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SYSTEMATICS OF THE HILDENBRANDIALES (RHODOPHYTA): GENE SEQUENCE AND MORPHOMETRIC ANALYSES OF GLOBAL COLLECTIONS[1]

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Fifty-seven collections of marine and freshwater *Hildenbrandia* from North America, South America, Europe, and Africa were compared with 21 type and historically important specimens using multivariate morphometrics. Additionally, phylogenetic analyses of 48 specimens of *Hildenbrandia* and two specimens of *Apophlaea* were carried out based on sequences of the *rbc*L chloroplast gene and the nuclear 18S rRNA gene. Morphometric analyses based on vegetative cell and filament dimensions distinguished two groups of freshwater *Hildenbrandia* specimens, the first corresponding to those collections from North America and the Philippines and the second to those from Europe and the Canary Islands. The first group had smaller mean cell and filament dimensions (cells 4.0 × 4.4 μm, filaments 46.5 μm) and corresponded to *H. angolensis*, whereas the second group had larger mean dimensions (cells 5.8 × 6.6 μm, filaments 55.3 μm) and represented *H. rivularis*. Marine specimens were morphometrically distinguishable into two groups based on tetrasporangial division pattern as well as other thallus characters. However, measurements and character determinations of some type specimens differed greatly from the original descriptions, and thus further work to determine the stability of these characters is required. Phylogenetic reconstruction based on the 18S rRNA gene and *rbc*L gene sequence data generally demonstrated separation of the marine and freshwater forms of *Hildenbrandia*, with some marine taxa forming monophyletic groups (e.g. *H. lecannellieri* and *H. occidentalis*) and others forming paraphyletic groups (e.g. *H. rubra*). The two specimens of *Apophlaea* formed a monophyletic group within the paraphyletic genus *Hildenbrandia*.

Key index words: **18S rRNA gene; *Apophlaea*; *Hildenbrandia*; Hildenbrandiales; morphometrics; *rbc*L gene; systematics**

The red algal order Hildenbrandiales contains two genera: *Apophlaea*, which is limited in distribution to the marine coastlines of New Zealand (Hawkes 1983), and *Hildenbrandia*, which is globally distributed in both marine and freshwater habitats (Rosenvinge 1917, Bourrelly 1955, Silva et al. 1996). *Hildenbrandia* is a crustose red alga, whereas *Apophlaea* possesses upright thallus portions in addition to a crustose basal thallus (Rosenvinge 1917, Hawkes 1983).

The Hildenbrandiales has traditionally been plagued with taxonomic problems due to a proliferation of taxonomic names where few morphological characters are available for their separation. Several characters commonly used within the genus *Hildenbrandia* for taxonomic purposes, such as thallus thickness and conceptacle dimensions (in marine species), are known to vary with the age of the plant (Pueschel 1982). In addition, cellular dimensions are variable in different parts of the thallus due to the branching filaments composing the crust (Starmach 1969), and the measurement of this character must be combined with representative sampling of cells along the lengths of the filaments. Although differences in these characters are evident for several species, such as the reportedly thicker thalli of *H. lecannellieri* and *H. occidentalis* (Hariot in Askenasy 1888, Gardner 1917), they must be interpreted with caution, given the variation present in these characters over the life of the alga. Other characters of doubtful validity (such as the presence of paraphyses to separate *H. dawsonii* from *H. canariensis* and *H. crouanii*) have been used previously to distinguish species (Hollenberg 1971). Reports of paraphyses are common in the *Hildenbrandia* literature (Hariot in Askenasy 1888, Womersley 1994), and their interpretation has been much debated. For example, the presence or absence of paraphyses was used, in part, to delimit sections within the genus *Hildenbrandia* by J. Agardh (1852), whereas more recent investigators have suggested that reports of paraphyses are actually empty sporangial walls in the conceptacle or fungal filaments (Ardré 1959, Denizot 1968). Pueschel (1982) provided ultrastructural evidence to support the view that they are empty sporangial walls.

Despite the widespread distribution of *Hildenbrandia*, little work has been conducted until now to clarify the systematics of the Hildenbrandiales by comparing results from modern molecular analyses with those from traditional morphometric analyses of type specimens. The present study uses a global taxon sampling approach within members of this order to compare phylogenetic relationships based on molecular data to phenetic relationships based on morphometric data. Such an approach is necessary with this order because the taxonomy of the group, until now, has been based

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on the few morphological characters available, and quite likely the evolutionary relationships within the order will not be elucidated using these techniques alone. Current systematic treatments in phycology generally use a combination of morphological and molecular analyses to obtain support for taxonomic decisions from several sources (Saunders et al. 1995 [Acrochaetiales-Palmariales complex], Vis and Sheath 1998 [*Batrachospermum*], Bailey 1999 [Corallinaceae], Pueschel et al. 2000 [*Audouinella*]), as is used in the current study.

The biogeography and systematics of *Hildenbrandia* in both North America and Europe have been previously examined (Sherwood and Sheath 1999, 2000). In this study we expand upon these analyses with the inclusion of collections of the Hildenbrandiales representing taxa not previously available for analysis, including *H. lecannellieri*, *H. patula*, *H. dawsonii*, and *Apophlaea sinclairii*. As well, collections from additional global regions are included from such diverse locations as the Philippines, Australia, the Canary Islands, Chile, Uruguay, and South Africa. DNA sequence analyses of the *rbc*L and 18S rRNA genes are presented here with phylogenetic reconstruction based on single gene and combined data sets, as well as morphometric analyses of global collections and the type specimens.

MATERIALS AND METHODS

Type specimens, historically significant specimens, and global collections analyzed. Type and historically significant specimens of the order Hildenbrandiales (*Hildenbrandia* and *Apophlaea*) were obtained and morphometrically analyzed (Table 1). The following type specimens were requested but were unavailable for examination (herbaria from which specimens were requested are given):

1. *Hildenbrandia* ("*Hildenbrandtia*") *arracana* ("*Arracana*") G. Zeller (1873:192); **MB**
2. *H. dawsonii* (Ardré) Hollenb. (1971:286) (basionym *H. canariensis* var. *dawsonii* Ardré [1959:230]); **PC**
3. *H. nardiana* Zanardini (1841:134); **Venezia**
4. *H. paroliniana* Zanardini (1841:135); **Venezia**
5. *H.* ["*Hildbrandtia*"] *prototypus* Nardo (1834:676); **Venezia**
6. *H. ramanaginaii* M. Khan (1974:238); **BHAV**, **BSD**, **BSIS**, **BURD**, **CAL**, **DD**
7. *H.* ["*Hildenbrandtia*"] *rivularis* spp. *chalikophila* Palik (1961: 151); **BP**
8. *H.* ["*Hildenbrandtia*"] *rosea* Kütz. (1843:384); **L**
9. *H.* ["*Hildenbrandtia*"] *sanguinea* Kütz. (1843:384); **KRAM**, **L**

The following type specimens of taxa previously synonomized with *H. rubra* were examined for verification of synonomy: *Palmella rubra* Hornem., *Erythroclathrus pellitus* Liebm., and *Rhododermis drummondii* Harv. No reproductive structures (gemmae or tetrasporangia) were visible on the specimen of *Palmella rubra*, and thus the synonomy of this species with *H. rubra* is uncertain. The type specimens of both *Erythroclathrus pellitus* and *Rhododermis drummondii* corresponded to *H. rubra*. Additional global collections of *Hildenbrandia* and *Apophlaea* included in the analyses are listed in Table 2. Coding of these samples follows standard state and provincial abbreviations for Canada and the United States.

Morphometric analyses. Type and historically significant specimens of *Apophlaea* were requested and examined, but only the upright portions of the thallus were present. Thus, morphometric comparisons to *Hildenbrandia* specimens could not be made due to the absence of the crustose portion of the thallus. Nonetheless, measurements of length and diameter of tetrasporangia were made, and tetrasporangial division pattern noted for comparison with *Hildenbrandia* specimens.

