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Characterization of a novel freshwater gigartinalean red alga from Belize, with a description of *Sterrocladia belizeana* sp. nov. (Rhodophyta)

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Freshwater representatives of the red algal order Gigartinales are extremely rare, with the monotypic genus *Sterrocladia* being the only truly freshwater example. This alga was described in 1850 from material originating from French Guiana, and although its affinities at the ordinal and familial levels have been debated based on morphological and anatomical characteristics, its placement has not been confirmed with the assistance of molecular analyses. Here we report several collections of a freshwater red alga from Belize that share many of the characteristics of the single described species, *Sterrocladia amnica*, but also display some important differences. Based on microscopic analysis, our collections were distinguished from *S. amnica* based on thickness of the cortical layer, cell size, possession of a loose versus entire medulla and absence of reproductive nemathecia. Transmission electron microscopy confirmed a pit plug anatomy consistent with placement of the new collections in the order Gigartinales, and a new method for resin infiltration into thick cortical tissue was described. Although recent material of *S. amnica* was unavailable, molecular phylogenetic analyses of the *rbcL* and SSU rRNA gene for our new samples indicated that it was indeed a member of the Gigartinales, and that it was positioned basal to the Gigartinaceae and Phyllophoraceae clades, likely representing a novel family. The COI-5P barcode sequence showed no close matches, but the vast majority of the closest hits were to representatives of the Phyllophoraceae. Here we describe a second species within the genus *Sterrocladia* to accommodate our Belizean collections, *Sterrocladia belizeana* sp. nov., and discuss the phylogenetic affinities of this unique genus of freshwater gigartinalean red algae. We also propose a lectotype of *Sterrocladia amnica* based on examination of syntype materials.

Key Words: Belize, Freshwater, Gigartinales, Phylogeny, Rhodophyta, *Sterrocladia belizeana*, Transmission electron microscopy

INTRODUCTION

The freshwater red algal genus *Sterrocladia* F. Schmitz is known only from northern South America, collected from Guyana and French Guiana. The sole described member of the genus, *S. amnica* (Montagne) F. Schmitz, is irregularly branched, uniaxial and terete, with a pseudoparenchymatous medulla and single-layered cortex. Plants are generally small (to 3 cm in length) and are attached by basal rhizoids. Sexual reproduction in the genus has not been definitively observed, with the only known reproductive structures being 'nemathecia' with tufts of short filaments and putative monosporangia (Bourrelly 1985).

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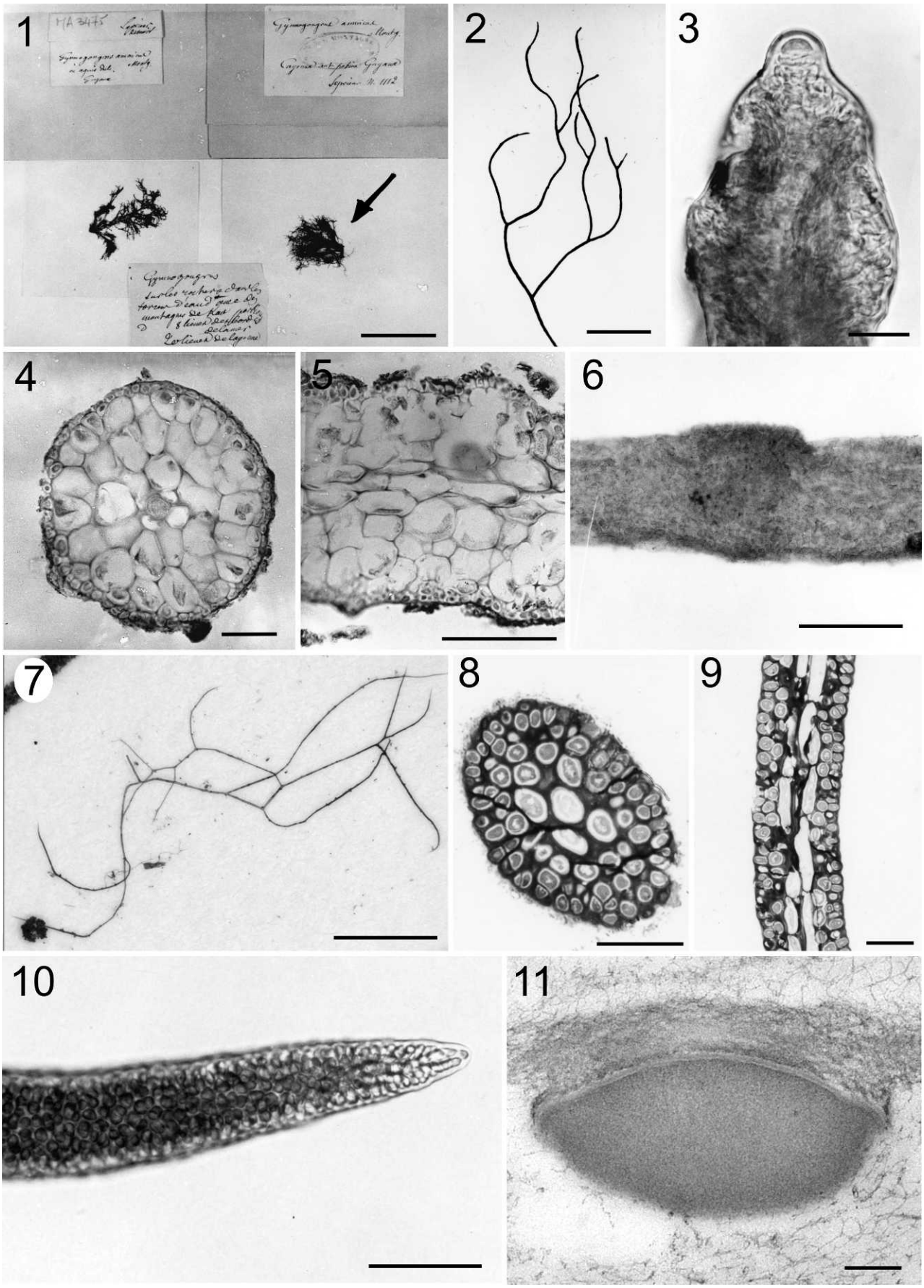
Since its description, the phylogenetic affinities of *Sterrocladia* have been elusive and under debate. Montagne (1850) first discovered the alga and reported the presence of nemathecia in samples from French Guiana and for this reason placed the alga in the marine genus *Gymnogongrus* (as *G. amnicus* Montagne), belonging to the family Phyllophoraceae of the Gigartinales. Schmitz (1893) subsequently noted that the alga had few other characteristics in common with *Gymnogongrus* and erected the genus *Sterrocladia* to accommodate it. Schmitz also came to a very different conclusion regarding the higher level taxonomic affinities of the alga and recommended that the genus be classified in an intermediate position between *Tuomeya* (Batrachospermaceae) and *Lemanea* (Lemaneaceae) of the Batrachospermales (then Nemaliales) (Schmitz 1893). Skuja (1944) examined the type material and additional specimens from Guyana and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concluded that *Sterrocladia* indeed belonged to the Gigartinales [close to the Caulacanthaceae (as Rhabdoniaceae) and Phyllophoraceae], as was originally proposed by Montagne, based on thallus construction and gross morphology. Kylin (1956) later placed *Sterrocladia* in the Batrachospermaceae of the Batrachospermales (again, as the Nemaliales), based primarily on vegetative thallus structure. Bourrelly (1985) followed Skuja (1944) and reclassified the genus within the Gigartinales but did not indicate a family level placement. Since Bourrelly's 1985 treatment *Sterrocladia* has received little attention, and no attempt has been made to elucidate the phylogenetic position of this unusual alga based on molecular phylogenetic analysis.

