

## **Nuclear 18S rRNA gene sequence analyses indicate that the Mastophoroideae (Corallinaceae, Rhodophyta) is a polyphyletic taxon**

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J.C. BAILEY, J.E. GABEL AND D.W. FRESHWATER. 2004. Nuclear 18S rRNA gene sequence analyses indicate that the Mastophoroideae (Corallinaceae, Rhodophyta) is a polyphyletic taxon. *Phycologia* 43: 3–12.

Nuclear small subunit ribosomal RNA (18S rRNA) gene sequences were determined for six species representing three genera classified within the Mastophoroideae, and also for two species of *Amphiroa* (Lithophylloideae). These data were combined with previously published 18S rRNA sequences for 38 other coralline species and analysed (1) to determine the phylogenetic position of the Mastophoroideae within the Corallinaceae and (2) to examine relationships among *Hydrolithon*, *Metamastophora*, *Neogoniolithon* and *Spongites*. Trees derived from parsimony and maximum likelihood analyses of these data indicate that the Mastophoroideae is not monophyletic. Instead, our data suggest that the group is polyphyletic and includes species belonging to four distinct evolutionary lineages. For example, *Neogoniolithon* spp. were associated with geniculate members of the Corallinoideae, and *Hydrolithon* was resolved as sister to the geniculate genus *Metagoniolithon*. We demonstrate that each of the four 'mastophoroid' lineages share a number of vegetative and reproductive features with their respective sister taxa. Together the molecular and morphological data imply that the Mastophoroideae is not monophyletic and that genera placed in this taxon might more naturally be classified in other subfamilies. Also, on the basis of our findings, *Porolithon pachydermum* is transferred to *Hydrolithon* as *H. pachydermum* (Foslie) Bailey, Gabel & Freshwater.

### **INTRODUCTION**

The subfamily Mastophoroideae (Corallinaceae, Rhodophyta) includes nongeniculate coralline red algae bearing uniporate tetrasporangial conceptacles that lack apical plugs. Fusions between cells of noncontiguous filaments are exclusively found in most species, although in one species, *Metamastophora flabellata* (Sonder) Setchell, both cell fusions and secondary pit connections are present (Woelkerling 1980a, b, 1988). As it is presently circumscribed, the subfamily contains eight genera: *Hydrolithon* Foslie, *Lesueuria* Woelkerling & Ducker, *Lithoporella* (Foslie) Foslie, *Mastophora* J. Decaisne, *Metamastophora* Setchell, *Neogoniolithon* Setchell & L.R. Mason, *Pneophyllum* Kützling and *Spongites* Kützling, that are delimited on the basis of vegetative and reproductive features (Penrose & Chamberlain 1993; Harvey & Woelkerling 1995).

Recent molecular studies have addressed phylogenetic relationships between families and subfamilies of coralline algae (Bailey & Chapman 1996, 1998; Bailey 1999). Because the Mastophoroideae had not been adequately sampled, the phylogenetic position of this taxon with respect to other coralline subfamilies remains uncertain. There are three conflicting hypotheses for the phylogenetic position of the Mastophoroideae: two based on differing interpretations of anatomical features and their morphogenesis and a third supported by molecular data for a single species.

In the phenetic classification of Harvey & Woelkerling (1995), the Mastophoroideae is recognized as one of eight subfamilies of coralline algae (see also Johansen 1981; Woelkerling 1988). Although the external morphology of genera and species may vary greatly, all nongeniculate species placed in

the taxon share a combination of features not found among other coralline taxa. Such features include the absence of secondary pit connections (cf. Woelkerling 1980a), the presence of cell fusions among certain vegetative cells, and uniporate tetrasporangial conceptacles lacking apical plugs.