Samples were fixed in 2.5% $CaCO_3$-buffered glutaraldehyde to prevent morphological distortion, or rehydrated after preservation in silica gel, and were analyzed to determine associations based on similarity. The following characters were measured or determined for all collections as well as type and historically significant specimens: cell diameter ($n = 30$), cell length ($n = 30$), filament length ($n = 30$), and basal layer height ($n = 30$). Where possible, marine collections were measured for the following reproductive characters: maximum conceptacle depth and diameter ($n = 10$) and maximum tetrasporangial length and diameter ($n = 10$). Presence (1) or absence (0) of protuberances on the thallus surface and the tetrasporangial division pattern for each sample were also noted. Tetrasporangial division pattern was coded as follows: divisions parallel (1) or some divisions not parallel (0). To avoid excessive influence on the analyses by very large or very small measurements, the data were ranged according to Dunn and Everitt (1982), which standardized the data so that quantitative measurements ranged between 0 and 1. For each of the following analyses, both cluster analysis (UPGMA algorithm) and ordination (principal coordinates [PCO] analysis or principal components [PCA] analysis) were performed on the data (however, only the cluster dendrograms are shown because both analyses revealed very similar trends):

- Analysis 1: All marine specimens for which reproductive data were available. PCO was the ordination method used in this instance because both quantitative and qualitative data were both present in the data matrix. The Gower similarity coefficient (Gower 1971) was used because it allows the incorporation of both data forms.
- Analysis 2: All freshwater specimens. PCA was used here because all data were quantitative.

Data were compared using Euclidean distances.

Significance of groups resulting from the analyses was tested using one-way analysis of variance ($P < 0.05$). Cluster and ordination analyses were carried out using MVSP 3.0 (Multi-Variate Statistical Package; Kovach Computing Services 1986–1998), and analysis of variance were carried out using Minitab (Ryan et al. 1985). Measurements of the type and historically significant specimens were compared with those from their protologues. A separate analysis including all specimens (both marine and fresh water) was run initially but is not shown here because it simply demonstrated a clear division between the marine and freshwater samples.

rbc*L and 18S rRNA gene sequence analyses.* For as many collections as possible the *rbc*L and 18S rRNA genes were amplified and sequenced (excluding types and historically significant specimens). PCR amplification of the *rbc*L and 18S rRNA genes, purification, sequencing, and alignment were carried out as described previously (Sherwood and Sheath 1999, 2000). Several bangiophyte DNA sequences were used as outgroups in the phylogenetic analyses (*Bangia*, *Porphyra*, *Erythrotrichia*, *Smithora*, and *Porphyridium*) as described in Sherwood and Sheath (2000). Both genes were analyzed separately and in a combined data set (for samples with both *rbc*L and 18S rRNA gene sequences available). Aligned sequences are available through TreeBase (Study Accession S798; *rbc*L matrix M1263; 18S rRNA matrix M1264). Phylogenetic analyses based on maximum parsimony (MP), neighbor-joining distance analysis (NJ), and the quartet puzzling variant of maximum likelihood (QP) were carried out to compare estimated relationships among samples using a variety of techniques. All phylogenetic analyses were carried out in PAUP*4.0b (Swofford 2000). MP analysis was performed with heuristic searches (100 replicates) under the conditions of random addition of taxa, steepest descent, and tree bisection-reconnection (TBR) branch swapping. Bootstrap resampling (2000 replicates) and decay analysis (AutoDecay v.4.0.2; Eriksson 1997) were used to determine support for nodes on trees. The DNA substitution model best suited for the alignments was determined using the program Modeltest (Posada and Crandall 1998); in both cases

TABLE 1. Type and historically significant specimens of the order Hildenbrandiales (*Hildenbrandia* and *Apophlaea*) examined.

Type specimen analyzed	Basionym and reference	Locality, collector, and date	Herbarium and specimen number
Apophlaea lyallii Hook.f. (syntype)	Same. 1855:244	On rocks, Preservation Harbour, Middle Island, New Zealand. D. Lyall (i.1851)	E (E00044471)
A. lyallii var. *gigartinoides* Hook. f. (syntype)	Same. 1855:244	Otago, New Zealand. D. Lyall (iii. 1850)	BM (000530643)
A. sinclairii Harv. ex Hook. f. et Harv. (syntype)	Same. 1845: 550	New Zealand. Sinclair (no date given)	BM (000530644)
Hildenbrandia ("*Hildenbrandtia*") *angolensis* Welw. ex W. West et G.S. West (syntype)	Same. 1897:3	Golungo Alto, Ad silices in rivulis sylv. primit. de Quibanga pr. Sange, Angola. Welwitsch (vi. 1857)	BM (Welwitsch Collection no. 150; BM 3435 slide collection)
H. canariensis Børgesen (syntype)	Same. 1929:15	Gran Canaria, south of Las Palmas near Christoballo. F. Børgesen (29.iii.1921)	C (3986)
H. ("*Hildenbrandtia*") *crouanii* ("*crouani*") (J. Agardh) J. Agardh (holotype)	*Haematophlaea crouani* (1852: 495). 1876:379	Sur les roches "Dit" ansi du Cortem, environs de Brest. "Frères" Crouan (no date given).	LD (27613)
H. ("*Hildenbrandtia*") *expansa* Dickie (syntype)	Same. 1874:357	St. Paul's Rocks (Challenger Expedition). H.N. Moseley (27.viii.1873).	BM (000530646)
H. fluviatilis Bréb. (historically significant specimen)	—	Specimen on a rock, glued to a card. Falaise, France.	PC (no number given)
H. fluviatilis Bréb. (historically significant specimen)	—	Specimen on a rock, glued to a card. Falaise, France.	S (no number given)
H. ("*Hildenbrandtia*") *galapagensis* Setch. et N.L. Gardner (holotype)	Same. 1937:91	Charles Island, Galapagos. J.T. Howell (26.iv.1932)	UC (236519)
H. kerguelensis (Askenasy) Y.M. Chamb. (slides of the holotype)	*H.* ("*Hildenbrandtia*") *prototypus* var. *kerguelensis* Askenasy (1888:30). 1962:372	Kerguelen (Gazelle Expedition). L. Askenasy (ix.1888)	BM (00530647)
H. ("*Hildenbrandtia*") *lecannellieri* ("*Le Cannellieri*"), "*Le Cannelieri*") Har. (slide of the holotype)	Same. 1887:74	Ad rupes maritimas Baie Orange (Fuegia). Hariot (viii.1883)	PC (no number given)
H. ("*Hildenbrandtia*") *occidentalis* Setch. ex N.L. Gardner (holotype)	Same. 1917:393	Land's End, San Francisco, California, U.S.A. N.L. Gardner (4.i.1916)	UC (188974)
H. ("*Hildenbrandtia*") *occidentalis* var. *lusitanica* Ardré (holotype)	Same. 1959:233	Supra rupes in oceano atlantico ad oras Lusitaniae, Parede. F. Ardré (26.vi.1957)	PC (no number given)
H. ("*Hildenbrandtia*") *occidentalis* var. *yessoensis* (Yendo) Ardré (slides of holotype)	*H.* ("*Hildenbrandtia*") *yessoensis* Yendo (1920:11). 1959:233	In rupibus maritimas ad oras Yesso, Oshoro, Hokkaido, Japan. K. Yendo (30.iii.1915)	SAP (no number given)
H. patula Womersley (isotype)	*H. expansa* Womersley (homonym) (1994:145). 1996:357	Apollo Bay, Victoria, Australia. H.B.S. Womersley (6.ii.1990)	AD (ADA60088)
H. ("*Hildbrandtia*") *rivularis* (Liebm.) J. Agardh (measurements from Sheath et al. 1993)	*Erythroclathrus rivularis* Liebm. (1839:174) 1852:495	Stream at Kiugs Mills, Sealand, Denmark. S. Hornemann (vi.1826)	C (no number given)
H. rivularis var. *drescheri* ("*Drescheri*") Lingelsh. (lectotype here designated)	Same. 1922:355	Mühlgraben bei Ellguth K. Othnackan. E. Drescher (1920)	BRSL (no number given)
H. ("*Hildenbrandtia*") *rubra* (Sommerf.) Menegh. (holotype)	*Verrucaria rubra* Sommerf. (1826: no pagination). 1841:426	Yaltdalen. Sommerfelt (vii.1822)	O (no number given)
H. sanjuanensis Hollenb. (slide of holotype)	Same. 1969:164	High intertidal one-half mile east of Friday Harbour Laboratories, San Juan Is., Washington, U.S.A. D. Russell (19.vi.1968)	US (00061194)
H. sanjuanensis Hollenb. (historically significant specimen)	—	About 100 yards south of Small Pox Bay, San Juan Island, Washington, U.S.A. G.J. Hollenberg (14.vi.1968)	US (066265, US slide #-1419)

Herbarium abbreviations are according to Holmgren et al. (1990).