In 2000 and again in 2010 we collected material of a freshwater red alga from Belize that bears morphological similarity to *Sterrocladia amnica*. Here we characterize these samples in terms of morphology and anatomy (and compare to the type material of *S. amnica*), illustrate the phylogenetic affinities of the alga based on analyses of multiple molecular markers and describe the second member of the genus *Sterrocladia*.

MATERIAL AND METHODS

A collection of a freshwater red alga was made 1 January 2000 from the stream outflow at Rio Frio Caves, Augustine, Mountain Pine Ridge, Belize (16°59′N, 89°00′W) by R.G. Sheath, and on 16 March 2010 and 20 July 2010 from the Stann Creek District (16°48′N, 88°30′W) by H.D. Laughinghouse. Morphologically, the alga bore resemblance to the unique gigartinalean freshwater red alga, *Sterrocladia amnica*, and syntype material of this taxon was borrowed from PC (Montagne collection, MA 3473, coll. Leprieur no. 1112, type locality French Guiana, *c*. 50 km from the sea) for comparison to our collections. Field collections of the new alga were fixed in 2.5% $CaCO_3$-buffered glutaraldehyde for light microscopy examination and also returned live to the lab for DNA extraction and transmission electron microscopy (TEM) fixation.

Light microscopy and TEM

Field-collected material (from the 2000 collection) was prepared for thick section microtomy by dehydration in a graded ethanol series and embedding in LR White resin (Ted Pella Inc., Redding, CA, USA). Light microscopy sections (*c*. 3–5 μm thick) were cut with glass knives and stained with 0.05% toluidine blue O or methylene blue, and sections were microscopically examined for vegetative anatomical structure and reproductive characteristics (Millonig 1976, Vercelli, Italy). Material for TEM (from the 2010 collection) was fixed in Karnovsky's fixative (Karnovsky 1965). Traditional preparation methods for TEM proved ineffective due to the thick cortex, which did not allow for complete infiltration of resin. To loosen these thick cortical cells, 4-cm-long pieces of material were soaked in dilute HCl for approximately 1 h. The soaked tissue was then crushed between two glass microscope slides until the cortex became separated from the medulla; this was confirmed by examining the crushed material on the light microscope. The crushed material was then embedded in 1% low melting point agarose blocks, which were fixed overnight in 2.5% EM-grade gluteraldehyde. The gluteraldehyde was subsequently removed and the blocks washed with Sorensen's buffer for 15 min six times. For all of the following steps until polymerization, the material was kept under vacuum. Agar blocks were postfixed with OsO_4 for 1.5 h (Palay *et al*. 1962) and then transitioned to 100% ethanol using a dilution series over a 5.5-h period. The blocks were kept in 100% ethanol on a rotary shaker overnight. The material was gradually transitioned to 100% Spurr's resin on a rotary shaker over a period of nine days, and then infiltrated with 100% Spurr's for three days (Spurr 1969). The infiltrated blocks were polymerized for 24 h at 67°C and were subsequently thin sectioned. Sections were examined using a Zeiss LEO 912 Energy Filtering Transmission Electron Microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) at 100 kV at the Biological

←

Fig. 1–11.

