues. The apical meristem and intercalary meristem produce both the medulla and the epithallium (Cabioch, 1969, 1971; Johansen, 1981) and a lateral meristem produces the cortex. The medullary filaments are surrounded by a photosynthetic cortex, covered by a unistratose layer of epithallial cells. Intercalary meristematic cells, located beneath the apical meristem, divide in the branch apex.

The medullary filaments are organized in tiers, with each tier consisting of a transverse row of parallel cells of a particular cell length. The arrangement of tiers has been used to distinguish genera of articulated corallines. The presence of alternating long- and short-celled tiers in the medulla of *Amphiroa* was first reported by Zanardini (1871) and later illustrated by Kützing (1858, pls 39–51, pp. 18–29). Weber-van Bosse (1904) and Yendo (1904) considered the number of tiers in genicula to be useful in recognizing the genus. Johansen (1976) also emphasized intergenicular and genicular medullary patterns as a generic character of *Amphiroa*. In *Amphiroa* the long- and short-celled tiers are separated by transverse lines, termed 'zonation' (Yendo, 1902), 'intra-nodal periods of growth' (Weber-van Bosse, 1904) and 'annual marking' or 'banding' (Taylor, 1945) (Fig. 2). In their scanning electron microscope study of morphogenesis of *Amphiroa*, Garbary & Johansen (1987) called these lines 'transverse marking', describing two modes of formation of this line on the intergenicular surface in the genus. The term 'zonation' will be used in this paper because the pattern is due to the underlying development of the thallus and is not just a surface feature.

The number and length of medullary tiers have been used as infrageneric taxonomic criteria in *Amphiroa* (Weber-van Bosse, 1904; Hamel & Lemoine, 1953; Dawson, 1953, 1964; Ganesan, 1967; Norris & Johansen, 1981; Srimanobhas, 1987; Srimanobhas & Masaki, 1987; Choi, 1989). Weber-van Bosse (1904) discriminated *A. anastomosans* from other species on the basis of cell length

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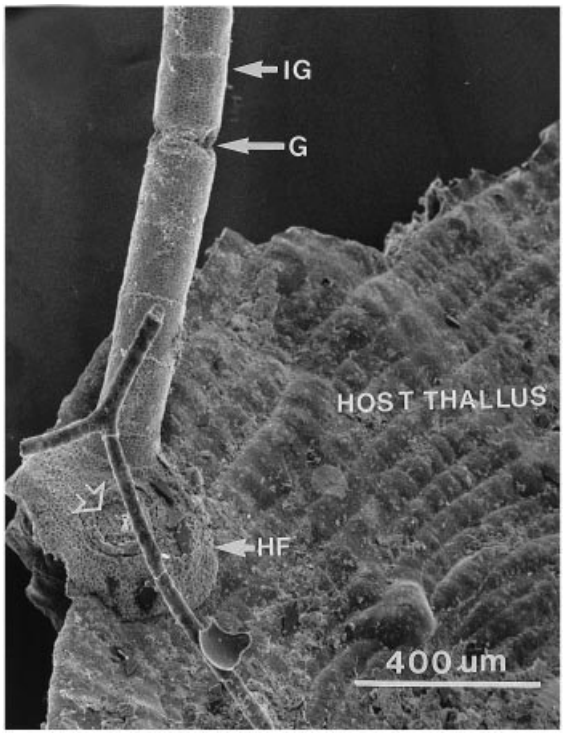


Fig. 1. *Amphiroa debilis*: the discoid-shaped holdfast (HF) attached to a crustose coralline alga (host thallus), a trace of a geniculum (G) and an intergeniculum (IG).

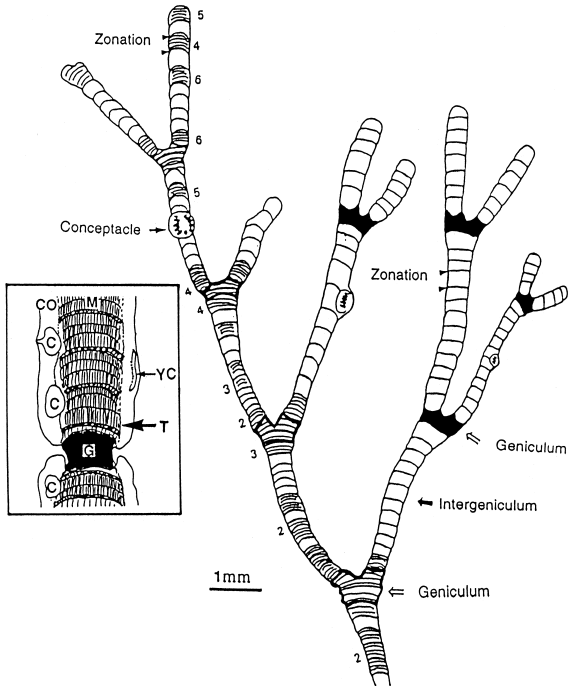


Fig. 2. Anatomy of *Amphiroa*. *Amphiroa annulata* showing the intergenicula and genicula. The zonation is the transverse lines on the surface of the decalcified whole thallus. In the branch on the left, tiers of cells (with numbers in parentheses) are shown between the zonation lines. Inset: *A. verruculosa*, showing a longitudinal section of the decalcified intergenicula, consisting of the medulla (M) and the cortex (CO), separated by a geniculum (G). A transverse tier of cells is arrowed (T). Tiers of cells continue through the geniculum, as shown in the decalcified branch of the thallus (main figure). Also shown are mature (C) and young conceptacles (YC), the sexual reproductive structures.

per tier, arrangement of tiers and shape of genicula. Recently, Riosmena-Rodriguez & Siqueiros-Beltrones (1996) studied five Baja California species of *Amphiroa* using medullary patterns of intergenicula and genicula in addition to conceptacle characters. They reported only range and number, and did not present any figures. They also described two types of gonimoblast filament arrangements in *Amphiroa*. These two types had already been used by Johansen (1976) in distinguishing *Amphiroa*, in which they arise from the margin, from *Calliarthron*, in which they arise from the surface.

Tier height and number thus appear to have potential for delimiting species. The size and arrangement of tiers can be said to form characteristic medullary units, corresponding to the surface zonation, in species of *Amphiroa*. A medullary unit consists of one short-celled tier and one to several long-celled tiers (and/or medium-celled tiers) per unit that make up the intergenicula and genicula of the thallus.

The present study describes these medullary unit patterns in the intergenicula and genicula of *Amphiroa* using scanning electron microscopy, light microscopy and statistical analysis. Both ultrastructure and filament anatomy under the light microscope reveal the mechanism of pattern formation and arrangement of intergenicular and genicular medulla. The differences in cell size and number between intergenicula and genicula within and among species were analysed statistically. The aims of this study were to describe the medullary unit pattern and to delineate four species of *Amphiroa* based on this medullary pattern formation.