TABLE 2. Collection or source information for specimens of marine and freshwater *Hildenbrandia* and *Apophlaea* used in this study.

Taxon	Collection or source information	Sample coding	GenBank Accession *rbc*L gene	GenBank accession 18S rRNA gene
H. rubra	Sherwood and Sheath (1999)	AKSW1	AF107811	AF108399
H. rubra	Sherwood and Sheath (1999)	BCSW1	—	AF108400
H. rubra	Sherwood and Sheath (1999)	BCSW2	AF107813	—
H. rubra	Sherwood and Sheath (1999)	BCSW3	—	—
H. occidentalis	Parkesville Bay, Vancouver Island, BC, Canada. Coll. S. Thompson & C. Sarzyzick, 5 October 1999.	BCSW4	AF534404	AF534412
H. rubra	Sherwood and Sheath (1999)	WASW1	—	—
H. rubra	Sherwood and Sheath (1999)	ORSW1	—	—
H. rubra	Sherwood and Sheath (1999)	ORSW2	AF107826	AF108414
H. rubra	Sherwood and Sheath (1999)	CASW1	AF107814	AF108401
H. rubra	Sherwood and Sheath (1999)	CASW2	—	—
H. occidentalis	Sherwood and Sheath (1999)	CASW3	AF107815	AF108402
H. rubra	Sherwood and Sheath (1999)	MEXSW1	—	AF108410
H. rubra	Sherwood and Sheath (1999)	MEXSW2	AF107823	—
H. dawsonii	Todos Santos, Baja California, Mexico. Coll. S. Fredericq, 24 October 1999.	MEXSW3	—	AF534413
H. rubra	Sherwood and Sheath (1999)	CRSW1	—	—
H. rubra	Sherwood and Sheath (1999)	CRSW2	AF107819	—
H. rubra	Water taxi dock at Belize City, Belize. Coll. R. Sheath & M. Koske, 31 December 1999.	BLZSW1	—	—
H. rubra	Sherwood and Sheath (1999)	NFSW1	AF107824	AF108411
H. rubra	Sherwood and Sheath (1999)	NSSW1	AF107825	AF108412
H. rubra	Sherwood and Sheath (1999)	MASW1	AF107821	AF108409
H. rubra	Sherwood and Sheath (1999)	RISW1	—	—
H. rubra	Sherwood and Sheath (1999)	CTSW1	AF107820	AF108408
H. angolensis	Sherwood and Sheath (1999)	TX7	—	AF108417
H. angolensis	Sherwood and Sheath (1999)	TX9	—	AF108418
H. angolensis	Sherwood and Sheath (1999)	CR20	AF107816	AF108404
H. angolensis	Sherwood and Sheath (1999)	CR24	AF107817	AF534414
H. angolensis	Sherwood and Sheath (1999)	SL2	—	—
H. angolensis	Sherwood and Sheath (1999)	SL9	—	AF108416
H. angolensis	Sherwood and Sheath (1999)	PR19	—	AF108415
H. angolensis	Hwy 301 N. of Sumterville, FL, USA. Coll. A. Sherwood & K. Müller, 29 March 1999.	FL63	AF534405	AF534415
H. rubra	Sherwood and Sheath (2000)	SWESW1	AF208812	AF208828
H. rubra	Sherwood and Sheath (2000)	SWESW1	AF208807	AF208826
H. crouanii	Sherwood and Sheath (2000)	SCOSW1	AF208808	AF534416
H. crouanii	Sherwood and Sheath (2000)	SCOSW3	—	—
H. crouanii	Sherwood and Sheath (2000)	SCOSW4	AF208809	—
H. rubra	Sherwood and Sheath (2000)	WALSW3	AF208815	AF208831
H. rivularis	Sherwood and Sheath (2000)	WAL2	AF208813	AF208829
H. rivularis	Sherwood and Sheath (2000)	WAL3	AF208814	AF208830
H. rubra	Sherwood and Sheath (2000)	NISW1	AF208799	AF208819
H. rivularis	Sherwood and Sheath (2000)	IR11	AF208805	AF208824
H. crouanii	Sherwood and Sheath (2000)	GERSW1	AF208803	AF534417
H. rivularis var. *drescheri*	Sherwood and Sheath (2000)	GER1	AF208804	AF208823
H. rubra	Sherwood and Sheath (2000)	NEDSW1	AF208801	AF208821
H. rubra	Sherwood and Sheath (2000)	FRASW1	AF208800	AF208820
H. rivularis	Sherwood and Sheath (2000)	FRA1	AF208802	AF208822
H. rivularis	Sherwood and Sheath (2000)	AT10	—	AF208816
H. rivularis	Sherwood and Sheath (2000)	AT14	AF208797	AF208817
H. rivularis	Sherwood and Sheath (2000)	AT15	AF208798	AF208818
H. rivularis	Sherwood and Sheath (2000)	SPA1	AF208810	—
H. rivularis	Sherwood and Sheath (2000)	SPA2	AF208811	AF208827
H. rivularis	Sherwood and Sheath (2000)	ITA1	AF208806	AF208825
H. lecannellieri	Pt. Bufnes, Chile. Coll. S. Fredericq. 11 May 1994.	CHISW1	AF534406	AF534418
H. crouanii	Beach at José Ignacios, Uruguay. Coll. G. Lemon, November 1998.	URUSW1	AF534407	AF534419
H. lecannellieri	Bellville, South Africa. Coll. G. Maneveldt, July 1999.	SASW1	AF534408	AF534420

(*continued*)

TABLE 2. (Continued).

Taxon	Collection or source information	Sample coding	GenBank Accession *rbc*L gene	GenBank Accession 18S rRNA gene
H. patula	Near Schnapper Point, west of Beachport, South Australia. Coll. R. Harvey & P. Mitrovski, February 2000	AUSSW1	—	AF534421
H. rubra	Near Schnapper Point, west of Beachport, South Australia. Coll. R. Harvey & P. Mitrovski, February 2000.	AUSSW2	—	AF534422
H. angolensis	Sto. Ninjo Cold Spring, Camiguin, Philippines. Coll. H.-G. Wagner, 1998.	PHI1	AF534409	AF534423
H. rivularis	Stream near Tenerife, Canary Islands. Coll. H.-G. Wagner, 1999.	CI1	—	—
Apophlaea sinclairii	Leigh Marine Station, Goat Island Bay, North Island, New Zealand (WELT A22671). Coll. R. Creese & W. Nelson, 6 April 2000.		AF534410	AF534424
Apophlaea lyallii	Brighton Beach, Otago New Zealand. Coll. C. Hurd, January 1997.		AF534411[a]	AF076996[b]

Samples without any GenBank accession numbers were used only in morphometric analyses.

MEX, Mexico; CR, Costa Rica; BLZ, Belize; SL, Saint Lucia; PR, Puerto Rico; SWE, Sweden; NOR, Norway; SCO, Scotland; WAL, Wales; NI, Northern Ireland; IR, Ireland; GER, Germany; NED, The Netherlands; FRA, France; AT, Austria; SPA, Spain; ITA, Italy; CHI, Chile; URU, Uruguay; SA, South Africa; AUS, Australia; PHI, Philippines; CI, Canary Islands. Samples followed by "SW" are salt water, or marine, collections.

[a] DNA sample of *Apophlaea lyallii* for *rbc*L gene amplification provided by G. W. Saunders.

[b] 18S rRNA gene sequence data for *Apophlaea lyallii* from Saunders and Bailey (1999).

the parameter-rich general time reversible (GTR) model was selected. Both NJ and QP trees were constructed based on this model and the appropriate parameters. For the NJ trees, 2000 bootstrap resampling replicates were used to assess support. QP reconstruction was carried out using 2000 puzzling steps as described by Strimmer and von Haeseler (1996).