Fig. 1. Herbarium sheet containing the lectotype (indicated by arrow) of *Gymnogongrus amnicus* [PC, Montagne collection (MA 3474), coll. Leprieur no. 1112] [= *Sterrocladia amnica* (Montagne) F. Schmitz]. Scale bar = 3 cm.

Fig. 2. Thallus overview of a syntype of *Gymnogongrus amnicus* [PC, Montagne collection (MA 3474), coll. Leprieur no. 1112] [= *Sterrocladia amnica* (Montagne) F. Schmitz]. Scale bar = 2 mm.

Fig. 3. An apex of a syntype of *Gymnogongrus amnicus* [PC, Montagne collection (MA 3474), coll. Leprieur no. 1112] [= *Sterrocladia amnica* (Montagne) F. Schmitz], demonstrating the uniaxial construction of the alga. Scale bar = 10 μm.

Fig. 4. Cross section of a syntype of *Gymnogongrus amnicus* [PC, Montagne collection (MA 3474), coll. Leprieur no. 1112] [= *Sterrocladia amnica* (Montagne) F. Schmitz], demonstrating the uniaxial construction and the one to two layers of small cortical cells. Scale bar = 30 μm.

Fig. 5. Longitudinal section of a syntype of *Gymnogongrus amnicus* [PC, Montagne collection (MA 3474), coll. Leprieur no. 1112] [= *Sterrocladia amnica* (Montagne) F. Schmitz], illustrating the medullary and cortical cells. Scale bar = 30 μm.

Fig. 6. Possible nemathecium from syntype material of *Gymnogongrus amnicus* [PC, Montagne collection (MA 3474), coll. Leprieur no. 1112] [= *Sterrocladia amnica* (Montagne) F. Schmitz]. Scale bar = 100 μm.

Fig. 7. Habit of *Sterrocladia belizeana* sp. nov.; image of the holotype specimen (US 21783). Scale bar = 3 mm.

Fig. 8. Cross section through resin-embedded filament of *Sterrocladia belizeana* sp. nov. demonstrating the two to three layers of cortical cells surrounding a loosely packed medulla with a central axial cell. Scale bar = 30 μm.

Fig. 9. Longitudinal section of resin-embedded material of *Sterrocladia belizeana* sp. nov. showing the two to three layered cortex and the medullary cells of only slightly larger size. Scale bar = 30 μm.

Fig. 10. Apex of a branch of *Sterrocladia belizeana* sp. nov. illustrating the general morphology of a branch. Scale bar = 30 μm.

Fig. 11. Transmission electron micrograph of half of a pit plug of *Sterrocladia belizeana* sp. nov. illustrating the typical gigartinalean pattern of no cap layer and the presence of a cap membrane. Scale bar = 200 nm.

Electron Microscope Facility (Pacific Biosciences Research Center, University of Hawaii at Manoa).

Molecular analyses and phylogenetic inference

DNA extraction, polymerase chain reaction amplification and DNA sequencing were carried out as described in Conklin *et al.* (2009) on material from the July 2010 collection. The *rbcL* gene was amplified using the primers and cycling conditions described in Rintoul *et al.* (1999) (except an annealing temperature of 55°C was employed), while the nuclear encoded SSU rRNA gene was amplified using the primers and cycling conditions listed in Milstein & Oliveira (2005). The COI-5P marker was amplified according to the protocols of Saunders (2005). Sequence alignments and all phylogenetic analyses except Bayesian analysis were completed in Geneious Pro 5.3.6 (Drummond *et al.* 2011). Alignments were assembled using the MUSCLE algorithm (Edgar 2004), with eight iterations and default parameter settings. Ambiguous regions of the SSU alignment were removed prior to phylogenetic analyses. Bootstrapping of the datasets using the maximum parsimony (MP) criterion was performed 1000 times with a heuristic search in PAUP*4.0 (Swofford 2002). The best-fit model of sequence evolution was determined using the Akaike Information Criterion implemented in ModelTest 3.7 (Posada & Crandall 1998), which is part of the PAUP*4.0 plugin for Geneious.

The *rbcL* and SSU sequences for the Belize freshwater red alga were compared to the content of the GenBank nucleotide database using the BLAST algorithm (Altschul *et al.* 1990) to confirm that the alga is a member of the Gigartinales. Subsequently, the *rbcL* and SSU sequences were analyzed as part of a detailed analysis of the Gigartinales. For these analyses the GTR+I+G model was determined to be the best fit for both the *rbcL* and SSU data sets. Maximum likelihood (ML) topologies and bootstrap values from 500 replicates were inferred using PhyML (Guindon & Gascuel 2003). Bayesian inference (BI) was completed in Mr. Bayes 3.1 (Huelsenbeck & Ronquist 2001) with three runs of five chains of Metropolis coupled Markov Chain Monte Carlo for 4×10^6 generations for the *rbcL* data set (which was partitioned by codon) and 6×10^6 generations for the SSU data set; trees were sampled every 100 generations. The first 500 trees were discarded from the *rbcL* analysis as burn-in, and the first 1000 trees were discarded from the SSU analysis. The COI-5P marker sequence was compared to other red algal barcodes in the Barcode of Life Data Systems (BOLD) database (Ratnasingham & Hebert 2007).