Materials and methods

Specimen collection

Specimens of the genus *Amphiroa* were collected by the author in Bermuda, Atlantic Ocean, at a depth of 2–5 m, during the periods 6–11 June 1986, 7–13 March 1987 and 5–28 June 1988. Materials were fixed in 5% formalin-seawater and sorted with respect to their external morphological structures. The specimens are housed at Clark University, Worcester. Slides are housed at the University of Massachusetts, Amherst and Clark University (8601-86060, 8701-8780, 8801-88105).

Other specimens examined

Additional specimens from the Caribbean islands, Japan, Korea, South Africa, South America, the Galapagos Islands and Hawaii, as detailed below, were examined for

comparative purposes. These specimens from other locations were used in identifying characters.

Herbarium abbreviations are as follows: BISH, Bishop Museum; CJU, Cheju University; CUW, Clark University, Worcester; FH, Farlow Cryptogamic Herbarium, Harvard University; SAP, Hokkaido University; WRT, William Randolph Taylor, University of Michigan.

Bermuda: Abbott's Cliff (CUW 569), Ireland Island (CUW 603), Tobacco Bay (CUW 587), Trunk Island (CUW 626), Walsingham Bay (CUW 561, 563), Whalebone Bay (CUW 565, 612, 613, 624, 630). **Costa Rica** (WRT 23050). **Cuba** (WRT 29476). **Hawaii** (BISH). **Florida**: Key West (FCH July 1895, Feb. 1898, May 1924). **Haiti**: Petit Goave (WRT 14052). **Jamaica** (FCH July 1894, 1905–1906). **Panama**: Point-a-Patra (WRT 22407, FH 39-206). **Santo Domingo** (FCH Jan.–Mar. 1871). **South Africa** (CUW 448, 449, 479, 504, 530, 534, 541, 548, 551). **Korea**: Cheju Islands (CUW). **Japan**: Hokkaido Islands (SAP).

Microtechnique and scanning electron microscopy

After fixation in formalin-seawater, specimens were decalcified in fresh 5% trichloroacetic acid for 2–30 days or until decalcification was complete. Following Jensen's method, after decalcification, specimens were dehydrated in consecutive dilutions of solution for paraffin embedding (branches were carefully marked in the plastic block to distinguish the different-aged regions) and serially sectioned (6–8 μm thick). Sectioned tissue was stained with Erhlich's haematoxylin, eosin and fast green and mounted in euparal (Jensen, 1962).

Fixed specimens for scanning electron microscopy (SEM) were rinsed with distilled water and sectioned with a razor blade. These clean sections were dehydrated using consecutive dilutions of 50% to 100% ethanol and glued to metal double stubs with silver paint. A critical-point drying machine (Tousimis Samdri 790-A) was used to prevent damage to specimens. Dried specimens were placed in a Hummer 5 sputter coater for 1 min and coated with silver and gold. Specimens were observed with an ECTA Auto Scan SEM and photographed with a Polaroid camera and T-Max film.

Statistical analysis

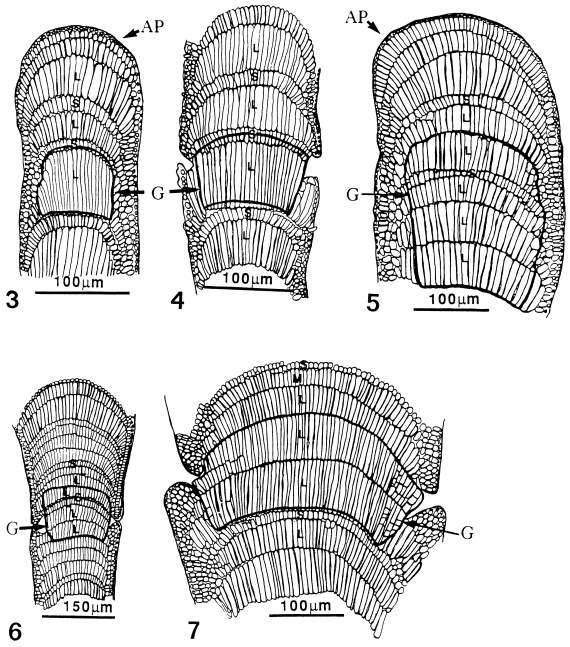
Representative data of the unit from the intergenicula and genicula were used from four Bermudan species: *Amphiroa annulata* Lemoine, *A. cuspidata* (Ellis *et* Solander) Lamouroux, *A. fragilissima* var. *debilis* Collins *et* Hervey and *A. rigida* var. *antillana* Børgesen. The sequence of formation of the medullary units was described from *A. cuspidata*. Anatomical measurements were made using an eyepiece micrometer (2–5 μm). Vegetative cell lengths were always measured to the centre of the primary pit-connections and diameters of cells were taken to the end of cell walls. Diameters of intergenicula were always scanned on the serial sections until the maximum lumen diameter was found, where all other measurements were taken ($n > 300$ for intergenicula, $n > 50$ for genicula for each species).

A Student's *t*-test (two-tailed analysis) was used to test differences in medullary tier height between intergenicula and genicula within species. Multivariate analyses were performed on proportional data from the four Bermudan species of *Amphiroa* using SYSTAT. One-way ANOVA (model I) was used to determine the significance of differences in number and height of intergenicular and genicular tiers among species (50 specimens in total of each species). Correlation analysis (Tukey multiple comparison) was applied to determine the relationships between intergenicular and genicular tier number and height in different species.