In a contrasting hypothesis, mastophoroid genera were not recognized as a separate subfamily by Cabioch (1971, 1972, 1988) because she considered the presence or absence of genicula to be a phylogenetically unreliable feature for delimiting taxa at the subfamilial rank. Instead, she placed greater emphasis on the vegetative development of the thallus, the presence or absence of secondary pit connections and the anatomy of tetrasporangial reproductive structures (i.e. conceptacles). For these reasons Cabioch classified mastophoroids, as well as the Australian endemic geniculate genus *Metagoniolithon* Weber-van Bosse and the nongeniculate parasite *Choreonema* Schmitz, within the subfamily Corallinoideae. Cabioch's concept of the Corallinoideae therefore includes both nongeniculate and geniculate species. Cabioch (1972, fig. 40) further postulated that the nongeniculate mastophoroids could be divided into two separate evolutionary lineages: one including *Neogoniolithon* and *Hydrolithon* (= *Porolithon* (Foslie) Foslie *sensu* Penrose & Woelkerling 1988) and the second including *Lithoporella*, *Mastophora* and *Metamastophora*.

A third hypothesis for the phylogenetic position of the Mastophoroideae has been erected on the basis of nuclear small subunit ribosomal RNA (18S rRNA) gene sequence analyses. However, this hypothesis is based on DNA sequence information for only a single mastophoroid species, *Spongites yendoi* (Foslie) Chamberlain. In the 18S rRNA trees, *S. yendoi* is robustly supported as sister to a clade including *Metagoniolithon* as well as species classified in the Lithophylloideae

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**Table 1.** Nuclear 18S rRNA GenBank accession numbers and collection information for nine species of coralline red algae examined in this study.

Subfamily/species	Accession number	Collection information
Lithophylloideae		
<i>Amphiroa hancockii</i> Taylor	AY234233	Roatan, Honduras
<i>A. tribulus</i> Foslie & Howe	AY234234	Turumote reef, La Parguera, Puerto Rico
Mastophoroideae		
<i>Hydrolithon onkodes</i> (Heydrich) Penrose & Woelkerling	AY234237	Summercloud Bay, New South Wales, Australia
<i>H. samoense</i> (Foslie) Keats & Chamberlain	AY234236	Beachport, South Australia, Australia
<i>H. pachydermum</i> (Foslie) Bailey, Gabel & Freshwater	AY234235	Media Luna reef, La Parguera, Puerto Rico
<i>Metamastophora flabellata</i> (Sonder) Setchell	AY234239 (clone 1)	Beachport, South Australia, Australia
	AY234240 (clone 2)	
<i>Neogoniolithon brassica-florida</i> (Harvey) Setchell & L.R. Mason	AY233346	Nora Creina Bay, South Australia, Australia
<i>N. spectabile</i> (Foslie) Setchell & L.R. Mason	AY234238	Little San Salvador Is., (Half Moon Cay), Bahamas
Incertae sedis		
Unidentified sp. (= <i>Sporolithon</i> sp.??)	AY247408	Beachport, South Australia, Australia

(Bailey & Chapman 1998; Bailey 1999). Although this observation supports Cabioch's contention that the mastophoroids and *Metagoniolithon* may share a recent common ancestor, these taxa were not resolved within (or sister to) the Corallinoideae (Bailey 1999). These preliminary data are therefore inconsistent with Cabioch's classification. However, phylogenetically accurate conclusions can rarely be drawn from examination of a single species, and this is particularly so for species-rich taxa such as the Mastophoroideae. Likewise, the available molecular data cannot be used to support or reject the subfamilial classification system of Harvey & Woelkerling (1995).

Taxonomic problems within the Mastophoroideae also exist. Morphological differences and the absence of comparative data for many species obscure boundaries between some genera and between many species within the Mastophoroideae. For these reasons, relationships between mastophoroid taxa, their geographic distributions and the approximate biodiversity of the group are poorly known. For example, four genera – some of which are recognized by some authorities but not by others – are included in the so-called 'Spongites complex'. This taxonomically difficult group of genera includes *Hydrolithon*, *Porolithon* and *Spongites* (Turner & Woelkerling 1982a, b; Penrose & Woelkerling 1988).

18S rRNA gene sequences were determined for six species representing three genera classified within the Mastophoroideae and also for two species of *Amphiroa* J.V.F. Lamouroux (Lithophylloideae). A ninth 18S rRNA sequence was obtained for a coralline alga that has yet to be conclusively identified. These data were used (1) to test the hypothesis of monophyly for the Mastophoroideae; (2) to test conflicting hypotheses for the phylogenetic position of the Mastophoroideae within the Corallinales; and (3) to re-examine relationships between selected genera within the group.

