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PHYLOGENETIC ANALYSES OF NORTH CAROLINA RHODYMENIALES. I. THE GENUS *ASTEROMENIA*

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Abstract: There are 16 species of the red algal order Rhodymeniales reported from North Carolina waters. One, *Asteromenia peltata*, is the only described species in its genus and is reported from the Atlantic, Pacific and Indian oceans. Sequences of the 18S rRNA gene from North Carolina and Bermuda specimens of *A. peltata* were different at five sites and differed from a sequence attributed to *A. peltata* from Western Australia at 74 and 73 sites respectively. Phylogenetic analyses of 18S rRNA gene sequences from 25 Rhodymeniales species resolved the North Carolina and Bermuda taxa in a monophyletic clade that was distinctly separated from the clade containing the Western Australian taxon. The type specimen of *A. peltata* is from Venezuela so the Atlantic taxon is retained within *Asteromenia*, but the large sequence divergence and separation of the Atlantic and Western Australia taxa in phylogenetic analyses indicates that a new genus name is required for the latter taxon. The number of differences between the North Carolina and Bermuda specimens is within the reported range of between species differences, and suggests that they represent separate *Asteromenia* species.

Key Words: 18S rDNA; *Asteromenia peltata*; Phylogenetic analyses; Rhodymeniales.

INTRODUCTION

The Rhodymeniales is an order of red algae characterized by the formation of auxiliary cells prior to fertilization of the carpogonium and their position on one- or two-celled filaments arising from the carpogonial branch supporting cell or epi-supporting cell (Schneider and Searles, 1991; Saunders et al., 1999). The order is divided into two informal 'groups,' four families, and 44 genera in the most recent taxonomic treatment (Saunders et al., 1999). There are 16 species from nine genera, with representatives of all four currently recognized families, reported in North Carolina waters (Kapraun, 1980; Schneider and Searles, 1991). Eleven of these species are reported only in offshore waters and many are rare. One of the species reported to occur infrequently offshore is *Asteromenia peltata* (Taylor) Huisman and Millar.

This species was originally described by Taylor (1942) as *Fauchea peltata* based on specimens from Venezuela, Brazil and Jamaica. The generic placement of this species was uncertain because all of these specimens, including the one later designated as the type (Huisman and Millar, 1996), were vegetative and no reproductive characters were observed. Schneider (1975) studied reproductive specimens collected from North Carolina

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offshore waters and reported that cystocarps lacked the cell filaments connecting the carposporophyte and pericarp called *tela arachnoidea* that are characteristic of *Fauchea* species. Schneider transferred *F. peltata* to *Weberella peltata* (Taylor) Schneider expanding the concept of *Weberella* to allow more latitude in the position of tetrasporangia and cystocarps, over both the lower and upper thallus surface. Huvé and Huvé (1977) subsequently sank *Weberella* into the genus *Halychrysis* (J. Agardh) Schmitz and transferred *W. peltata* to *Halychrysis peltata* (Taylor) Huvé et Huvé. Schneider and Searles (1991) accepted this transfer in their seaweed flora of the southeastern United States.

Huisman and Millar (1996) examined specimens from North Carolina, Norfolk Island and Western Australia in their study leading to the description of the new genus *Asteromenia* Huisman et Millar represented by a single species, *Asteromenia peltata* (Taylor) Huisman et Millar. They found that *A. peltata* has a four-celled carpogonial branch, and intercalary tetrasporangia that develop within an unmodified cortex. These characteristics make this species different from all other species in the genera within which it had been previously placed. Cystocarps from a Western Australian specimen had adventitious filaments arising from the inner pericarp that were not observed in North Carolina specimens, but they were unable to discern from the limited study material if the filaments were a consistent or variable feature of these cystocarps. The specimens were therefore considered to represent a single, geographically widespread species.