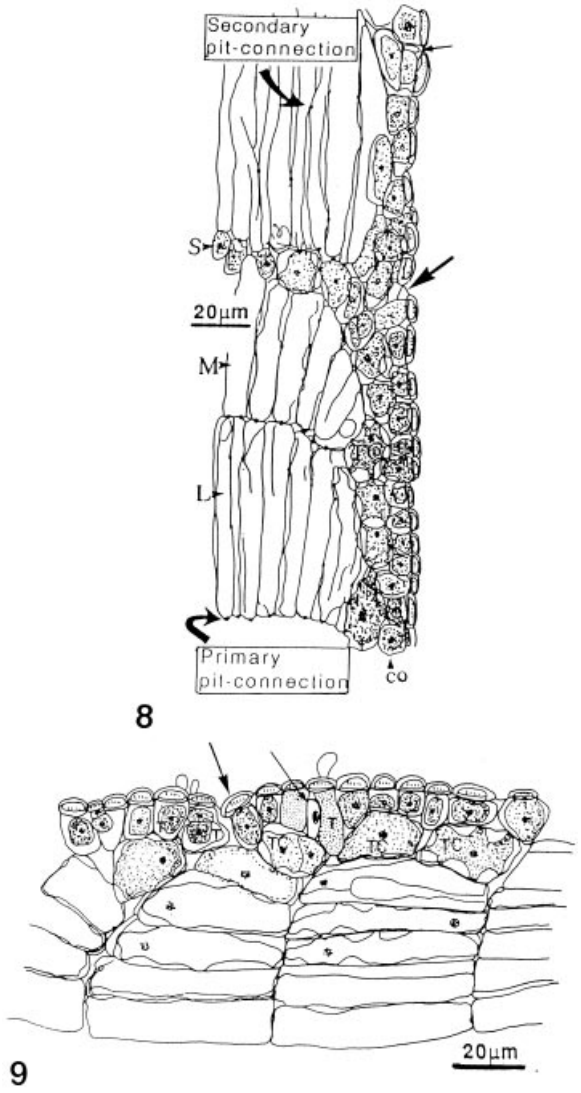
Results

External and internal morphology

Amphiroa grows attached to the substratum by a holdfast (Fig. 1). The most common substrata are crustose coralline algae, congeneric articulate coralline algae (e.g. *A. itonoi* growing on *A. beauvoisii*)



Figs 3–7. Young and old branches of *Amphiroa* spp. in longitudinal section (S, short-celled tier; M, medium-celled tier; L, long-celled tier). Fig. 3. *Amphiroa itonoi*: a young geniculum (G) near the branch apex (AP) and the alternation of long (L) with short cells (S) in each unit. Bold arrow indicates intercalary meristematic cells surrounded by epithallial cells. Fig. 4. *A. itonoi*: old branch showing the arrangement of unit patterns in a geniculum (G) (1L + 1S = a geniculum) and two intergenicula (1L + 1S = a unit). The old geniculum includes more of the intergenicular cells than the young geniculum. Fig. 5. *Amphiroa crustiformis*: young branch showing a geniculum (G; 3L + 1S + 1L) at the branch apex (AP) and the alternation of long- with short-celled tiers. Bold arrow indicates intercalary meristematic cells. Fig. 6. *A. crustiformis*: the arrangement of unit patterns in an old geniculum (G) and intergeniculum (3L + 1S + 1L = a geniculum). Fig. 7. *A. annobonensis*: an old geniculum (G) and the alternation of long- with short-celled tiers (1L + 1L = geniculum; 3L + 1 medium-long tier (M) + 1S = a unit in intergeniculum).



Figs 8, 9. *Amphiroa cuspidata*: decalcified branches and longitudinal sections. Fig. 8. Lateral initials having many pit-connections, filamentous medullary cells (S, short-celled tier; M, medium-celled tier; L, long-celled tier) connected by primary and secondary pit-connections. Also shown is the space (bold arrow) between epithallial cells near a short-celled tier from a longitudinal section of an intergeniculum and an elongated trichocyte initial cell (arrow) differentiating from the lateral initial filled with cytoplasm. Fig. 9. Longitudinal fracture of the cortex showing the arrangement of epithallial cells, trichocyte and trichocyte initials in *Amphiroa cuspidata* (drawings from camera lucida). A declined epithallial cell (bold arrow) causing a different elevation of epithallial cells on the surface from a longitudinal section of the cortex and an elongated trichocyte initial cell (arrow) differentiating from the lateral initial (TC) are also seen. T, trichocytes bearing hairs.

or rocks in coral reefs. Thallus branches consist of alternating calcified intergenicula and fibrous genicula formed by apical medullary filaments (Figs 2–4). The medullary filaments which constitute a branch core elongate by synchronous divisions of subapical meristems after having given rise to epithallial cells at the branch apex (Figs 3, 5). Meristematic cells that generate epithallial cells are termed the intercalary meristem (Cabioch, 1969), while primary meristems, called the terminal meristem, do not produce epithallia and are not covered with any other cells. In *Amphiroa*, because meristematic cells give rise to epithallial cells, there is an intercalary meristem (Figs 3, 5) although it occurs in the apical meristem. Some cells elongate (long cells) while others (small cells) stay the same size as primary initials (Figs 3, 5). Divisions of the primary and intercalary meristem result in precisely arrayed tiers of cells with different medullary cell sizes and shapes (Figs 5–7). After branches reach a certain length, which is highly variable between species, medullary cells differentiate into cortical cells (Fig. 8). Trichocyte initials differentiate from lateral initials located at the periphery of the medulla and become large cells bearing hairs (Fig. 9). Arrangement of the medullary cells, division patterns of lateral initials in the cortex and arrangement of epithallial cells are factors that determine particular medullary patterns in *A. cuspidata* (Figs 8, 9).

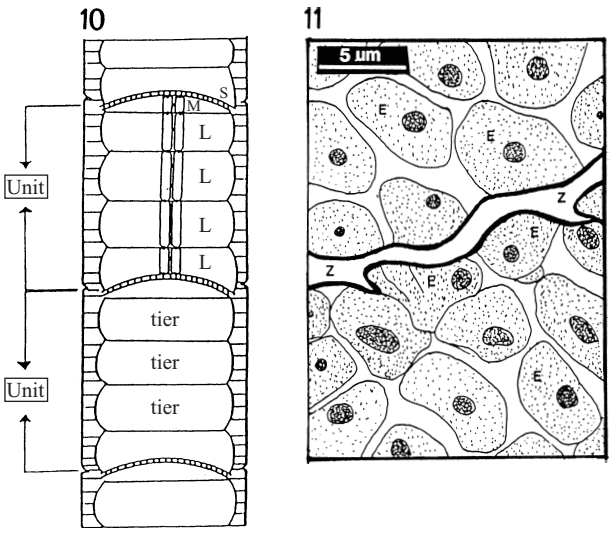
Formation of the medullary unit

Branches in *Amphiroa* consist of medullary tiers of various heights surrounded by photosynthetic cortical cells (Figs 2, 8). Long medullary cells are interconnected by primary and secondary pit-connections (Fig. 8). These long cells contain a large amount of floridean starch produced from cortical cells while cortical cells and lateral initials have dense cytoplasm (Figs 8, 9). When medullary cells are arranged in a transverse row, they form a tier (Fig. 10). Three categories of medullary tiers may be recognized on the basis of cell length: (1) short tiers of cells (5–)8–25(–28) μm long; (2) medium-length tiers of cells 30–40(–45) μm long; and (3) long tiers of cells 45–120(–130) μm long. These tiers are organized into successively produced groups or 'medullary unit patterns' indicated as a 'unit' in Fig. 10. A group of several of these medullary unit patterns and the associated cortical filaments make up intergenicula and genicula (Figs 2, 8). Thus, a basic pattern is generated as new medullary units are produced during growth at the branch apices. The association of growth patterns with the medullary unit patterns can be seen by the consistency of the number of tiers per unit, with the following condition: the younger the branches, the greater the number of medullary tiers per unit in decalcified whole fronds (e.g. *A. annulata*, Fig. 2). The consistency in the length of intergenicular medullary cells (i.e. tier height) of four species is shown in Table 1. The medullary unit pattern can be observed using the following five criteria: (1) A single short-celled tier is the last formed in every unit and marks the distal boundary of the unit (Figs 2, 10). This