Character analysis. Tetrasporangial morphology was mapped onto both the *rbc*L and the 18S rRNA gene phylogenies resulting from parsimony analysis using the program MacClade v.3 (Maddison and Maddison 1992) to examine the evolution of this character within the Hildenbrandiales. Tetrasporangial morphology is the only character from the morphometric data set that lends itself to this kind of analysis because it is the only one with discrete character states.

RESULTS

Morphometric analyses. Mean tetrasporangial dimensions of *Apophlaea sinclairii*, *A. lyallii*, and *A. lyallii* var. *gigartinoides* type and historically significant specimens (length 23.9–26.1 μm; diameter 6.0–7.5 μm) were well within the range of dimensions of marine *Hildenbrandia* specimens (length 15.0–45.0 μm; diameter 4.3–13.5 μm) (Table 3). The tetrasporangial divisions of all *Apophlaea* specimens were parallel.

Analysis 1. Cluster analysis (Fig. 1) and PCO analysis (not shown) of all marine specimens for which reproductive character data were obtainable demonstrated two groups, which were distinguishable based on tetrasporangial division pattern. The two groups are significantly different based on cell length ($P < 0.034$), filament height ($P < 0.001$), conceptacle diameter ($P < 0.023$), conceptacle depth ($P < 0.002$), and tetrasporangial diameter ($P < 0.010$). Within group A, the type specimens of *H. occidentalis* and *H. lecannellieri* are distinct from the remainder of the specimens in that group, based on their large filament length ($P < 0.001$) and large cell length ($P < 0.003$).

Few biogeographic trends are evident from the morphometric analyses of marine specimens, because collections from such geographically distinct locations as the Atlantic and Pacific oceans (e.g. RISW1 and BCSW1; CTSW1 and CASW1) and Australia and the North Sea (AUSSW1 and GERSW1) associate in the analyses.

Comparison of our measurements of the type and historically significant specimens to their protologues indicated some large differences between the two sets of measurements (Table 3). For example, we observed the thallus thickness of the type specimens of *H. lecannellieri* and *H. occidentalis* to be substantially smaller than reported in the protologues of these two species (e.g. approximately 930 μm vs. 5000–8000 μm, and approximately 670 μm vs. 1000–2000 μm, respectively) (Hariot 1887, Gardner 1917).

Analysis 2. The cluster dendrogram (Fig. 2) and PCA biplot (not shown) based on analyses of all freshwater samples and type specimens demonstrated two groups

TABLE 3. Means of morphometric characters of type and historically significant specimens of *Hildenbrandia* and *Apophlaea* (protologue measurements, where available, are listed in parentheses).

Specimen name	Cell diameter (μm)	Cell length (μm)	Filament height (μm)	Basal layer height (μm)	Conceptacle diameter (μm)[a]	Conceptacle depth (μm)[d]	Tetrasporangial length (μm)[a]	Tetrasporangial diameter (μm)[a]
H. angolensis[b]	5.5	4.6	44.4	8.5	—	—	—	—
	(3.5–5.0)	(—)	(—)	(—)	—	—	—	—
H. canariensis	5.1	4.5	175.1	12.3	94.6	87.5	25.6	8.9
	(4.0–5.0)	(6.0)	(<250)	(—)	(~100)	(~100)	(26.0)	(8.0)
H. crouanii	4.4	4.7	329.3	12.9	114.2	83.6	30.4	11.6
	(—)	(—)	(—)	(—)	(—)	(—)	(—)	(—)
H. fluviatilis (PC)[b,c]	5.6	8.3	35.5	10.2	—	—	—	—
	(—)	(—)	(—)	(—)	—	—	—	—
H. fluviatilist(S)[b,d]	7.1	7.3	40.3	6.4	—	—	—	—
	(—)	(—)	(—)	(—)	—	—	—	—
H. galapagensis	3.8	4.2	128.7	14.6	74.6	83.8	27.5	12.3
	(3.5–4.0)	(3.5–4.0)	(300–350)	(—)	(—)	(—)	(22.0–28.0)	(10.0–14.0)
H. kerguelensis	3.8	5.2	355.8	13.3	117.1	161.4	22.9	6.6
Chamberlain	(—)	(—)	(370.0)	(—)	(100)	(200)	(25.0)	(6.0)
(1962)[e]	(4.0–7.0)	(4.0–7.0)	(<560)	(—)	(100–145)	(130–185)	(25.0–30.0)	(3.0–7.0)
H. lecannellieri	4.8	7.0	928.5	11.4	75.4	70.1	30.9	7.4
	(5.0–10.2)	(5.0–10.2)	(5000–8000)	(—)	(—)	(—)	(—)	(—)
H. occidentalis	4.3	6.6	667.5	13.7	121.0	201.2	33.6	9.0
	(3.0–4.5)	(3.0–13.5)	(1000–2000)	(—)	(100–150)	(200–800)	(25.0–32.0)	(9.0–10.0)
H. occidentalis var. *lusitanica*	3.5	5.0	292.1	12.3	90.6	126.1	40.9	9.4
	(3.0–4.5)	(3.0–8.0)	350–500)	(—)	(100–150)	(200–300)	32.0–42.0)	(9.0–10.0)
H. occidentalis var. *yessoensis*	3.7	6.0	304.8	14.2	98.7	157.7	17.4	5.1
	(3.8–4.0)	(3.0–4.0)	(200–500)	(—)	(—)	(70–90)	(—)	(—)
H. patula	3.8	5.6	295.5	15.0	63.3	64.6	26.1	8.7
	(3.0–7.0)	(3.0–10.0)	(250–800)	(—)	(40–60)	(80–120)	(20.0–30.0)	(7.0–10.0)
H. rivularis[b]	8.4	8.6	38.4	5.5	—	—	—	—
	(—)	(1.5–2× diameter)	(—)	(—)				
H. rivularis var. *drescheri*[b]	6.0	6.4	52.8	9.2	—	—	—	—
	(—)	(—)	(—)	(—)	—	—	—	—
H. rubra	4.3	4.6	102.2	12.4	88.5	93.0	30.9	10.2
	(—)	(—)	(—)	(—)	(—)	(—)	(—)	(—)
H. sanjuanensis (type)	3.4	4.8	238.5	13.4	64.2	94.8	13.6	3.9
	(3.0–5.0)	(1.2–1.5× diameter	(<540)	(—)	(45–90)	(<180)	(9.0–11.0)	(2.5–3.5)
H. sanjuanensis (collection from type locality)	4.2	5.7	322.1	12.8	74.1	107.4	11.0	3.6
Apophlaea lyallii[f]	—	—	—	—	—	—	23.9	6.0
							(—)	(—)
A. lyallii var. *gigartinoides*[f]	—	—	—	—	—	—	26.1	7.5
							(—)	(—)
A. sinclairii[f]	—	—	—	—	—	—	25.2	6.0
							(—)	(—)

[a] Measurements of conceptacle and tetrasporangial dimensions are not applicable to freshwater specimens.
[b] Freshwater specimens (no measurements of tetrasporangia or conceptacles).
[c] Possible type specimen of *H. fluviatilis* from herbarium PC.
[d] Possible type specimen of *H. fluviatilis* from herbarium S.
[e] Although not the protologue, independent measurements of the type specimen of *H. kerguelensis* are reported in this publication.
[f] Only tetrasporangial division pattern and measurements of tetrasporangial dimensions could be obtained for *Apophlaea* specimens (see text).

that corresponded to the European/Canary Islands collections and the North American/Philippines collections. The two groups are significantly different based on cellular dimensions ($P < 0.001$), filament length ($P < 0.032$), and basal layer height ($P < 0.001$), with the European group having larger measurements for all characters than the North American group.

Strong biogeographic trends are evident from this data set in that all collections from the continents of North America and Europe are distinct from one another. The geographically distant Philippines freshwater collection morphometrically corresponds to the North American *H. angolensis*, which may represent a continuum of this taxon across the Pacific because it has also been identified from Hawaii and Fiji (Vis et al. 1994, Sheath and Cole 1996). The Canary Islands collection more closely resembles the European collections of *H. rivularis* than *H. angolensis*, which was originally described from Africa.