## MATERIAL AND METHODS

Species examined in this study are listed in Table 1. Methods for DNA extraction and polymerase chain reaction (PCR) amplification of the 18S rRNA gene(s) have been previously described (Bailey & Chapman 1998; Bailey 1999). PCR prod-

ucts were sequenced according to the manufacturer's suggested specifications for the BigDye cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) using primers given by Saunders & Kraft (1994). DNA sequences were determined on an Applied Biosystems (ABI) 377 or ABI 3100 automated DNA sequencer and the electropherograms were edited by eye, if necessary.

Material for *Metamastophora flabellata* was contaminated by adherent invertebrates and epiphytes; therefore, PCR products for the 18S rRNA gene of this species were cloned using the PinPoint Xa-1 T-Vector cloning kit (Promega, Madison, WI, USA). Transformed clones with incorporated coralline 18S rRNA genes were initially identified by comparison of sequences derived from primers G03 (18S rRNA variable region) and G06 (conserved region) with those of other coralline algae (Bailey 1999). Each was also confirmed using nucleotide (BLASTn) searches of the GenBank database ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). Two cloned 18S rRNA genes for *M. flabellata* were fully sequenced.

The nine sequences determined in this study were aligned by eye with those for 38 other species of coralline algae; GenBank accession numbers and collection information for these taxa are given in Bailey & Chapman (1998) and Bailey (1999). A total of 101 sites were excluded from subsequent analyses because they could not be confidently aligned. The data matrix is available from the corresponding author on request. The data set was analysed using parsimony and maximum likelihood (ML) methods as implemented in PAUP\* (v. 4.010b, Swofford 2002). Trees were rooted on the 18S rRNA sequence for *Rhodogorgon caribbowensis* J.N. Norris & K.E. Bucher (Rhodogorgonales; Norris & Bucher 1989; Fredericq & Norris 1995), which has previously been identified as sister to the Corallinales (Saunders & Bailey 1997). For parsimony, characters were unordered, equally weighted and gaps were treated as missing data. Trees were constructed using the heuristic tree-bisection-reconnection search algorithm with 500 random sequence addition replicates. Bootstrap proportion (BP) values for nodes of the parsimony tree(s) were calculated based on analyses of 10,000 pseudoreplicate data sets using the 'fast step-wise' addition option (Felsenstein 1985). ML analyses were performed using the HKY85 model of nucleotide substitution with nucleotide frequencies and the transi-

tion–transversion ratio estimated directly from the data (Hasegawa *et al.* 1985). The optimal ML tree was obtained using 10 random sequence addition replicates, and bootstrap values for the ML tree were based on 70 replicates.

## RESULTS

The 18S rRNA sequences determined for eight species of coralline algae have been deposited in GenBank (Table 1).

Nucleotide sequences for the two cloned 18S rRNA PCR amplification products from *M. flabellata* differed from one another at three sites. The three single-nucleotide polymorphisms observed between the clones indicate that the repeated 18S rRNA genes within the nuclear genome of *M. flabellata* are not all identical. We did not determine whether this variation occurs within individuals, between different plants or possibly both. The 18S rRNA sequences for *Neogoniolithon brassica-florida* and *N. spectabile* differed at 26 of 1779 comparable sites. The mean sequence divergence estimated among the three species of *Hydrolithon* (*H. onkodes*, *H. pachydermum* and *H. samoënsense*) was 2.2%. This mean, however, does not convey the fact that *H. onkodes* and *H. pachydermum* are very closely related and differ at only six sites over 1753 comparable positions (= 0.34% divergence). On the other hand, the sequence for *H. samoënsense* differs from those for *H. pachydermum* by 2.4% and *H. onkodes* by 3.9%. Nevertheless, mean nuclear 18S rRNA sequence divergence values for *Neogoniolithon* spp. (1.5%) and *Hydrolithon* spp. (2.2%) examined here are within the range (0.53–2.90%) reported for species belonging to other genera of coralline algae (Bailey 1999).

The 18S rRNA sequence alignment contained 1815 aligned positions, of which 329 were parsimony-informative. Cladistic analysis of these data resulted in 66 equally parsimonious trees of 1229 steps (CI = 0.52, RI = 0.76). The 50% majority rule consensus of these 66 trees is depicted in Fig. 1. Maximum likelihood analysis of these data yielded the tree (–ln L = 10361.16779) depicted in Fig. 2. The parsimony and ML trees are topologically consistent with one another, although bootstrap values and resolution within some clades differ.