RESULTS

The type material of *Gymnogongrus amnicus* Montagne consisted of multiple syntypes [located in the Muséum National d'Histoire, Paris, France (PC)], none of which was designated the holotype at the time of description. The syntypes were from the Montagne collection (MA 3474 and MA 3475) and the Thuret collection (TA 10970, TA 10971 and TA 10972). Collection MA 3474 is here designated as a lectotype, since there is evidence that it was the key specimen in the proposal of *G. amnicus* by Montagne (1850): (1) inside the envelope, there is a hand-written label from Camille Montagne, with data from the type locality ('20 lieus de Cayenne, 8 lieus de la mer, montagnes de "Kau", sur les rochers dans le torrents d'eau douce'); (2) on one of the sheets with the alga (it has two parts) he wrote in pencil: '1112 puzza die mare; aquae dulcis'. Therefore, we designate here the specimen MA 3474, Herbarium PC, Montagne collection, coll. Leprieur no. 1112 as the lectotype for *G. amnicus* Montagne (Ann. Sci. Nat., Bot., ser. 3, 14: 289, 1850).

Sterrocladia F. Schmitz

TYPE SPECIES: *Sterrocladia amnica* (Montagne) F. Schmitz, Flora 77: 388–389, 1893.

BASIONYM: *Gymnogongrus amnicus* Montagne, Ann. Sci. Nat., Bot., ser. 3, 14: 289, 1850.

TYPE LOCALITY: French Guiana, Kaw, *c.* 50 km from the sea, 04°29′N, 52°02′W.

LECTOTYPE: Herbarium PC, Montagne collection (MA 3474), coll. Leprieur no. 1112.

Vegetative features previously described for *Gymnogongrus amnicus* and *Sterrocladia amnica* by Montagne (1850), Skuja (1944) and summarized by Kylin (1956) and Bourrelly (1985) were re-evaluated in this study. The thalli were pseudoparenchymatous, cylindrical and irregularly branched, with main axes and branches of similar size, 150–215 μm in diameter (Figs 1, 2). Apices were rounded and slightly mucronated due to the occurrence of a prominent apical cell, which undergoes divisions in early stages to form axial and pericentral cells (Fig. 3). The cross section of the thallus revealed an obvious uniaxial structure, with the presence of an axial cell surrounded by two to three inner layers of large and irregular medullar cells, 25–40 × 24–36 μm, and one, rarely two, outer layers of small cortical cells (Fig. 4). The medulla was entire, with all cells abutting in cross section (Fig. 4). The axial cell was adherent to the outer cortex. Longitudinal section of the thallus showed one row of cylindrical to elliptical axial cells, 40–67 × 13–22 μm, surrounded by medullar and cortical cells (Fig. 5). A surface view of the thallus showed the presence of small, irregularly shaped, polygonal and densely arranged cortical cells, 6.0–11.5 × 4.5–8.5 μm (Fig. 6).

Reproductive structures developed in nemathecia, forming wart-like protuberances on the thallus surface (Fig. 6). Details of the nemathecial internal structure were not clearly discernible in the dried type specimens observed. However, we roughly confirmed their characteristics as previously described by Skuja (1944). Nemathecia were composed of short, branched filaments, producing terminal 'sporangia'; it was not clear if these structures were actually sporangia or spermatangia. According to Skuja (1944), their large size (16–20 μm long, 15–19 μm in diameter) and presence of chloroplasts suggest they were sporangia. In this case, they could be asexual monosporangia, sexually related carposporangia or young (undivided) tetrasporangia. However, the nature of the reproductive structures has