Millar et al. (1996) were the first to use DNA sequence data in a phylogenetic study of Rhodymeniales species. Saunders et al. (1999) subsequently published more extensive DNA sequence based phylogenetic analyses of the Rhodymeniales. This study analyzed nuclear-encoded 18S ribosomal RNA gene sequences, and included a single specimen of *A. peltata* that had been collected in Western Australia. This taxon was resolved within their 'Group 1,' a set of species referable to the family Rhodymeniaceae sensu stricto and characterized by having mostly four-celled carpogonial branches and intercalary, cruciately divided tetrasporangia. A second sequence generated from a Bermuda specimen of *Asteromenia peltata* was published in a study of Gigartinales species (Saunders et al., 2004), but too few Rhodymeniales species were included in their analyses to infer relationships, and the Bermuda and Western Australia sequences were not compared. The purposes of this study were to verify the phylogenetic relationships of *Asteromenia*, and the conspecificity of *A. peltata* specimens from North Carolina, Bermuda, and Western Australia by analyzing nuclear-encoded 18S ribosomal RNA gene sequences.

MATERIALS AND METHODS

A specimen of *Asteromenia peltata* was collected in Onslow Bay (33°57.878′N 77°01.715′W, 36 m depth) by SCUBA during July 2004 as part of UNCW's Coastal Oceans Research and Monitoring Program. Total genomic DNA was extracted from the specimen following the method of Hughey et al. (2001) with a final cleaning step using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). The nuclear-encoded 18S ribosomal RNA gene (18S) was amplified in reactions containing 10–50 ng template DNA; 80 μM each dNTP; 1 mM $MgCl_2$; 0.2 μM each amplification primer, and Hot Star Taq DNA polymerase and buffer per manufacturer's suggestions (Qiagen, Valencia, CA, USA). The thermocycling protocol of amplification reactions consisted of an initial denaturing and enzyme activation step of 95°C for 15 min, followed by 30 cycles of 94°C for 30 sec; 50°C for 30 sec; 72° for 1 min 30 sec (with a 0.2°C/sec ramp rate between the

annealing and extension steps), followed by a final extension step of 72° for 5 min. Amplification products were cleaned with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and used as templates in Big Dye (Applied Biosystems, Foster City, CA, USA) sequencing reactions. Oligonucleotide primers for amplification and sequencing reactions were those published in Saunders and Kraft (1994). Sequencing reactions were run on an ABI 3100 Genetic Analyzer (DNA Analysis Core Facility, Center for Marine Science, UNCW) and the reaction results edited and sequence contigs created using Sequencher (Gene Codes Corp., Ann Arbor, MI, USA). The resulting sequence has been deposited in GenBank under the accession number AY880245.

The North Carolina *A. peltata* sequence was aligned with 18S sequences from 24 Rhodymeniales species available in GenBank using MacClade (v. 4, Maddison and Maddison, 2000). *Plocamium angustum* (J. Agardh) Hooker et Harvey (accession# U09620) and *Plocamiocolax pulvinata* Setchell (accession# U09618) were included as outgroup taxa following Saunders et al. (1999). GenBank accession numbers for the included Rhodymeniales species are given in Figure 1. Regions of the alignment where site homology was uncertain were excluded from analyses. Data set characteristics and rate models were determined using MacClade, Modeltest (Posada and Crandall, 1998), and PAUP (Swofford, 2002). The aligned sequence data set and model parameters are available from the first author. Phylogenetic analyses using maximum likelihood (ML) and distance algorithms were performed using PAUP. Maximum-likelihood searches consisted of 10 random sequence additions with the MulTrees setting and tree bisection reconnection (TBR) branch swapping using the evolutionary model derived with Modeltest. Maximum-likelihood bootstrap analyses consisted of 100 replications of 1 random sequence addition with MulTrees and TBR branch swapping. Distance analyses consisted of neighbor-joining (NJ) tree building using distances corrected with the same model used in the ML analyses. NJ bootstrap analyses were based on 1,000 replications of NJ tree building with the same distance correction. One-tailed Shimodaira-Hasegawa (SH) tests with 1,000 fully optimized bootstraps were used to test *a priori* hypotheses of relationships and the NJ tree topology against the ML tree topology.

RESULTS

A small, prostrate, multi-lobed blade with a peltate holdfast was collected from a hard-bottom reef in North Carolina's offshore waters (Fig. 1). Sections of the blade showed that the vegetative structure consisted of a 2–4 cell layer cortex of small cells and a 3–5 cell layer medulla of much larger cells (Fig. 2). This combination of characters identified this specimen as *Asteromenia peltata* (Schneider, 1975; Schneider and Searles, 1991).