*Heydrichia* Townsend, Chamberlain & Keats and *Sporolithon* Heydrich (Sporolithaceae) are resolved as an early diverging lineage (BP = parsimony 64%, ML 84%) sister to all other species examined. The unidentified species collected from South Australia diverges next and occupies an isolated but well-supported (BP = 97%, 99%) position sister to species belonging to the Melobesioideae. The melobesioidean taxa, which possess multiporate bi- or tetrasporangial conceptacles, do not form a monophyletic taxon. Instead, these taxa are resolved as members of a grade (Fig. 1) or unresolved polytomy (Fig. 2). Within the Melobesioideae only three lineages (*Phymatolithon* Foslie, *Lithothamnion* Heydrich and *Leptophytum* W.H. Adey) received strong support in both analyses (Figs 1, 2). Otherwise, internal nodes associated with the Melobesioideae are poorly supported and the relationships between genera within this group are unresolved (Figs 1, 2). The two 18S rRNA clones for *M. flabellata* form a clade (BP = 100%, 100%), and this species is placed sister to all other taxa bearing uniporate tetrasporangial conceptacles with strong support (BP = 96%, 99%). The remaining coralline species are divid-

ed between two large clades. The first includes two mastophoroid taxa (*Hydrolithon* and *Spongites*) as well as members of Metagoniolithoideae and Lithophylloideae (BP = 83%, 91%). Within this clade *S. yendoi* diverges first. *Hydrolithon* spp. (Mastophoroideae) are resolved as sister to *Metagoniolithon* spp. (Metagoniolithoideae) with moderate support (BP = 81%, 75%) and, in turn, this clade is resolved as sister to species classified in the Lithophylloideae (Figs 1, 2). The second clade is only moderately supported (BP = 53%, 66%) and includes *Neogoniolithon* spp. (Mastophoroideae) that are positioned sister to geniculate taxa placed in the Corallinoideae (Figs 1, 2). The Corallinoideae is divided into two clades corresponding to the tribes Janieae [*Cheilosporum* (J. Decaisne) G. Zanardini, *Halitilon* (J. Decaisne) J. Lindley and *Jania* J.V.F. Lamouroux] (BP = 88%, 93%) and Corallineae (BP = 68%, 58%), which includes all other corallinoidean species examined.

Our parsimony and ML analyses imply that the Mastophoroideae is polyphyletic (Figs 1, 2). We interpret our tree(s) as including four mastophoroid lineages (*Hydrolithon*, *Metamastophora*, *Neogoniolithon* and *Spongites*). Our phylogenetic reconstruction(s) indicates that each taxon is derived from a most recent common ancestor that is not shared with any other mastophoroid taxon examined in this study (Figs 1, 2).