Table 1. Numerical data relating to tier height and number of intergenicular and genicular medullary cells of four species of *Amphiroa* in Bermuda

	Tier height		No of tiers per unit in intergenicula and genicula	
	M	(n)	M	(n)
A. annulata				
Intergenicula	51±23	365	5±1·5	68
Genicula	52±24	59	3±1·1	27
A. cuspidata				
Intergenicula	67±29	335	8±1·8	41
Genicula	60±26	116	4±0·8	16
A. fragilissima var. *debilis*				
Intergenicula	41±26	370	3±1·0	147
Genicula	51±27	129	6±2·3	16
A. rigida var. *antillana*				
Intergenicula	44±30	386	3±0·6	159
Genicula	138±28	54	2±0·0	10

Mean (M, unit = μm)±standard deviation (SD) with number of samples (n).



Figs 10, 11. Medullary unit patterns. Fig. 10. Diagram of the medullary unit pattern from the long- and short-celled tiers showing some cells in a unit consisting of four long, one medium and one short tier (curved band of short cells). Fig. 11. Fronds of *Amphiroa cuspidata* showing the formation of the medullary unit from the surface under light microscopy. The grooved zonation (Z) between epithallial cells (E) can be seen on the highly magnified intergenicular surface.

phenomenon is called 'zonation' in this study. (2) The distal boundary of the unit appears as a gap between epithallial cells when seen using light microscopy (Figs 8, 9). It appears as a groove in the decalcified intergenicular surface under high magnification (Fig. 11). (3) Regularly spaced arching wrinkles in the epithallium coincide with the boundaries of the short-celled tiers (Figs 8, 9). (4) Units are similar in construction and range of tier number within a single frond (Fig. 10). (5) Units differ between species in the tier number and height per unit (Figs 12, 14, 16). Short-celled tiers extend at regular intervals into the cortex where each tier is ringed by enlarged cells just below the branch surface. A unit includes medullary cells and cortical filaments at the lateral boundary (Figs 13, 15).

Three medullary unit pattern types in intergenicula

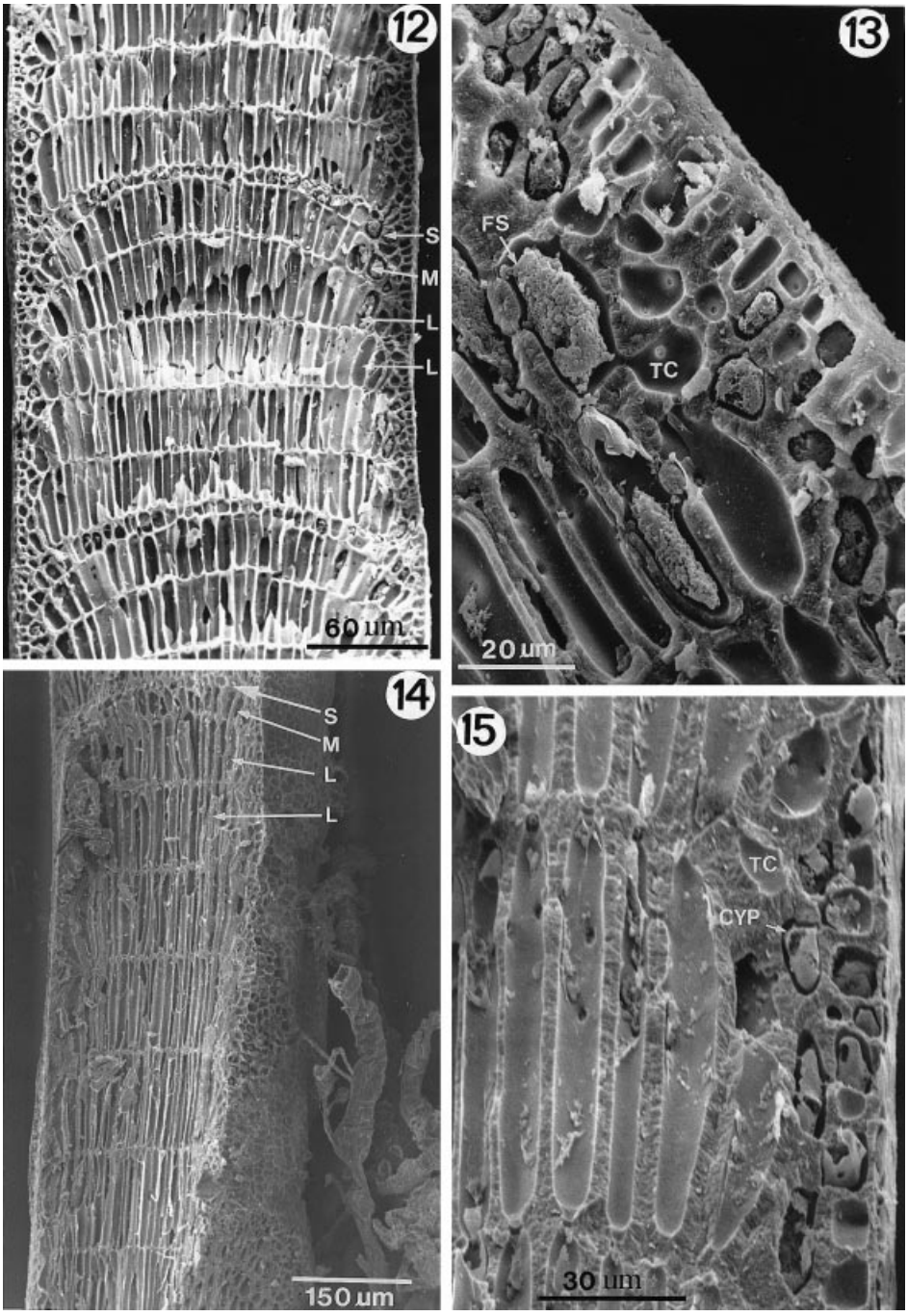
In longitudinal section of the intergenicula, the shape of the medullary unit shows the relationship between frond cortex and medulla. The unit shape is determined by lateral initials and cortical cell orientation and organization as well as by the resulting arrangement of medullary tiers (Figs 13, 15, 16).