The full 27 taxa 18S data set included 1,658 aligned sites. The homology of positions was uncertain at 36 sites within the alignment and they were excluded from further analyses. Insertion/deletion mutations (indels) were inferred for at least one taxon at 70 of the 1,622 analyzed sites, and 323 (19.9%) sites were variable. The largest indel required the coding of gaps at six contiguous sites, but the majority were only one to three contiguous sites in size. Mean base use within the data set was 24.7% A; 20.8% C; 28.4% G, and 26.1% T. The inferred transition:transversion ratio was 1.83 with a slight bias towards pyrimidine-pyrimidine transitional changes (170 to 158). A general time reversible + invariable sites + gamma (shape = 0.6329) distribution model was selected as the best fit to these data under the Akaike Information Criterion.

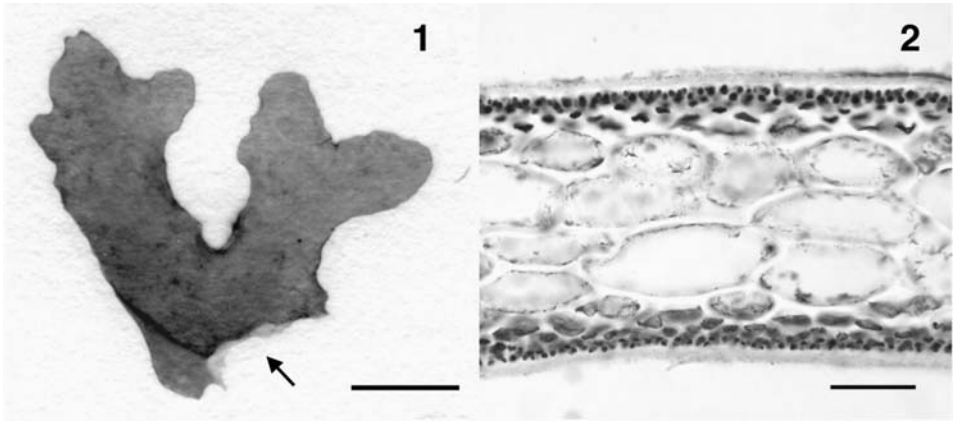

FIG. 1. *Asteromenia peltata* specimen collected in Onslow Bay, North Carolina (WNC 2004-037). The arrow indicates where two lobes of the blade were removed for sectioning and DNA extraction. Scale = 5 mm.

FIG. 2. Section of *Asteromenia peltata* blade showing small-celled cortex and medulla of larger cells (WNC 2004-S006). Scale = 50 μm.

The 18S sequences for specimens of *Asteromenia peltata* from North Carolina and Bermuda were different at five sites (0.3%). These sequences in contrast were different from that for a Western Australian specimen identified as *A. peltata* at 74 sites (North Carolina specimen, 4.5%) and 73 sites (Bermuda specimen, 4.5%).

Ten separate maximum-likelihood searches resulted in the same tree topology (LnLi = –6158.08417, Fig. 3). Neighbor-joining distance analysis resulted in a topology that only differed in the position of the Champiaceae clade as sister to the Faucheaceae/ Lomentariacea clade rather than the clade containing the Atlantic Ocean specimens of *Asteromenia*. The position of these clades received very weak to no bootstrap support in either ML or NJ analyses and a SH test showed no significant difference between the two trees ($p = 0.219$).

Species of the families Lomentariaceae and Champiaceae are resolved in strongly supported monophyletic clades [bootstrap proportions (BP) = 100/100; 90/90, Fig. 1]. The Faucheaceae is paraphyletic with respect to the Lomentariaceae, and species of both are resolved in a strongly supported clade (BP = 100/100). The Rhodymeniaceae is resolved as a monophyletic group in the ML tree, but this clade only received bootstrap support in the NJ analyses (BP = 70). Specimens of *Asteromenia peltata* from North Carolina and Bermuda are strongly resolved (BP = 100/100) as the sister taxon to a clade of uncertain familial affiliation containing the species *Erythrymenia minuta* and *Hymenocladia chondricola*. The specimen identified as *A. peltata* from Western Australia occupies a well-supported position within the clade of Rhodymeniaceae species. A SH test comparing the topology resulting from a maximum-likelihood analysis constrained to include a monophyletic *Asteromenia* containing specimens from North Carolina, Bermuda and Western Australia was found to be significantly different from the unconstrained maximum-likelihood topology ($p = 0.000$).

