Molecular Phylogenetics and Evolution 94 (2016) 730–736

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Multigene phylogeny of the red algal subclass Nemaliophycidae ☆

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ARTICLE INFO

Article history:
Received 4 March 2015
Revised 11 October 2015
Accepted 13 October 2015
Available online 27 October 2015

Keywords:
Red algae
Tree of life
Freshwater macrophytes
Seaweeds
Simulation analysis
Hard polytomy

ABSTRACT

The red algae (Rhodophyta) are a lineage of primary endosymbionts whose ancestors represent some of the first photosynthetic eukaryotes on the planet. They primarily inhabit marine ecosystems, with only ~5% of species found in freshwater systems. The subclass Nemaliophycidae is very diverse in ecological and life history features and therefore a useful model to study these traits, but the phylogenetic relationships among the orders are, for the most part, poorly resolved. To elucidate the phylogeny of the Nemaliophycidae, we constructed a nine-gene dataset comprised of nuclear, plastid, and mitochondrial markers for 67 red algal specimens. The resulting maximum likelihood (ML) phylogeny confirmed the monophyly of all orders. The sister relationship of the Acrochaetiales and Palmariales received high support and the relationship of the Balliales with Balbianiales and Entwisleiales with Colaconematales was moderately supported. The Nemaliales, Entwisleiales, Colaconematales, Palmariales and Acrochaetiales formed a highly supported clade. Unfortunately, all other relationships among the orders had low bootstrap support. Although the ML analysis did not resolve many of the relationships, further analyses suggested that a resolution is possible. A Phycas analysis supported a dichotomously branching tree and Bayesian analysis showed a similar topology with all relationships highly supported. Simulations extrapolating the number of nucleotide characters beyond the current size of the dataset suggested that most nodes in the phylogeny would be resolved if more data become available. Phylogenomic approaches will be necessary to provide a well-supported phylogeny of this subclass with all relationships resolved such that the evolution of freshwater species from marine ancestors as well as reproductive traits can be explored.

1. Introduction

The red algae (Rhodophyta) are a diverse group of ca. 7000 species of photosynthetic eukaryotes with a fossil record dating back 1.2 billion years (Butterfield, 2000; Guiry and Guiry, 2015). Their chloroplasts derive from a primary endosymbiosis event (Delwiche, 1999) and possess light-harvesting complexes called phycobilisomes that contain the red and blue pigments phycoerythrin, phycocyanin and allophycocyanin (Gantt, 1990). These pigments allow marine red algae to tolerate a sizeable range in light levels and live in deeper waters as compared to other photoautotrophic organisms (Kain and Norton, 1990). Red algae lack typical microtubule structures such as flagella and centrioles (Woelkerling, 1990). Many red algae have pit connections between adjacent cells that may function to facilitate cell-to-cell communication (Pueschel, 1990). These connections feature a capped plug comprised primarily of protein, whose shape is an important diagnostic feature at the subclass and order level (Saunders and Bailey, 1997, 1999; Saunders and Hommersand, 2004).

Of the five subclasses of Florideophyceae, the Nemaliophycidae is the most biologically diverse providing an ideal test case to study red algal evolution. Traits of interest include, among others, habitat transitions between marine and freshwater ecosystems, evolution of complex thalli from filamentous forms and vice versa (Saunders and Kraft, 1997; Saunders and Hommersand, 2004), species with and without calcification (Huisman et al., 2004), and uncharacteristic variability in phycoerythrin type (e.g., Saunders et al., 1995). Although there are other freshwater red algae (e.g., *Bangia*, *Compsopogon*, *Hildenbrandia*), only the Nemaliophycidae contains orders comprised strictly of freshwater taxa (Balbianiales, Batrachospermales, Thoreales) along with strictly marine orders (Balliales, Colaconematales, Entwisleiales, Nemaliales, Palmariales,

☆ This paper was edited by the Associate Editor Elizabeth Zimmer.

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http://dx.doi.org/10.1016/j.ympev.2015.10.015
1055-7903/

and Rhodachlyales), while Acrochaetiales is primarily marine with a few freshwater species in the genus *Audouinella* (Kumano, 2002). Reproductively, life histories have transitioned between alternations of heteromorphic and isomorphic generations (e.g., Huisman et al., 2004), and vary from the standard florideophycean triphasic pattern (a haploid gametophyte, a diploid carposporophyte, and a diploid tetrasporophyte; e.g., Huisman et al., 2004), to patterns lacking production of tetrasporangia (site of meiosis in most red algae), to biphasic patterns lacking a carposporophyte with (Van der Meer and Todd, 1980) and without (e.g., Stegenga, 1978) stark sexual dimorphism, and finally monophasic species, in which the gametophytic stage directly produces diploid tetrasporangia following fertilization (e.g., DeCew and West, 1982). In addition asexual reproduction in the midst of sexual lineages has been documented at the population (e.g., *Rhodophysema elegans*; Saunders et al., 1989), species (e.g., *Rhodophysema georgii*; Saunders and Bird, 1989), genus (e.g., *Camontagnea*; Womersley, 1994) and family (e.g., Meiodiscaceae; Clayden and Saunders, 2010) levels of taxonomy. Most nemaliophycidaean species are free living and grow attached to a variety of substrata, however, some taxa grow exclusively inside of other algae (e.g., *Rhododrewia porphyrae*; Clayden and Saunders, 2014) or in marine invertebrates (e.g., *Rubrointrusa membranacea* inside the hydroid *Dynamena pumila*; Clayden and Saunders, 2010) while *Rhodophysema kjellmanii* is unusual among red algal parasites by occurring in a lineage (Clayden and Saunders, 2014) that is essentially devoid of secondary pit connections (see Blouin and Lane, 2012).