Three medullary unit patterns shown diagrammatically in Fig. 18 are recognized: type A, angled medullary tiers arranged in a straight horizontal row with lateral initials cut off abruptly from peripheral medullary cells (Figs 12, 18*A*) and cortical cells in old branches aligned perpendicularly; type B, the height of medullary tiers gradually reduces (cell length is variable depending on species) as they curve into the cortex (Figs 16, 18*B*), with most species forming a thick cortex (Fig. 17) and a thickly calcified intergenicular surface (due to calcification and cortical thickness, zonations are less apparent in older branches); type C, rounded medullary tiers arranged in slightly irregular horizontal rows (Figs 14, 18*C*) with lateral initials or cortical cells surrounding circularly peripheral medullary cells that are cut off from the medulla (Figs 8, 15). On the basis of the three types of medullary unit pattern, species from this and other studies are grouped in Table 2.

Structure of intergenicula and genicula

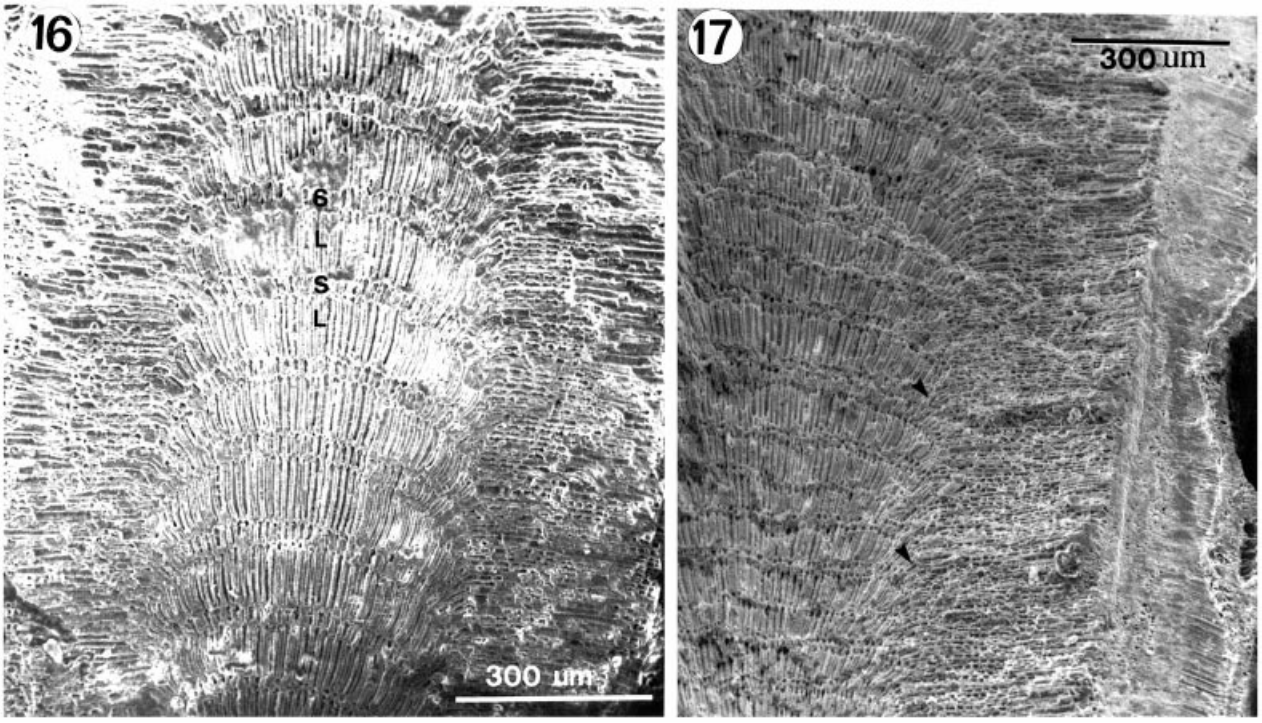
Branches consisting of intergenicula and genicula ramify on either the genicula (Fig. 2) or the intergenicula (Fig. 19). Genicular cell walls, which are filled with mucopolysaccharides and stain intensely, are clearly distinguished from decalcified intergenicula on light microscopy (Fig. 2). Genicular medullary cells are narrower and longer than intergenicular medullary cells (Figs 4, 7). As genicula age, part of the genicular cortex cracks off (Figs 4, 7) or swells (Fig. 20) and results in a changed genicular shape. Genicula are convex from the cracking off of the genicular cortex (Figs 4, 7).

Genicular tiers constitute all or part of a unit which, in many taxa, extends into the neighbouring intergeniculum (Figs 4, 5, 7). On the basis of the



Figs 12–15. The change in medullary unit patterns (S, short-celled tier; M, medium-celled tier; L, long-celled tier) from young to old intergenicula. Fig. 12. *Amphiroa annulata*: transversely aligned cells in the centre of the medulla and abruptly cut off lateral initials in a young plant. Fig. 13. *A. annulata*: longitudinal fracture of the medulla and cortex in an old intergeniculum showing the abruptly cut off lateral initials (TC) connected to many pit-connections from the medulla, and peripheral medulla containing floridean starch (globular structures, FS). Fig. 14. *Amphiroa cuspidata*: straight lines of cells in the centre of the medulla and the curved rows of peripheral medulla in a young intergeniculum. Fig. 15. An old intergeniculum in *A. cuspidata* showing lateral initials (TC) containing cytoplasm (CYP) and curved rows of peripheral medulla.

medullary unit patterns, the growth pattern of intergenicula is easily interpreted from the association of intergenicula with genicula (Figs 19, 20). Genicular medullary cells from the initial and later stages clearly show either part of a unit in the intergenicular medulla or the same number of intergenicular tiers per unit (Figs 5, 6). Weber van-Bosse (1904, p. 84) indicated that 'The regularity with which these rows of long and short cells alternate is liable to much variation in each species and in each plant. Still a certain rule may be detected.' It is variable within species. However, since young and old branches produce different numbers and lengths of intergenicular medullary



Figs 16, 17. Fig. 16. *Amphiroa rigida* var. *antillana*: arched medulla in the centre of the intergenicular medulla and perpendicularly arranged cortical cells from a medium-aged intergeniculum. Fig. 17. *A. rigida* var. *antillana*: slightly wavy medullary cells in the centre of the old intergenicular medulla, curved peripheral medullary cells towards the cortex (arrows).

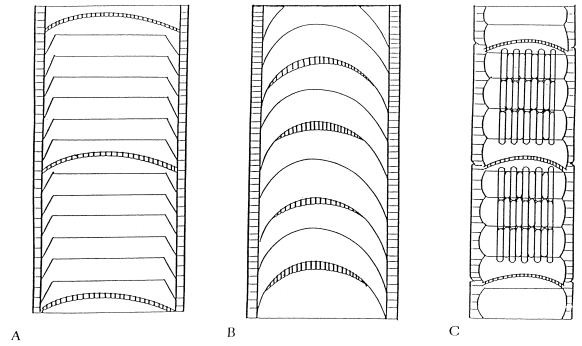


Fig. 18. *Amphiroa* species: the three different types of medullary unit patterns: type A, a straight horizontal row with lateral initials cut off abruptly from peripheral medullary cells; type B, medullary tiers gradually reduced and curving into the cortex; type C, lateral initials or cortical cells are cut off from the medulla. (See Table 1 for the distribution of *Amphiroa* species forming these unit patterns.)

cells, the unit pattern in each species can be useful for unravelling the taxonomy of the genus (Fig. 18).