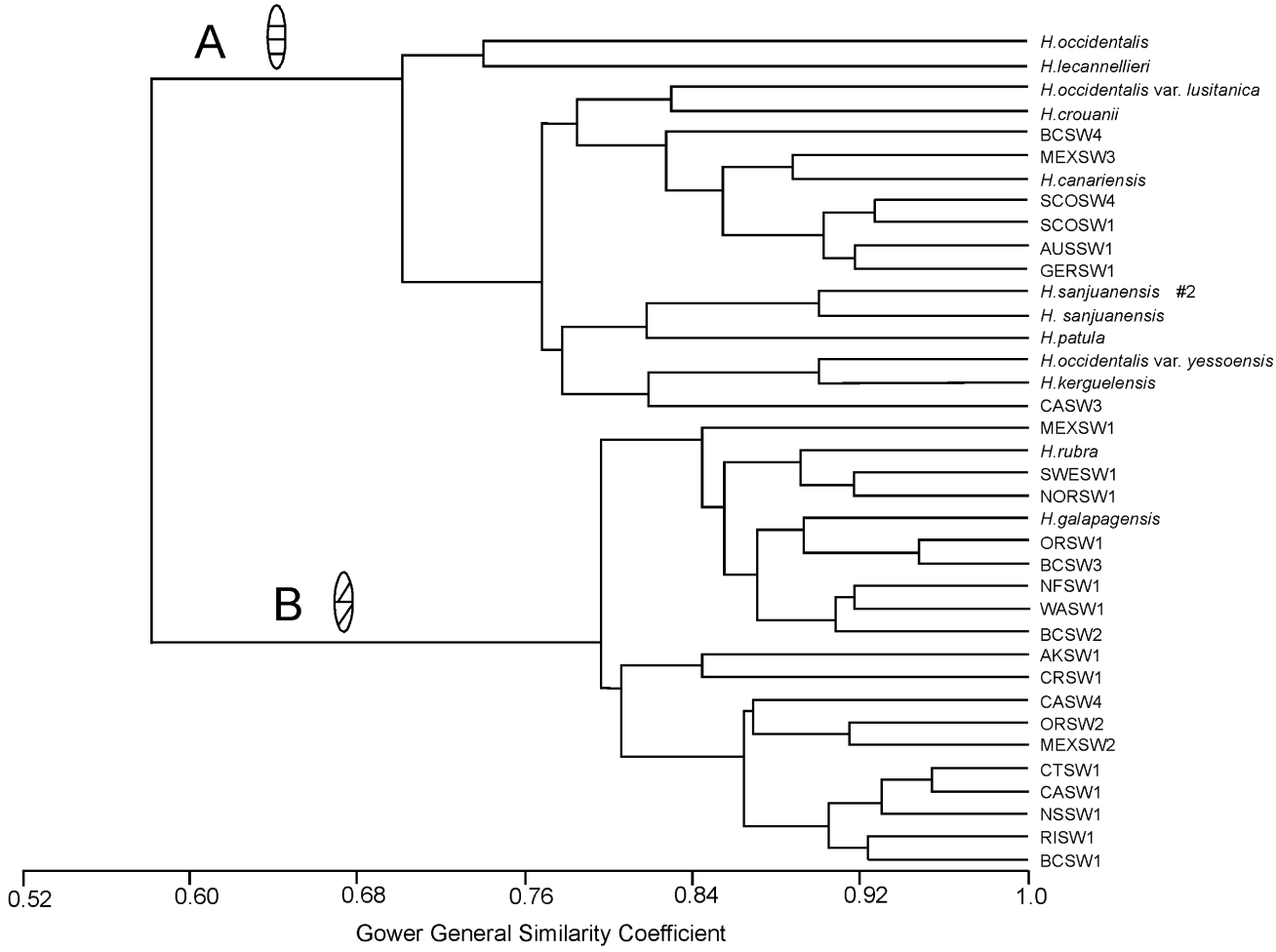

FIG. 1. Cluster dendrogram of all marine specimens (including type and historically significant specimens) for which reproductive character data were available, based on all characters. Two groups are evident, corresponding to specimens with parallel tetrasporangial division pattern (A) and those with nonparallel tetrasporangial divisions (B). The numerical scale indicates the level of similarity at which clusters are formed, according to the Gower similarity coefficient.

18S rRNA gene sequence analyses. Parsimony analysis of representatives of the Hildenbrandiales for the 18S rRNA gene yielded four most-parsimonious trees. One of the four trees is shown in Figure 3; the others differed in the position of the WALSW3 sample within the *H. rubra* group and the position of several other North American and European *H. rubra* samples with respect to one another (these positions were all equivocal in the analyses). Very few topological differences were observed among the different analyses of the 18S rRNA gene sequence data, and thus only the parsimony tree is shown, with support measures from other analyses included on this figure. The ingroup (the Hildenbrandiales) is well supported as monophyletic (100% bootstrap proportion [BP], 154 decay steps). *Hildenbrandia rubra* is a paraphyletic taxon (three separate clades), and there is little support for the relationships among these groups. Several *H. rubra* collections from both Europe and North America are identical in their phylogenetically informative sites (NSSW1, NORSW1, and FRASW1), paradoxically indicating a closer relationship among these samples from different continental coastlines than among some from the same coastline. Another *H. rubra* clade contains four Pacific North America samples (BCSW1, MEXSW1, CASW1, and AKSW1) as well as one collection from eastern Canada (NFSW1), and from Figure 3 it can be seen that these collections are phylogenetically very close despite the fact that some of them do not share the same ocean basin (i.e. NFSW1 and AKSW1).

Some taxa are strongly supported by the analyses. For example, the two collections morphologically corresponding to *H. lecannellieri* (SASW1 and CHISW1) are monophyletic (98%–100% BP, 8 decay steps), as are the two collections of *H. occidentalis* (BCSW4 and CASW3, identical in 18S rRNA gene sequence). Several marine species with the same (parallel) tetrasporangial divisions form a clade, including *H. crouanii*, *H. patula*, and *H. dawsonii*. However, one additional sample of *H. crouanii* (GERSW1) is in an unsupported position

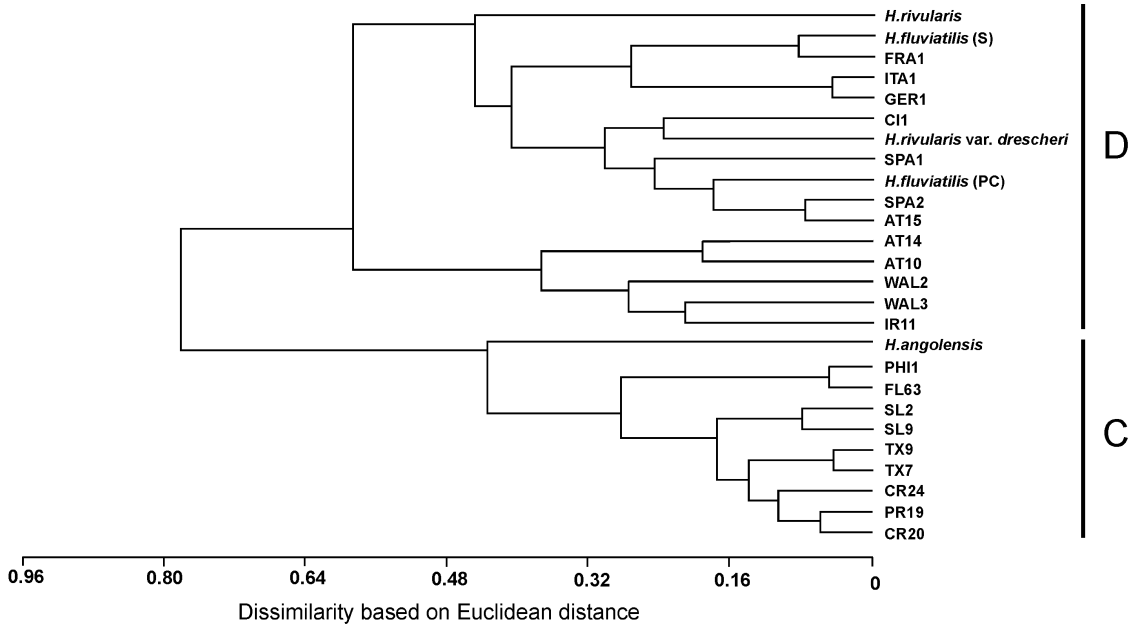

FIG. 2. Cluster dendrogram of all freshwater specimens (including type specimens and historically significant specimens). Two groups are evident, corresponding to those specimens from North America/Philippines (C) and Europe/Canary Islands (D). The numerical scale indicates the level of dissimilarity based on Euclidean distances.

near the base of the tree, which is most likely due to the large number of undetermined bases (N's) in this sequence. The two collections of the second genus within the Hildenbrandiales, *Apophlaea*, group tightly together (97%–100% BP; 6 decay steps); however, the phylogenetic position of *Apophlaea* with respect to the genus *Hildenbrandia* is unresolved because there is little or no support for the positioning of the major clades with respect to one another.