To understand the evolutionary diversification of these traits, a solid phylogeny needs to be reconstructed. An association between the Acrochaetiales and Palmariales, and a complex of the previous two with the Colaconematales and Nemaliales was shown by the earliest SSU + LSU analyses of Harper and Saunders (2001, 2002), but remarkably few improvements have been made to the phylogeny since. The phylogenetic relationships among orders in the Nemaliophycidae (as currently defined) were not well-supported in the three-gene phylogeny by Le Gall and Saunders (2007) except for additional support for the relationships outlined previously, moderate evidence for an alliance between the Balbianiales and Balliales, and variable indications that the Batrachospermales were the deepest diverging lineage in this subclass. A data mining approach targeted at identifying research priorities for red algal phylogenetics based on 14 loci mined from GenBank highlighted the radiation among orders in the Nemaliophycidae as one of five unresolved regions (Verbruggen et al., 2010). Simulations suggested that the lack of support in this region was probably due to the lack of informative data (Verbruggen et al., 2010) and added nothing new to our understanding of relationships among the constituent orders. In adding the new order Entwisleiales to the Nemaliophycidae, Scott et al. (2013) provided the best phylogenetic resolution among orders in this subclass to date, but even their analyses only served to strengthen some of the previous results while providing an indication that this new order was sister to the Colaconematales. In short, previous studies have fallen short at resolving interordinal relationships in this diverse subclass of red algae.

The goal of this study was to bring more taxa and more gene regions to the conundrum of inferring relationships among orders of Nemaliophycidae. We present a nine-gene data set representing the plastid, nuclear and mitochondrial genomes of 67 specimens representing all of the constituent orders in an effort to resolve a molecular phylogeny. We use several data exploration methods including fast site removal and simulations studies to determine if a well-resolved phylogeny among the orders can be achieved.

2. Materials and methods

2.1. Taxon sampling

A total of 67 specimens representing each of the 10 Nemaliophycidae orders were selected for study (Table S1). The species richness of each order (based on the current taxonomy) was used to determine our final taxon sampling (Table 1). All genera were sampled for the Balbianiales and Entwisleiales. The freshwater order Thoreales has two species-rich genera, so multiple species were sequenced per genus to capture the diversity. When possible, all genes were sequenced from the same specimen. Voucher information is given in Table S1.

2.2. DNA extraction, amplification and sequencing

DNA was extracted from field-collected samples or culture strains (Table S1) using various standard methods. Extraction methods for Acrochaetiales, Balliales, Colaconematales, Entwisleiales, Nemaliales, Palmariales and Rhodachlyales were reviewed in Saunders and McDevit (2012a,b). For the Batrachospermales, Balbianiales and Thoreales the NucleoSpin® Plant II (Macherey-Nagel, Düren, Germany) kit was used according to the manufacturer's protocol. For *psa*A, *psb*A, *psa*B, EF2, *cox*1, *cob*, 18S rDNA, and 28S rDNA genes, the primers and amplification conditions outlined in Saunders and Moore (2013) were followed with a few exceptions. For the EF2 gene, the primer set (BatEF2F 5′ CTCGTATCATCGAGAC GGCGAATGT 3′ and BatEF2R 5′ GGAAGATCMGCYGGRTTYTTCGGCT 3′) was utilized for amplification of the batrachospermalean taxa and (ThorEF2F 5′ CAAGAATTATAGAATCTGCTAATGT 3′ and ThorEF2R 5′ GGAAGATCKGCRGGRTTYTTCGGCT 3′) for *Thorea hispida*. A few of the taxa in the Batrachospermales did not amplify using the standard *cob* primers and the following primer set was designed (COBdwlF 5′ AGCAYRTWATGMGVGAYGTDAAYTT 3′ and COBdwlR 5′ CWATWACHCCHCCYAATTTRTGWGG 3′). For marine taxa, the amplification of the *rbc*L gene followed Saunders and Moore (2013), for Balbianiales and Batrachospermales it followed (Vis et al., 1998), and for Thoreales it followed (Johnston, 2012). For freshwater specimens, PCR products were prepared for sequencing using UltraClean™ PCR Clean-up DNA purification kit (Mo Bio, Carlsbad, CA, USA) according to manufacturer's protocols. If multiple PCR bands were obtained, the product with the correct length was gel purified using GelElute extraction kit (5 Prime, Gaithersburg, MD). Sequence data for the freshwater Batrachospermales, Balbianiales and Thoreales were obtained using an ABI 3100 Genetic Analyzer (Applied Biosystems). We sequenced with PCR primers and internal primers to have adequate coverage for sense and antisense strands. Contigs were assembled and edited in Sequencher 4.10.1 (Gene Codes Corp., Ann Arbor, Michigan, USA). For the marine specimens, all PCR products were sent to Genome Quebec for cleaning and sequencing. All new sequence data generated were submitted to GenBank (Table S1).

2.3. Phylogenetic analyses

The nine genes were aligned with MAFFT version 7.058beta (Katoh et al., 2002) each gene under the following options: – localpair – maxiterate 1000. These alignments were checked by eye for errors. Most genes aligned easily without indels, but 18S rDNA and 28S rDNA had indels. There were unalignable regions in the 28S rDNA primarily caused by taxa in the Thoreales. These regions were removed. There was also a 6 bp gap in EF2 due to the outgroup taxa having two extra codons; this gap was left in the alignment. The Nemaliales had unalignable introns in EF2 and