Genicula in *A. itonoi* consist of a long-celled tier and a part of a short-celled tier, most of which is in the intergeniculum (Figs 3, 4). However, genicula in *A. spina*, *A. annobonensis* and *A. rigida* var. *antillana* are composed of long-celled medulla (part of a unit: one or two long-celled tiers) (Figs 7, 19). In *A. rigida* var. *antillana* units are composed of two long- and one short-celled tiers (a total of two to four tiers per unit). Unlike intergenicula, a geniculum often consists of two or three units of medulla, e.g. in *A. annulata*, *A. cuspidata* and *A. fragilissima* var. *debilis* (Figs 2, 20). The number of genicular cells rarely changes as the fronds age although genicular structures may differ in old genicula due to the development of the genicular cortex (Figs 4, 6, 20). The demarcation between calcified intergenicula and decalcified genicula cells sometimes passes through the peripheral or central parts of the tiers (Fig. 20). The height and number of tiers per unit in four species of intergenicula and genicula are presented in Table 1.

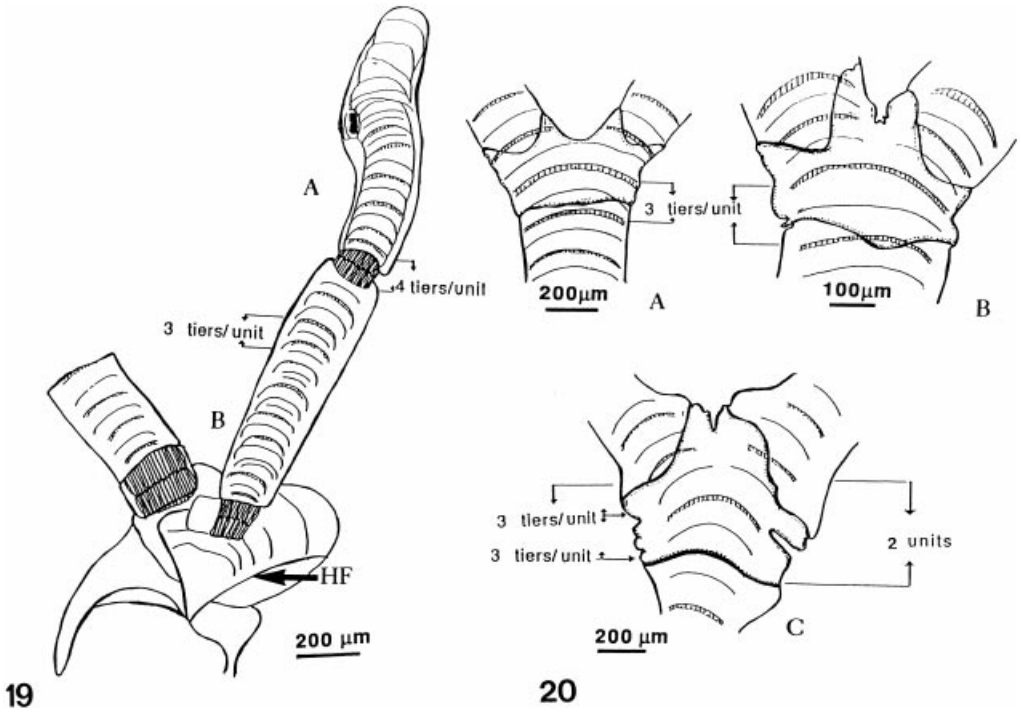
Each of the four species of *Amphiroa* collected in Bermuda has its own particular qualitative medullary unit pattern. The characters identifying this pattern in each species are the following (the cell lengths are approximate for each species):

Amphiroa annulata: six tiers, one short and five long (e.g. 20, 55, 70, 75, 73, 65 μm long) per unit in intergenicular medullary cells and six partially stained genicular tiers, one long, one short and four long (e.g. 55, 15, 43, 68, 70, 63 μm long) per geniculum. Part of two units forms a geniculum. The first long-celled tier after a short-celled tier when a unit starts in the intergenicula is shorter than the next long-celled tier.

Amphiroa cuspidata: ten tiers, one short, one medium and eight long (e.g. 10, 30, 78, 85, 90, 100, 103, 110, 113, 113 μm long) per unit in inter-

Table 2. *Amphiroa* species: three different types of unit patterns in the intergenicular medulla

Type of unit	Species	Reference
Type A		
Centre of medullary tiers arranged straight and peripheral medullary cells abruptly cut off		
	A. anastomosans	Weber-van Bosse (1904), Srimanobhas (1987), Choi (1989) Personal observation
	A. annulata	Lemoine (1929), personal observation
	A. beauvoisii	Norris & Johansen (1981), Choi (1989) Personal observation
	A. cyathifera	Personal observation
	A. tribulus	Personal observation
Type B		
Medullary cells curved into the cortex		
	A. anceps	Johansen (1969)
	A. annobonensis	Pilger (1919), personal observation
	A. bowerbankii	Johansen (1969), personal observation
	A. capensis	Johansen (1969), personal observation
	A. crustiformis	Dawson (1963), personal observation
	A. dilitata	Srimanobhas (1987), personal observation
	A. foliacea f. *procumbens*	Weber-van Bosse (1904), personal observation
	A. fragilissima var. *debilis*	Present study
	A. itonoi	Srimanobhas & Masaki (1987), Choi (1989) Personal observation
	A. misakiensis	Norris & Johansen (1981), Srimanobhas (1987), personal observation
	A. rigida var. *antillana*	Present study
	A. verruculosa	Present study
Type C		
Centre of medullary cells arranged straight and peripheral medullary cells cut off lateral initials forming an arch		
	A. cuspidata	Present study
	A. debilis	Present study
	A. fragilissima	Weber-van Bosse (1904), personal observation



Figs 19, 20. *Amphiroa spina* and *A. fragilissima* var. *debilis*: the development of genicula based on the medullary unit pattern. Fig. 19. *A. spina*: the old genicula (B) which are close to the holdfast (HF) and the young genicula (*A*) which are located between intergenicula. Fig. 20. *A. fragilissima* var. *debilis*: longitudinal section of genicula from young (*A*) to medium age (*B*) to old (*C*) showing a partially decalcified geniculum consisting of two units (2L + 1S = unit).

genicular medullary cells and five partially stained genicular tiers, two long, one medium, one long and one short (83, 58, 30, 70, 20 μm long) per geniculum. Part of two units forms a geniculum in *A. cuspidata*.