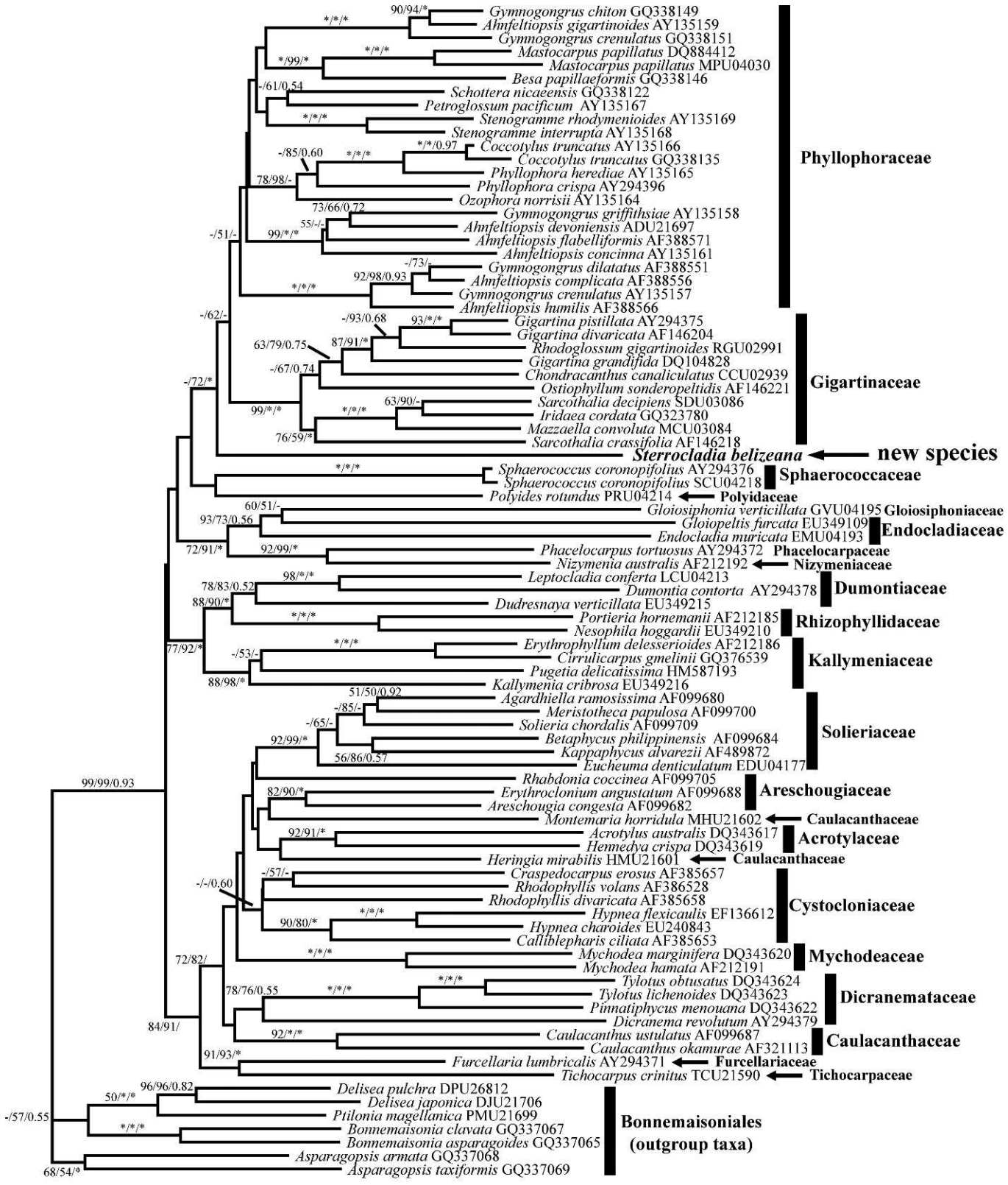

Fig. 12. Maximum likelihood phylogeny of the Gigartinales (with representatives of the Bonnemaisoniales included as outgroups) based on chloroplast *rbcL* gene sequences. Support values for individual nodes are indicated with maximum parsimony bootstrap as the first value, maximum likelihood bootstrap as the second value and Bayesian posterior probability as the third value. Asterisks indicate full support. *Sterrocladia belizeana* sp. nov. is positioned sister to the Phyllophoraceae and Gigartinaceae clades, with low support. Scale bar = 0.02 substitutions/site.

never been unequivocally described in the genus (Skuja 1944; Kylin 1956; Bourrelly 1985; this study).

Freshwater red alga from Belize

Samples were collected in both the wet and dry seasons and during two different years. Light microscopic examination of our field-collected material demonstrated a construction superficially similar to *Sterrocladia amnica* but sufficiently different that we propose a new species, *Sterrocladia belizeana* sp. nov., to accommodate our samples from Belize. Thalli were cylindrical and gracile, up to 3 cm in length, mostly dichotomously branched (Fig. 7). The branched thallus was covered with cortical cells and had a uniaxial construction (Fig. 8). The highly pigmented and round to irregularly shaped cortical cells (3.0–5.0 × 6.0–7.5 μm) were typically two to three layers thick, with the medullary cells (5.0–7.5 μm diameter) occupying only a fraction of the volume of the thallus, and medullary cells not abutting in cross section (Fig. 8). Longitudinal sections through the thallus revealed a construction of medullary cells arranged in filaments, surrounded by smaller (and more densely staining) cortical cells (Fig. 9). Comparison of the Belize collection and the type material of *S. amnica* revealed strong morphological and anatomical similarity between the two samples in gross habit (Figs 2, 7) and structure of the apical region (Figs 3, 10), but also some unique features of the Belize collection. In cross section, *S. amnica* was observed to have a much smaller cortex (Fig. 4) than the Belize collection (Fig. 8), with the central axial and medullary cells occupying the vast majority of the volume of the centre of the thallus in the former. Additionally, the cortical cells of *S. amnica* were proportionately much smaller than the axial cells (Fig. 4), as compared with the Belize collection (Fig. 8). While structures that may represent nemathecia were observed in the lectotype material of *S. amnica* (Fig. 6), no corresponding structures were found for the new collection from Belize. The principal morphological and anatomical features of *Sterrocladia amnica* and our new collection (*S. belizeana*) are summarized and contrasted in Table 1.