The freshwater collections of *Hildenbrandia* form a monophyletic group with the exception of two North American samples of *H. angolensis* (TX9 and PR19), which are poorly supported near the base of the tree. Support for the main freshwater clade is variable but is stronger with the omission of *H. angolensis* samples SL9 and TX7 (80%–95% BP; decay 1 step). As previously reported (Sherwood and Sheath 2000), many of the European *H. rivularis* samples are identical in sequence for the 18S rRNA gene, and the relationships among these samples are unresolved due to this lack of a phylogenetic signal, resulting in a polytomy of *H. rivularis* samples in these analyses. The North American freshwater collections are supported as being basal to the European collections and the one sample of *H. angolensis* from the Philippines groups with a North American collection of the same species (FL63) with weak support (58%–67% BP; 1 decay step).

rbc*L gene sequence analyses.* Parsimony analysis of the *rbc*L gene for representatives of the Hildenbrandiales yielded one most-parsimonious tree (Fig. 4). Again, the resulting topologies from the different forms of analysis were very similar to one another, and so only the parsimony tree is shown, but with the measures of support from MP, NJ, and QP superimposed. The associations seen in the 18S rRNA gene sequence analyses were largely seen in the *rbc*L analyses as well. As in the 18S rRNA gene analyses, the common marine taxon *H. rubra* forms several clades and is therefore not monophyletic, and many of the same associations are evident (e.g. AKSW1 and NFSW1; MASW1, NISW1, and NORSW1). In contrast to the 18S rRNA gene results, a clade containing most of the marine *H. crouanii* samples is not formed, and these samples are scattered throughout the tree. The North American freshwater taxon, *H. angolensis*, is not monophyletic, which was also illustrated by the 18S rRNA gene results, and the collections from the Philippines and Florida (PHI1 and FL63) are not associated, which was indicated by the previous analyses. The European freshwater collections of *H. rivularis* (and *H. rivularis* var. *drescheri*) once again form a monophyletic group with high support (100% BP; decay 3 steps). The well-supported monophyletic taxa in the 18S rRNA gene sequence analyses are also indicated here in the *rbc*L gene sequence analyses (*H. lecannellieri*, *H. occidentalis*, *H. rivularis* [and its variety, *drescheri*], and *Apophlaea*). Again, although *Apophlaea* forms a monophyletic group, the position of the clade with respect to *Hildenbrandia* is unresolved.

The combined data set of *rbc*L and 18S rRNA sequences was analyzed but resulted in phylogenetic trees with much less resolution than those shown (Figs. 3 and 4). Because these analyses did not further resolve the relationships among the samples, they are not shown.

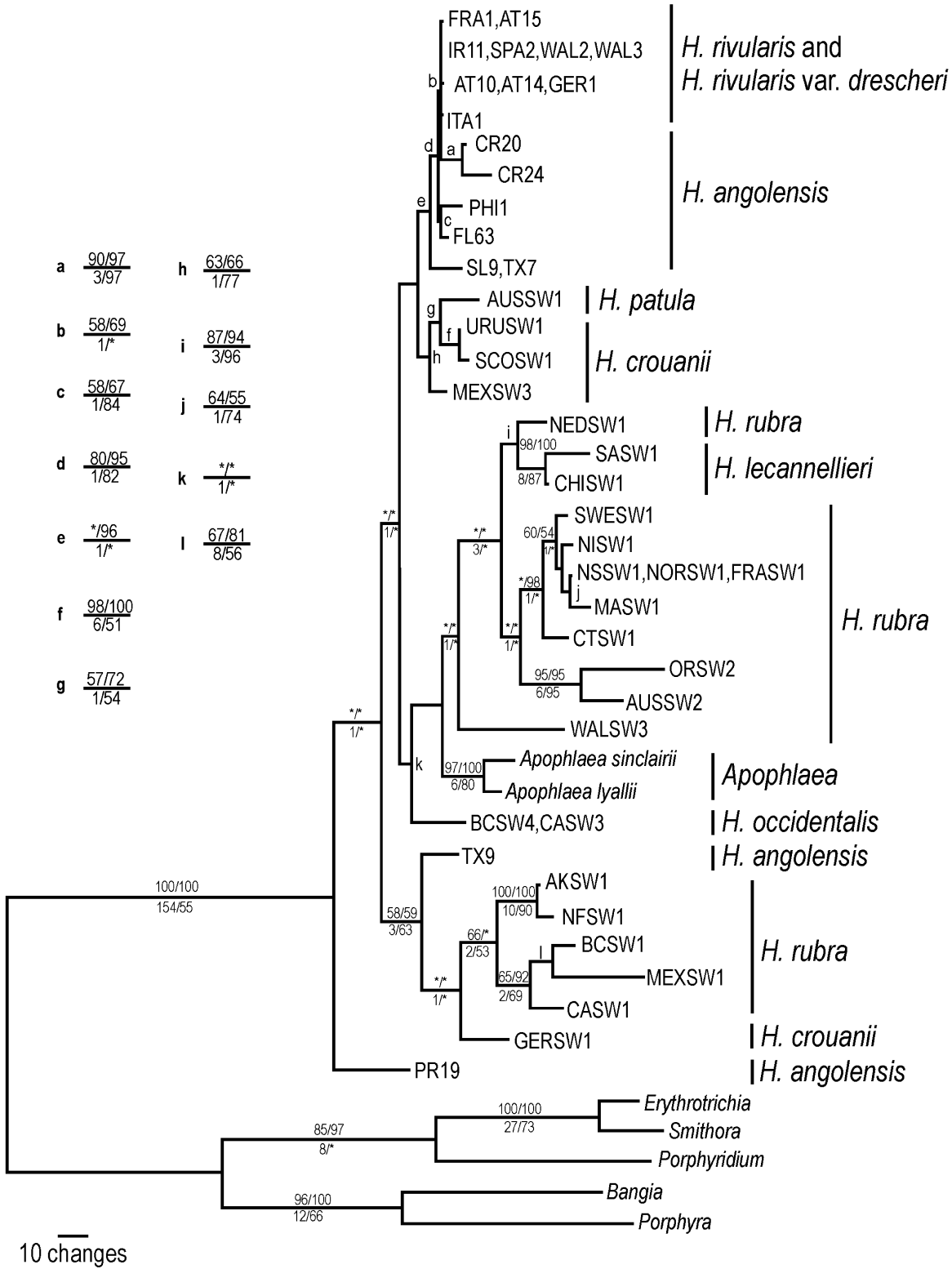

FIG. 3. One of four most-parsimonious trees based on analysis of the 18S rRNA gene for representatives of the Hildenbrandiales. Branch lengths are indicated above branches. Support measures are included for all forms of phylogenetic analysis (MP, NJ, and QP), where appropriate, as follows: MP bootstrap values above branch and left of slash, decay values below branch and left of slash, NJ bootstrap values above branch and right of slash, QP values below branch and right of slash. Some sets of support values are indicated on the tree as a letter, and corresponding values are shown to the upper left of the diagram. Asterisks indicate a lack of support at that node for the corresponding measure.