Amphiroa fragilissima var. *debilis*: four tiers, one short, one medium and two long (e.g. 8, 45, 70, 75 μm long) per unit in intergenicular medullary cells and eight partially stained genicular tiers, one long, one medium, one short, one medium, one long, one short and two long (e.g. 15, 45, 10, 30, 70, 13, 50, 65 μm long) per geniculum. One complete unit and parts of two units are involved in forming a geniculum in *A. fragilissima* var. *debilis*.

Amphiroa rigida var. *antillana*: three tiers, one short, one medium and one long (e.g. 8, 43, 113 μm long) per unit in intergenicular medullary cells and two tiers, both long (e.g. 145, 150 μm long), per geniculum. Two long-celled tiers comprise a geniculum in *A. rigida* var. *antillana*.

The arrangement of long- and short-celled tiers and the configuration of peripheral medullary cells (lateral initials) characterize a medullary unit pattern (Fig. 18). After filamentous medullary cells primarily differentiate into subapical meristem and epithallia at the apex, branches elongate until they reach a certain length and/or they ramify on or off the genicula. Intergenicula in the upper part of fronds are longer and thinner than those in the basal part. The filamentous medullary cells in the upper part of a frond in *A. cuspidata* (mean 110 μm) are longer than those at the lower part of the frond. Medullae in *A. annulata* (7 tiers/unit) and *A. cuspidata* (10 tiers/unit) have a greater number of tiers per unit than those in the basal part, whereas cells in *A. fragilissima* var. *debilis* (mean 63 μm; 3 tiers/unit) and *A. rigida* var. *antillana* (mean 50 μm; 3 tiers/unit) produce shorter and fewer medullary tiers per unit in the upper part of a frond. Statistically the tier height and number in *A. cuspidata* are significantly different from those in *A. fragilissima* var. *debilis* and *A. rigida* var. *antillana* (Tukey multiple comparison, $p < 0{\cdot}001$). Even though tier height of intergenicula of *A. fragilissima* var. *debilis* and *A. rigida* var. *antillana* is similar, genicular structure and number of genicular tiers per unit show the difference between these two species. The following correlation exists between the branch size (length, diameter) and medullary tier size (number, length) per unit in *A. cuspidata*: the longer the intergenicular size, the longer the medullary tiers and the greater the number of medullary tiers per intergeniculum (personal observation).

Tier height and number of tiers in genicula within all four Bermudan species are significantly different from those in intergenicula. Even though genicular medullary cells are continuations of intergenicular medullary cell filaments, they are much longer than intergenicular cells in *A. rigida* var. *antillana* (Table 1). Among the four species examined, tier height of intergenicula in *A. cuspidata* is significantly different from that of the other three species (Tukey multiple comparison, $p < 0{\cdot}001$). Tier height and number of tiers in intergenicula in *A. fragilissima* var. *debilis* are similar to those in *A. rigida* var. *antillana* (Table 1; one-way ANOVA, $p < 0{\cdot}09$), but tier height and number of tiers in genicula differ significantly between those two species (one-way ANOVA, $p < 0{\cdot}001$). In addition to numerical data for genicula in these two species, genicular structure is also very different: genicular cells are partially decalcified and swollen in aged *A. fragilissima* var. *debilis* (Fig. 20*C*), while those in *A. rigida* var. *antillana* are consistently composed of two cells per geniculum from young to old branches. Mean and standard deviation for tier height and number of tiers in intergenicula and genicula of four species are presented in Table 1.

Discussion

Basis of zonation

The external morphology of *Amphiroa* is distinguished by transverse lines on the intergenicular surface, here called 'zonations' but previously given various names (Yendo, 1902; Weber-van Bosse, 1904; Taylor, 1945). Zonations on the intergenicular surface of either calcified or decalcified fronds are associated with the alternation of long- and short-celled tiers. As Garbary & Johansen (1987) remarked, the ridges at branch apices result from meristematic activity in the short-celled tiers. This study confirms the association of the short-celled tiers with zonation formation. In decalcified tissue zonation lines correspond to grooves between epithallial cells at the level of short-celled tiers. Because haematoxylin stains the spaces between heavily calcified cells, the dark staining of this groove on the decalcified thallus indicates it is more calcified than other areas of the intergenicular surface. These grooves are more regularly distinguished in younger intergenicula than in older ones because more meristematic divisions cover them in the old intergenicula. The cortex is also indented at this zonation line at the level of the short-celled tiers.

Even though Garbary & Johansen (1987) observed two types of zonations from the branch apex (*A. bowbanskii*-type with overlapping of epithallial and cortical cells, and *A. beauvoisii*-type with irregularly organized epithallial cells), both types are seen on the same thallus. The *A. bowbanskii* type occurs on medium-aged and older branches while the *A. beauvoisii* type appears on the branch apices. From actively dividing meristematic

cells in the cortex of old intergenicula the epithallial cells are asymmetrically distributed with irregular spaces between them. The mechanism by which these spaces form is unclear.

Establishment of the unit pattern

These surface zonations are manifestations of the underlying development of the medulla. Characteristic number and length of medullary cells have long been used to characterize species of *Amphiroa*. *A. anastomosans*, with a pattern of four or five tiers of long cells (36, 56, 72, 76 μm long) was accepted and discriminated from other species on the basis of medullary cell pattern (Weber-van Bosse, 1904). The unit configuration analysed here is evident in the figures of Lemoine (1929, pp. 73–78; *A. annulata* and *A. fragilissima*) and Weber-van Bosse (1904, pp. 92–93; *A. foliacea*) as an alternating pattern. Due to the statistical significance of tier height and number of tiers in interspecific comparisons, documentation of medullary unit patterns from young, medium-aged and old thalli is potentially of great importance in *Amphiroa* species identification.

Multivariation of intergenicular and genicular medullary cells has been introduced as a generic character (Johansen, 1976). Although using these characters in *A. rigida* is unreliable because of the irregularity of cell number and length in the intergenicular medulla, Weber-van Bosse (1904) accepted and discriminated *A. anastomosans* (four or five tiers of long cells, 36, 56, 72, 76 μm long) from other species. In addition to the Bermudan species analysed here, a comparison of the medullary unit patterns and their configurations at the different locations in *A. anastomosans*, *A. annobonensis*, *A. annulata*, *A. foliacea*, *A. itonoi* and *A. valonioides* confirms the medullary unit pattern in the intergenicular medulla as a genetic character.