Complete resin infiltration of *S. belizeana* sp. nov. material for transmission electron microscopy proved to be exceptionally difficult and necessitated the development of a novel protocol (see Material and Methods). Although several partial pit plugs with a consistent morphology were viewed, no complete pit plugs were evident in the material. Transmission electron microscopy revealed a pit plug with a cap membrane and no cap layers (Fig. 11), a structure that is common to a number of florideophyte orders and is consistent with placement of the alga in the order Gigartinales (Pueschel 1990).

The phylogenetic position of the freshwater red alga from Belize was evaluated based on comparisons of *rbcL* and SSU sequences. The alignment for the *rbcL* gene sequence analysis consisted of 87 sequences (including seven outgroup sequences) and was 1319 nt in length. Phylogenetic reconstruction returned a tree topology that was consistent with current understanding of relationships within the order Gigartinales (Fig. 12). The new collection from Belize was positioned in a clade with the Gigartinaceae and Phyllophoraceae, but with low support (MP lacking/72% ML/1.00 BI support), and was not clearly supported within any currently recognized gigartinalean family for which sequence data are available.

The alignment for the SSU gene sequence analysis contained 74 sequences (including six outgroup sequences) and was 1714 nt in length after removing ambiguous regions. As for the *rbcL* analyses, the SSU tree topology was consistent with current phylogenetic understanding of the Gigartinales (Fig. 13). The sample from Belize was positioned similarly to the *rbcL* analyses; in a clade with the Gigartinaceae and Phyllophoraceae, albeit with much higher support (94% MP/98% ML/1.00 BI). As for the *rbcL* analyses, the *S. belizeana* sequence was not clearly allied with any existing family of the Gigartinales for which sequence data were available.

The COI-5P DNA barcode sequence of the Belize collection was submitted to the BOLD database to check for matches or close matches to any existing sequences. No matches were returned, and 98 of the closest 99 barcodes were members of the gigartinalean family Phyllophoraceae (the remaining single barcode, a member of the Gigartinaceae, likely represents a misidentification) but the highest similarity was only 87.46%.

Sterrocladia belizeana A.R. Sherwood, Necchi, Carlile, Laughinghouse & Sheath *sp. nov.*

Figs 7–11

Found in montagne, lotic freshwater habitats. Thalli gracile, mostly dichotomously branched, attached, to 3 cm in length, 50–120 μm in diameter. Thalli uniaxial, with highly pigmented cortical cells in two to three layers, and medullary cells arranged in filaments that were not closely abutting when viewed in cross section. Cortical cells small, round to irregular in shape, 3.0–5.0 × 6.0–7.5 μm. Axial cells (*c.* 10 μm in cross section) and medullary cells (5.0–7.5 μm in cross section) slightly larger than cortical cells. Pit plug anatomy with a cap membrane and lacking cap layers. Reproduction unknown.

HOLOTYPE: US 21783

ISOTYPES: US 21784, BISH 751591, 751592

TYPE LOCALITY: Stann Creek District (16°48′N, 88°30′W), Belize

DISTRIBUTION: Known only from the locations in Belize reported in this manuscript.

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Fig. 13. Maximum likelihood phylogeny of the Gigartinales (with representatives of the Bonnemaisoniales included as outgroups) based on nuclear SSU rRNA gene sequences. Support values for individual nodes are indicated with maximum parsimony bootstrap as the first value, maximum likelihood bootstrap as the second value and Bayesian posterior probability as the third value. Asterisks indicate full support. *Sterrocladia belizeana* sp. nov. is positioned sister to the Phyllophoraceae and Gigartinaceae clades, with high support. Scale bar = 0.02 substitutions/site.