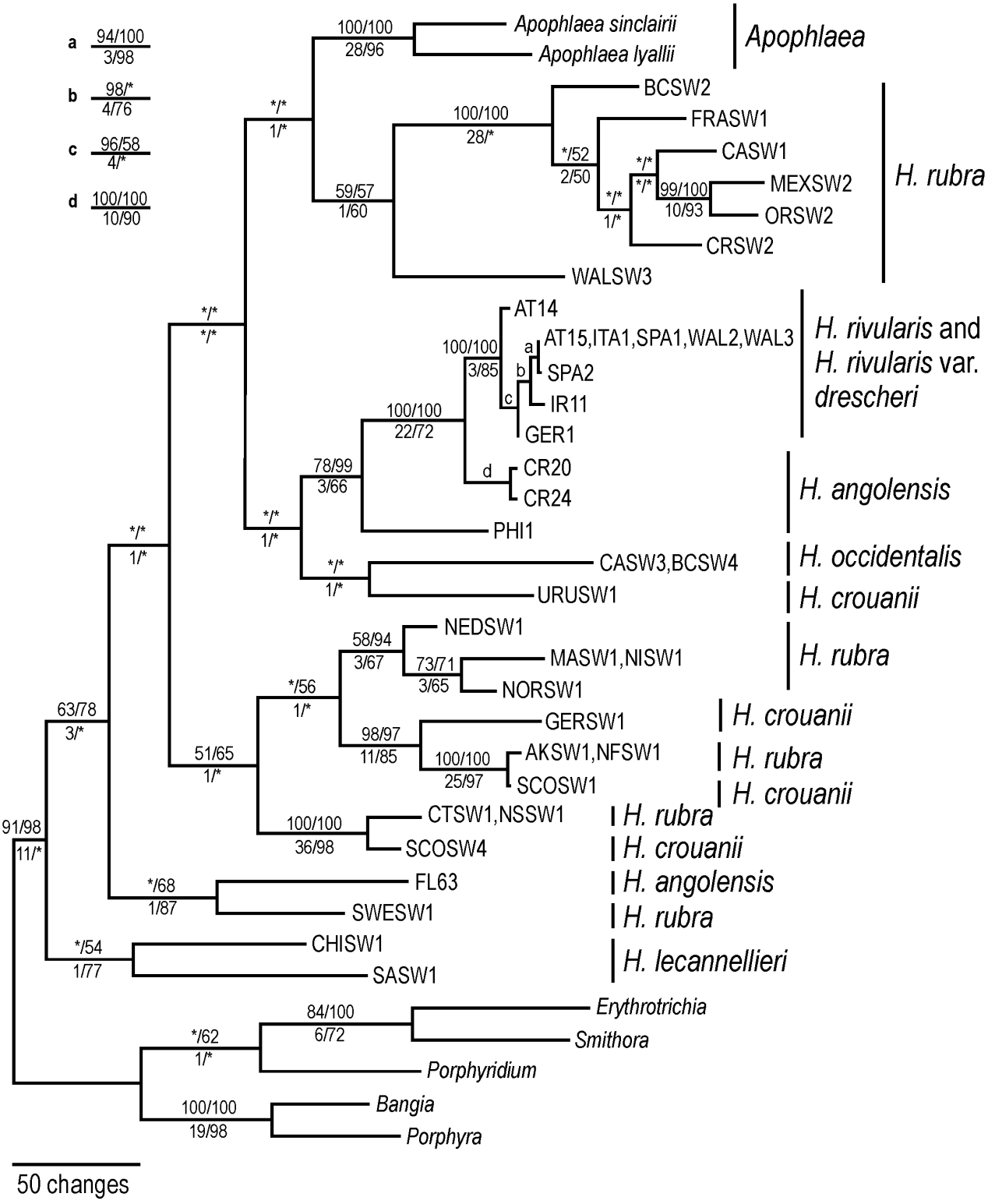

FIG. 4. The single most-parsimonious tree (showing branch lengths) generated by parsimony analysis of the *rbc*L gene for representatives of the Hildenbrandiales. Support measures are included for all forms of phylogenetic analysis (MP, NJ, and QP), where appropriate, as follows: MP bootstrap values above branch and left of slash, decay values below branch and left of slash, NJ bootstrap values above branch and right of slash, QP values below branch and right of slash. Some sets of support values are indicated on the tree as a letter, and corresponding values are shown to the upper left of the diagram. Asterisks indicate a lack of support at that node for the corresponding measure.

Evolution of tetrasporangial morphology in the Hildenbrandiales. Because most morphological characters used to distinguish species within the Hildenbrandiales are known to vary with environmental factors and the age of the alga, the taxonomically critical character of tetrasporangial morphology was mapped onto the topologies of the 18S rRNA gene tree (Fig. 5a) and the *rbc*L gene tree (Fig. 5b). Neither form of tetrasporangial morphology has uniquely arisen according to these analyses (except for the parallel division morphology in the *rbc*L analysis), indicating that these morphologies have most likely evolved several times within the order. Although relationships of the freshwater samples within the 18S rRNA gene tree are equivocal, the *rbc*L gene tree illustrates the large freshwater clade (containing almost all freshwater collections) as arising from a marine lineage with parallel tetrasporangial divisions (Fig. 5b).

DISCUSSION

The present combination of morphometric and gene sequence analyses applied to representatives of the Hildenbrandiales has provided a necessary framework for establishing hypotheses regarding the evolutionary relationships within the order, which until now were not well understood. Because the traditional taxonomy of the Hildenbrandiales was based on morphology, a reevaluation of the order would not be advisable without a morphological data set gathered in a standardized fashion. Thus, the comparison of traditional morphology with the results of modern molecular methods has yielded the strongest data set yet gathered on the Hildenbrandiales for systematic purposes.

The analyses presented here based on the 18S rRNA gene, the *rbc*L gene, and combined gene data sets point to a monophyletic *Apophlaea* within a paraphyletic *Hildenbrandia*. Previous molecular analyses (Saunders and Bailey 1999) have also supported the continued placement of *Apophlaea* within the Hildenbrandiales. The morphometric analyses of the type specimens could not include the types of *Apophlaea* because only the upright portions of the *Apophlaea* thalli were preserved, yet the basal crustose portion is the only part of the plant directly comparable with *Hildenbrandia*. This shortcoming of the type method was compensated for, in part, by including *Apophlaea* in the molecular analyses. Within *Apophlaea*, sequence divergence values between the two species (8.9% for the *rbc*L gene and 1.2% for the 18S rRNA gene), in combination with the different morphologies of the two species, also supports continued recognition of both *A. sinclairii* and *A. lyallii*.

Comparison of the phenetic analyses (cluster and ordination) with the 18S rRNA and *rbc*L gene sequence analyses for the Hildenbrandiales demonstrates some similar trends for the freshwater taxa but few similarities for the marine taxa. In all analyses (cluster, PCA/PCO, *rbc*L gene sequences, and 18S rRNA gene sequences), *H. rivularis* forms a very distinct group that is separate from *H. angolensis*. This supports the use of the cell dimensions, filament height, and basal layer height as good characters to distinguish these species. The two species are also biogeographically distinct, with *H. rivularis* found in Europe and the Canary Islands and *H. angolensis* found in North America and the Philippines. The marine samples, however, are separated in the cluster dendrogram and PCO biplot (not shown) by tetrasporangial morphology, and this distinction is only partially congruent with the phylogenies produced. Other relationships that are evident from the phylogenetic analyses, especially biogeographic patterns, are not discernible from the phenetic results.

Although several species of *Hildenbrandia* included in the molecular analyses are monophyletic and demonstrate relatively small levels of sequence variation (e.g. *H. occidentalis* and *H. rivularis*), others are genetically heterogeneous and do not form monophyletic groups (e.g. *H. rubra* and *H. angolensis*). Taxonomically, this presents a problem because these "unnatural" groupings should not be interpreted as species in a cladistic sense (Wiley et al. 1991). This raises the question "What are *H. angolensis*, *H. rivularis*, and *H. rubra*?" Neither *H. angolensis* nor *H. rubra* is monophyletic in any of the gene sequence analyses, which raises the question of whether these "species" are actually comprised of a number of morphologically similar yet evolutionarily distinct lineages. This is certainly a possibility given the very simple morphology of members of the genus. *Hildenbrandia rivularis* is rendered paraphyletic in all gene sequence analyses by the inclusion of *H. rivularis* var. *drescheri* within the *H. rivularis* clade, and separation of the two taxa is not supported by the molecular data. Given that *H. rivularis* var. *drescheri* was described based only on the subjective character of a slightly darker color than *H. rivularis*, the synonymy of the two taxa is here recommended.