A. annobonensis has never been reported since Pilger (1919) but it has been treated as a synonym. Price *et al.* (1986, p. 11) synonymize *A. annobonensis* from west Africa with *A. beauvoisii* Lamouroux following Norris & Johansen's identification (1981) and Johansen's comment. Even a specimen from South Africa (Johansen, 1981, p. 70, fig. 12*E*) and Korea (Choi, 1989) in this species is named as a new species. However, the unit pattern in *A. annobonensis* from South Africa and Korea is the same as Pilger's illustration and description in 1919 (no type specimen available). Even though the number of genicular tiers in *A. annobonensis* is the same as in *A. rigida*, thallus formation and tier height and number of intergenicula are significantly distinct to keep this species rather than create a new species (Norris & Johansen, 1981).

The finding of genicular cells associated with intergenicular cells in terms of the unit pattern resolves the taxonomic problem in *A. annulata*. In general, genicula in *Amphiroa* appear at the part of the unit in the intergenicular medullary tiers. Also, genicular cells are generally longer than intergenicular ones (Table 1). Norris & Johansen (1981, p. 20) synonymized *A. annulata* in the Gulf of Mexico with *A. valonioides* because of a unizonal genicular tier. The present data show a significant difference in the number of genicular cells and their configuration for *A. annulata* compared with those in authentic *A. valonioides* (Yendo, 1904; Norris & Johansen, 1981; Srimanobhas, 1987; Choi, 1989; personal observation). Genicula in *A. annulata*, unlike *A. valonioides*, are partially composite and consist of four to seven tiers per geniculum (Table 1). Genicula in *A. valonioides* consist of one complete genicular medullary tier (Norris & Johansen, 1981; Srimanobhas, 1987; Choi, 1989). Genicula in *A. itonoi*, *A. spina*, *A. annobonensis* and *A. rigida* var. *antillana* consist of two tiers. However, genicula in *A. itonoi* are composed of a long-celled tier and a part of a short-celled tier while others form two long-celled tiers.

Even though Riosmena-Rodriguez & Siqueiros-Beltrones (1996) indicated genicular cortex to be absent in *A. rigida* and *A. valonioides*, cortical cells in their genicula have been observed in this study. During development of genicula in those two species, cortical cells became cracked with age (Fig. 4). Thus, genicular cortication among species should be re-examined based upon morphogenesis. The structure and size of cortical cells in intergenicula are, however, unique for each species (unpublished data).

Johansen (1969, 1981) described two types of genicular development based on external morphology (presence or absence of cracking at the margin of the cortex): type I in which the calcified cortex cracks and breaks off, exposing the uncalcified genicular medulla cells, and type II (*A. ephedraea*) in which decalcification starts in the centre of the thallus and proceeds to the surface with the decalcified cortex remaining as part of the geniculum. Here, two groups of species are delineated by the arrangement of the genicular medullary cells: (1) group I is characterized by two tiers per geniculum and complete genicular cells; (2) group II has many tiers per geniculum, partially stained at the genicular margin or centre and sometimes forming a swollen genicular cortex in old branches (Johansen, 1969). Although *A. annobonensis* belongs to group I, the intergenicular medullary unit pattern (two or four long- and one short-celled tiers per unit) is different from that of *A. itonoi* (two tiers, one long- and one short-celled per unit) and *A. rigida* var. *antillana* (two or three

long-and one short-celled tier per unit). Intergenicular medullary cells in *A. rigida* var. *antillana* curve into the cortex and form a typical arch-shaped unit pattern (Figs 16–18*B*). However, this species has been placed in synonymy with *A. rigida* by Norris & Johansen (1981) and Riosmena-Rodriguez & Siqueiros-Beltrones (1996) based upon the number of tiers per genicula (two tiers per geniculum). According to Srimanobhas (1987) and Riosmena-Rodriguez & Siqueiros-Beltrones (1996), however, there are one to three tiers in intergenicula in *A. rigida*; those in *A. rigida* var. *antillana* contain two to four tiers per unit in intergenicula (Table 1). Even though external anatomy is variable, according to environment, branching in those two species is unique. Branching in *A. rigida* appears irregular while branches in *A. rigida* var. *antillana* clearly divide dichotomously. Silva *et al.* (1996) keep *A. rigida* var. *antillana* as a distinct variety of *A. rigida* in their study of the Indian Ocean algae.

Although *A. itonoi* and *A. rigida* both contain two tiers per geniculum, Srimanobhas & Masaki (1987) named *A. itonoi* as a new species because of its different habitat (grows on the same genus, *Amphiroa*) and very small size of thallus (less than 5 mm high). Species which have the same number and shape of tiers in genicula should be reconsidered on the basis of the medullary unit pattern in intergenicula. *A. annulata*, *A. cuspidata*, and *A. fragilissima* var. *debilis* belong to group II.

While external changes in genicular morphology have been studied (Johansen, 1969; Riosmena-Rodriguez & Siqueiros-Beltrones, 1996), the analysis of internal structural development presented here is a crucial point. Although the three species have similar external and internal genicular structures, each shows a distinct unit pattern. A unit of the intergenicular medullary cells in *A. annulata* is composed of three to seven tiers with the type A shape (Figs 12, 18*A*). In *A. fragilissima* var. *debilis* the intergenicular medulla form an arch shape (type B; Fig. 18*B*) but a unit of the intergenicular medulla consists of two to five tiers (one short, one medium, and two or three long-celled tiers per unit) (Figs 17, 18). Short- and medium-celled tiers are present more often than long-celled tiers. A unit in *A. cuspidata* consists of seven or eight long-celled tiers in young branches and three to five long-celled tiers with one short- and one medium-celled tier in old branches of type C (Figs 14, 18*C*). These three types of medullary unit patterns can distinguish between species (Table 2).

Riosmena-Rodriguez & Siqueiros-Beltrones (1996) emphasize as a character the position of the gonimoblast filament in delimiting the genus *Amphiroa* as well as its species. However, Johansen (1976) had already described these two types: superficial in *Calliarthron* and peripheral in *Amphiroa ephedrea*. Gonimoblast filaments in *Tenarea* arise peripherally from the fusion cell. In *A. rigida*, Srimanibhas (1987) and Choi (1989) reported a superficial position while Riosmena-Rodriguez & Siqueiros-Beltrones (1996) found it to be peripheral. Although Riosmena-Rodriguez & Siqueiros-Beltrones (1996) and Choi (1989) indicated that the position of the gonimoblast filament in *A. misakiensis* is peripheral, Srimanobhas (1987) described it as superficial in that species. Thus, the use of gonimoblast filament position as a character distinguishing these two species should be reexamined. Since this character has been found in a genus of crustose coralline algae, *Tenarea*, its use in discriminating among subfamilies should also be reconsidered.

Conclusion

Examination of medullary growth patterns in *Amphiroa* based upon young to old branching has revealed a fundamental repeating pattern of intergenicular and genicular medulla that is introduced here as the medullary unit pattern. This pattern is a particularly homogeneous character of the genus compared with other articulated coralline algae but is heterogeneous among species of *Amphiroa*.

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