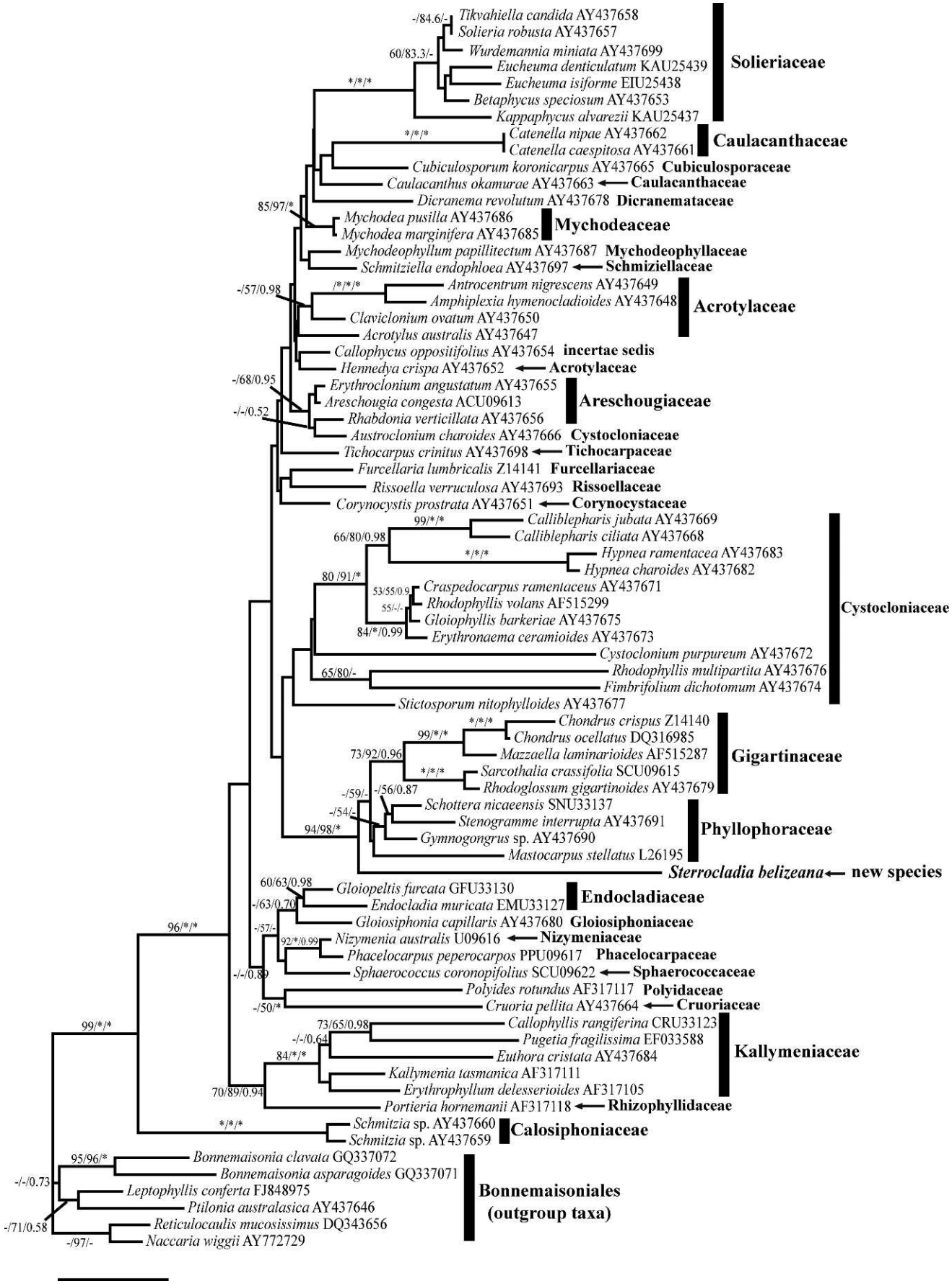
-/84.6/-
Tikvahiella candida AY437658
Solieria robusta AY437657
60/83.3/-
Wurdemannia miniata AY437699
Eucheuma denticulatum KAU25439
//*
Eucheuma isiforme EIU25438
Betaphycus speciosum AY437653
Kappaphycus alvarezii KAU25437
Solieriaceae
//*
Catenella nipae AY437662
Catenella caespitosa AY437661
Caulacanthaceae
Cubiculosporum koronicarpus AY437665 Cubiculosporaceae
Caulacanthus okamurae AY437663 ← Caulacanthaceae
Dicranema revolutum AY437678 Dicranemataceae
85/97/*
Mychodea pusilla AY437686
Mychodea marginifera AY437685
Mychodeaceae
Mychodeophyllum papillitectum AY437687 Mychodeophyllaceae
Schmitziella endophloea AY437697 ← Schmiziellaceae
-/57/0.98
/*/*/*
Antrocentrum nigrescens AY437649
Amphiplexia hymenocladioides AY437648
Acrotylaceae
Claviclonium ovatum AY437650
Acrotylus australis AY437647
Callophycus oppositifolius AY437654 incertae sedis
Hennedya crispa AY437652 ← Acrotylaceae
-/68/0.95
Erythroclonium angustatum AY437655
Areschougia congesta ACU09613
Areschougiaceae
-/-/0.52
Rhabdonia verticillata AY437656
Austroclonium charoides AY437666 Cystocloniaceae
Tichocarpus crinitus AY437698 ← Tichocarpaceae
Furcellaria lumbricalis Z14141 Furcellariaceae
Rissoella verruculosa AY437693 Rissoellaceae
Corynocystis prostrata AY437651 ← Corynocystaceae
99/*/*
Calliblepharis jubata AY437669
66/80/0.98
Calliblepharis ciliata AY437668
//*
Hypnea ramentacea AY437683
Hypnea charoides AY437682
80 /91/*
53/55/0.9
Craspedocarpus ramentaceus AY437671
55/-/-
Rhodophyllis volans AF515299
Gloiophyllis barkeriae AY437675
84/*/0.99
Erythronaema ceramioides AY437673
Cystocloniaceae
Cystoclonium purpureum AY437672
Rhodophyllis multipartita AY437676
65/80/-
Fimbrifolium dichotomum AY437674
Stictosporum nitophylloides AY437677
//*
Chondrus crispus Z14140
99/*/*
Chondrus ocellatus DQ316985
73/92/0.96
Mazzaella laminarioides AF515287
Gigartinaceae
//*
Sarcothalia crassifolia SCU09615
Rhodoglossum gigartinoides AY437679
-/59/-
-/56/0.87
Schottera nicaeensis SNU33137
-/54/-
Stenogramme interrupta AY437691
94/98/*
Gymnogongrus sp. AY437690
Mastocarpus stellatus L26195
Phyllophoraceae
Sterrocladia belizeana ← new species
60/63/0.98
Gloiopeltis furcata GFU33130
Endocladiaceae
-/63/0.70
Endocladia muricata EMU33127
Gloiosiphonia capillaris AY437680 Gloiosiphoniaceae
96/*/*
-/57/-
92/*/0.99
Nizymenia australis U09616 ← Nizymeniaceae
Phacelocarpus peperocarpos PPU09617 Phacelocarpaceae
-/-/0.89
Sphaerococcus coronopifolius SCU09622 ← Sphaerococcaceae
Polyides rotundus AF317117 Polyidaceae
-/50/*
Cruoria pellita AY437664 ← Cruoriaceae
99/*/*
73/65/0.98
Callophyllis rangiferina CRU33123
-/-/0.64
Pugetia fragilissima EF033588
Euthora cristata AY437684
84/*/*
Kallymenia tasmanica AF317111
70/89/0.94
Erythrophyllum delesserioides AF317105
Kallymeniaceae
Portieria hornemanii AF317118 ← Rhizophyllidaceae
//*
Schmitzia sp. AY437660
Schmitzia sp. AY437659
Calosiphoniaceae
95/96/*
Bonnemaisonia clavata GQ337072
Bonnemaisonia asparagoides GQ337071
-/-/0.73
Leptophyllis conferta FJ848975
Ptilonia australasica AY437646
-/71/0.58
Reticulocaulis mucosissimus DQ343656
-/97/-
Naccaria wiggii AY772729
Bonnemaisoniales
(outgroup taxa)