So few morphological characters are available for the Hildenbrandiales that the groups indicated in the phylogenetic trees cannot be reliably related to a morphologically based taxonomic scheme. The reasons for incongruency in groupings from the different analyses must be further investigated. Some possibilities include use of molecular markers of limited informative ability for the group and convergent morphology of several independent lineages. As previously mentioned, it is not an unreasonable postulation that the simple morphology of *Hildenbrandia* arose multiple times (by reduction of upright thallus portions to the crustose base or by uncoupling from an upright gametophytic generation), resulting in paraphyly or polyphyly of the genus and/or some genetic lineages within it. If such an event can be demonstrated, then eventually, with data from more taxa worldwide and the use of additional molecular markers to ascertain the phylogenetic relationships within the Hildenbrandiales, *Hildenbrandia* may be split up into several genera.

In contrast to more variable morphological characters, tetrasporangial morphology appears to be uni-

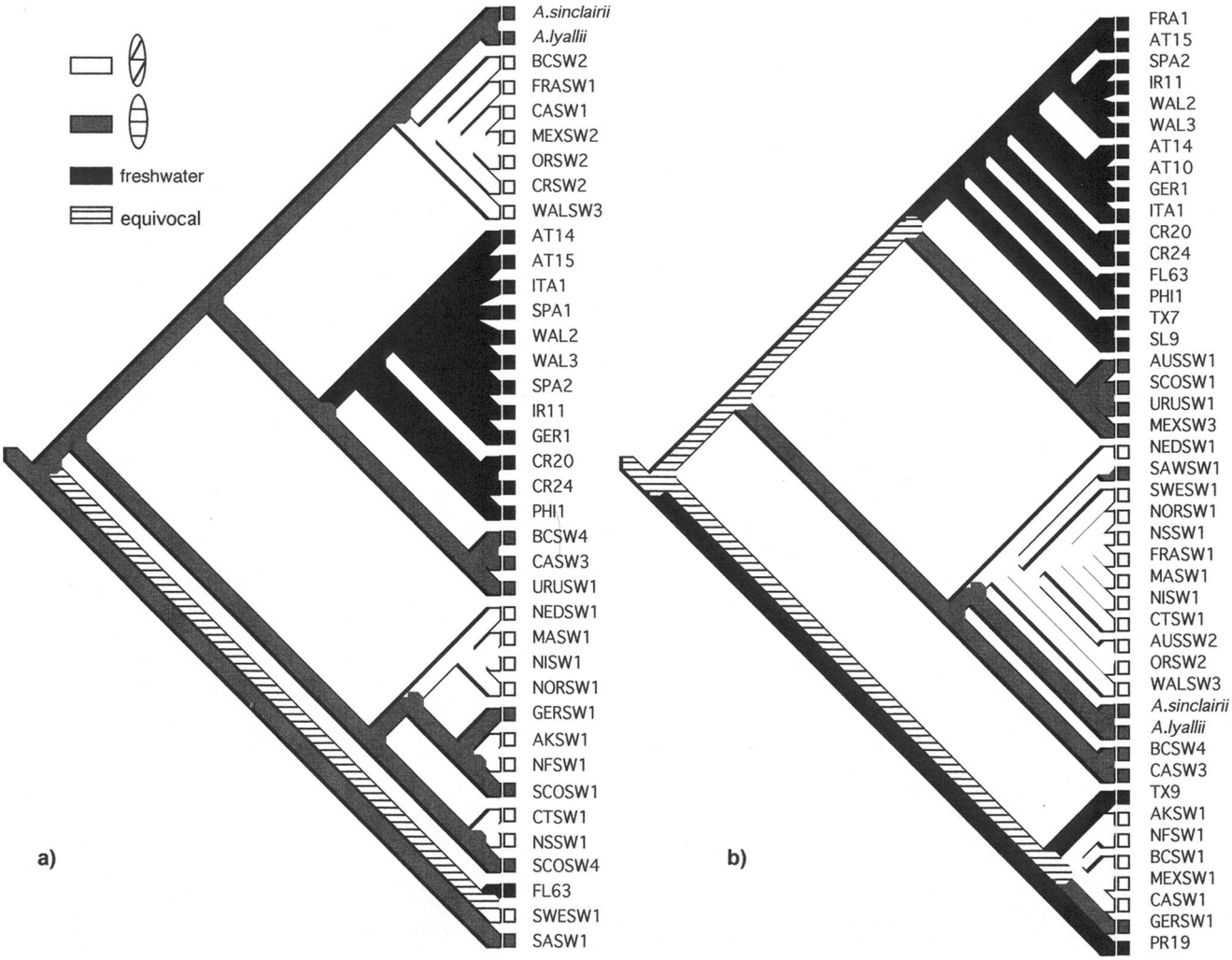

FIG. 5. (a) Cladogram based on the 18S rRNA gene for representatives of the Hildenbrandiales with tetrasporangial morphology mapped on the topology of one of the four most-parsimonious trees. (b) Cladogram based on the *rbc*L gene for representatives of the Hildenbrandiales with tetrasporangial morphology mapped on the topology of the single most parsimonious tree.

form within and among collections and thus is probably the most appropriate character for morphological distinction of the marine species. Overall, the paucity of useful morphological characters within *Hildenbrandia* means that tetrasporangial morphology must be one of the primary characters used to distinguish species of *Hildenbrandia*, given its relative stability as a character. Taxonomic conclusions in this study have been hindered by several discrepancies in tetrasporangial morphology observed between descriptions of taxa and observations of their type specimens. For example, the type specimen of *H. canariensis* was observed to have oblique parallel divisions rather than transverse parallel divisions (Børgesen 1929). Examination of the type specimen of *H. crouanii* in this investigation revealed that all tetrasporangia had transverse parallel divisions. This is in direct contrast to observations by Rosenvinge (1917), who examined the same specimen and reported oblique tetrasporangial divisions. The possibility that oblique versus transverse cleavage could result from a preservation artifact was not established because no live material was examined containing oblique cleavages, and this, in combination with the differences observed in the type specimens from their descriptions, means that reevaluation of the taxonomy of the marine taxa would be premature.

Several taxa represented in the morphometric analyses by their type specimens were not included in the molecular analyses due to sample unavailability (e.g. *H. kerguelensis*, *H. occidentalis* var. *lusitanica*, *H. occidentalis* var. *yessoensis*, and *H. sanjuanensis*). Based on morphometry, several of the marine taxa appear to be closely related to one another and may in fact be synonymous, such as *H. crouanii* and *H. occidentalis* var. *lusitanica*. However, these marine taxa all have tetrasporangial divisions that are parallel to one another and thus are distinguishable based only on the more subtle characters of conceptacle size and shape and average thallus thickness. Therefore, taxonomic changes involving these

taxa will await analysis of additional specimens and/or corresponding molecular data to further evaluate these proposals. Major taxonomic changes will not be proposed until the phylogenetic histories of these taxa can be better resolved with different markers or the reasons for the lack of monophyly elucidated for the different lineages within the Hildenbrandiales.

Taxonomic proposals. Hildenbrandia rivularis (Liebm.) J. Agardh (1852:495) emend. A.R. Sherwood et Sheath

Basionym: Erythroclathrus rivularis Liebm. (1839: 174).

Synonyms: Hildenbrandia rivularis var. *drescheri* Lingelsh. (1922: 355), *H. fluviatilis* Bréb. (nomen nudum), *H. rosea* var. *fluviatilis* Kütz. (nomen nudum), *H. paroliniana* Zanardini (1841:155).

Taxonomic Notes: Lingelsheim (1922) described a variety of *H. rivularis* (var. *drescheri*), that he distinguished from the nominate variety as being a dark blood-red color rather than the lighter red to pink color often reported for *H. rivularis* (Budde 1926, Geitler 1932). Both our morphometric and molecular analyses indicate that *H. rivularis* var. *drescheri* is indistinguishable from *H. rivularis*, and their synonymy is recommended.

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