DISCUSSION

Analyses of 18S sequences generated from geographically distant specimens identified as *Asteromenia peltata* indicate that the Atlantic Ocean and Western Australian specimens

FIG. 3. Maximum-likelihood tree resulting from analyses of 18S rRNA gene sequences for 25 Rhodymeniales and two outgroup species. Bootstrap proportion values for maximum-likelihood and neighbor-joining analyses respectively are given for branches when greater than 50.

represent different species that do not belong in the same genus or probably the same family. The Western Australian taxon is well resolved within the Rhodymeniaceae while the Bermuda and North Carolina specimens are resolved within a clade that is not a part of any currently recognized family.

Huisman and Millar (1996) observed cystocarps of a Western Australian specimen with adventitious filaments arising from surface cells of the inner pericarp. They were unable to determine if this was a consistent characteristic because of the limited number of

cystocarpic plants studied from the area. Adventitious filaments have not been seen in cystocarps of North Carolina specimens (Schneider, 1975; Huisman and Millar, 1996), but the similarity of all other studied characters convinced Huisman and Millar to place Western Australian specimens in *A. peltata*. The molecular evidence suggests that either the morphological similarities between the Atlantic and Western Australian taxa do not represent truly homologous characters, or that the generated sequence was derived from another contaminating species.

The amount of sequence divergence between taxa of different taxonomic rank is variable for different DNA loci and different red algal lineages (Bailey and Freshwater, 1997). The range of between species 18S sequence differences within the Gelidiales and Gracilariales is 0.0–0.4% and 0.2–3.5% respectively (Bird et al., 1992; Bailey and Freshwater, 1997). Four Rhodymenialean species pairs were included in the study of Saunders et al. (1999) and may be compared. The 18S sequence differences for these pairs ranged from 0.17 to 0.97%. The amount of 18S sequence divergence between the North Carolina and Bermuda *A. peltata* specimens (0.3%) falls within the range known for Rhodymeniales, as well as Gelidiales and Gracilariales species. This indicates that the North Carolina and Bermuda specimens represent different species. Saunders et al. (G.W. Saunders, pers. comm. January 2005) have identified morphological characters that they feel may separate the two taxa, and these molecular analyses support their findings.

The two *Asteromenia* specimens from the Atlantic Ocean were strongly allied with *Erythrymenia minuta* Kylin and *Hymenocladia chondricola* (Sonder) Lewis in 18S analyses. All three species have solid thallus construction, intercalary tetrasporangia, and where known, four-celled carpogonial branches (Sparling, 1957; Huisman and Millar, 1996; Womersley, 1996). As discussed by Saunders et al. (1999), these characters variously suggest a relationship with the Champiaceae or Rhodymeniaceae. The familial relationships of *Asteromenia* as well as *Hymenocladia* J. Agardh and *Erythrymenia* Schmitz *ex* Mazza will require more detailed morphological studies of the Rhodymeniales.

The 18S data presented here clearly indicates that taxa identified as *A. peltata* from widely separated geographic locations do not represent a natural monophyletic species. The designated type of *A. peltata* is a specimen from Venezuela, so the Atlantic Ocean taxa should be retained within *Asteromenia*. A new genus name is required for the Western Australia taxon that was sequenced by Saunders et al. (1999). The 18S data also indicate that North Carolina and Bermuda *A. 'peltata'* specimens represent different species, and therefore *Asteromenia* is not a monotypic genus. Determining the true number of species in, and geographic distribution of, *Asteromenia* will require molecular and morphological analyses of *A. 'peltata'* specimens from many locations, and such a study is in progress (G.W. Saunders, pers. comm. January 2005).

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