Table 1. Comparison of morphological and anatomical features of *Sterrocladia amnica* and *S. belizeana* sp. nov.

Taxon	Overall thallus construction	Internal thallus construction	Reproductive structures	Cortical construction	Medullary construction
Sterrocladia amnica (Mont.) F. Schmitz	branched, filamentous thallus	uniaxial	nemathecia with 'sporangia'	cortical cells in 1–2 layers	medullar cells occupy most of cross sectional volume and are abutting
Sterrocladia belizeana sp. nov.	branched, filamentous thallus	uniaxial	none observed	cortical cells in 2–3 layers	medullar cells occupy small fraction of cross sectional volume and are not abutting (loose)

ETYMOLOGY: The specific epithet recognizes the only known location of this freshwater red alga.

GENBANK ACCESSIONS FROM ISOTYPE MATERIAL: JQ963342 (*rbcL*), JQ963343 (SSU), JQ963344 (COI)

DISCUSSION

Although several hundred species of freshwater red algae are known worldwide, *Sterrocladia* is the only completely freshwater genus belonging to the order Gigartinales (Bourrelly 1985; Kumano 2002). Here we describe the second species in the genus *Sterrocladia*, which has a unique combination of anatomical features that clearly distinguishes it from *S. amnica*.

The red algal order Gigartinales is a large and diverse group that has undergone much assessment and taxonomic revision in recent years to approach monophyletic status (e.g. Saunders *et al*. 2004). A number of lineages have been removed from the Gigartinales and elevated to ordinal status in recent years, including the Acrosymphytaceae (Withall & Saunders 2007), Plocamiaceae (Saunders & Kraft 1994), Halymeniaceae (Saunders & Kraft 1996) and Peyssonneliaceae (Krayesky *et al*. 2009) and more work remains to be done. Although phylogenetic relationships among many families of the Gigartinales are not well supported in current reconstructions (e.g. regions D & E of Verbruggen *et al*. 2010), the alliance of the families Gigartinaceae and Phyllophoraceae is strongly documented (Saunders *et al*. 2004; Verbruggen *et al*. 2010).

Our phylogenetic analyses suggest that *Sterrocladia* is representative of a novel lineage of the Gigartinales that is in a clade also containing the Gigartinaceae and Phyllophoraceae (Figs 12, 13), although support values were equivocal in some analyses as they commonly are in comparable positions in the current understanding of the Gigartinalean phylogeny (Verbruggen *et al*. 2010). The sequence divergences between *Sterrocladia* and members of the Phyllophoraceae and Gigartinaceae are high based on all three molecular markers analyzed in the present study (*rbcL*, SSU and COI-5P), and the former is uniaxial while the latter two families are both multiaxial in construction (Abbott 1999), highlighting some major differences that support *Sterrocladia* as a separate lineage. The suggestion that *Sterrocladia* represents an undescribed family was previously made by Skuja (1944), who, however, believed the alga to be closely related to the batrachospermalean genus *Lemanea*. We defer formal recognition of *Sterrocladia* as a distinct family (Sterrocladiaceae) until definitive material of *S. amnica* (the type of the genus) can be included in phylogenetic analyses and is shown to also be positioned with our collections from Belize. Unfortunately, these analyses are unlikely to occur in the near future. *Sterrocladia amnica* is a rare species, known only from Guyana and French Guiana. Two separate attempts to collect this species in French Guiana by our colleague, M.L. Vis (Ohio University) were unsuccessful, leading us to believe that acquisition of *S. amnica* material will not be a straightforward task. Thus, we advocate taking a conservative approach and recommend *incertae sedis* status of *Sterrocladia* at the familial level for the present time, until a more thorough phylogenetic analysis of the genus can be completed.

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