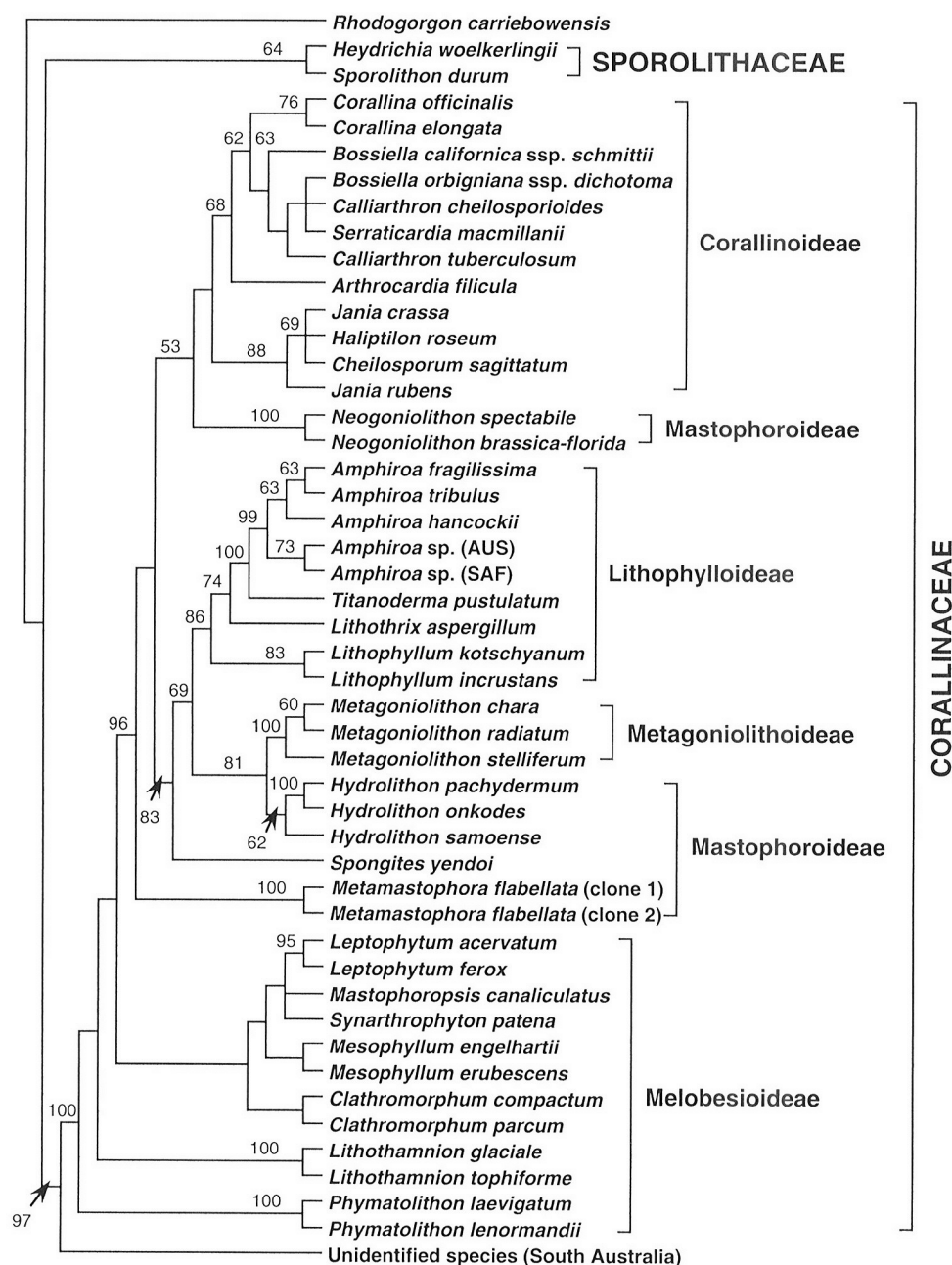
## DISCUSSION

Our molecular data indicate that the subfamily Mastophoroideae, as it is presently defined, is a polyphyletic taxon comprising four evolutionarily distinct lineages. Each of the four lineages is discussed below, and molecular and morphological (reproductive) data are used to compare each of the four mastophoroid lineages with their respective sister taxa. Our objective is to identify characters or combinations of characters that may indicate a phylogenetic link between taxa previously classified in different subfamilies. In particular, our focus is on comparisons of reproductive features that have supplanted vegetative characteristics as the primary bases for identifying and classifying coralline algae (e.g. Townsend 1981; Penrose & Woelkerling 1992). These include the ontogeny and anatomy of tetrasporangial, male and female or carposporangial conceptacles. Finally, we discuss briefly the taxonomic implications of our results, the status of the Melobesioideae and the phylogenetic position of an unidentified species included in this study.

### The phylogenetic position of *Neogoniolithon*

Two species of *Neogoniolithon*, *N. spectabile* and *N. brassica-florida*, were examined in this study. Our molecular data indicate that the two are closely related (BP = 100%, 100%), and in both parsimony and ML trees these taxa were resolved as sister to the Corallinoideae (Figs 1, 2). This implies that the nongeniculate genus *Neogoniolithon* is more closely related to geniculate species placed in the Corallinoideae than it is to other nongeniculate taxa. Weak to moderate character support was obtained for the node that *Neogoniolithon* and the Corallinoideae share, depending on the method used to infer the trees (BP = 53%, 66%).

Cabioch (1972, 1988) classified *Neogoniolithon*, as well as

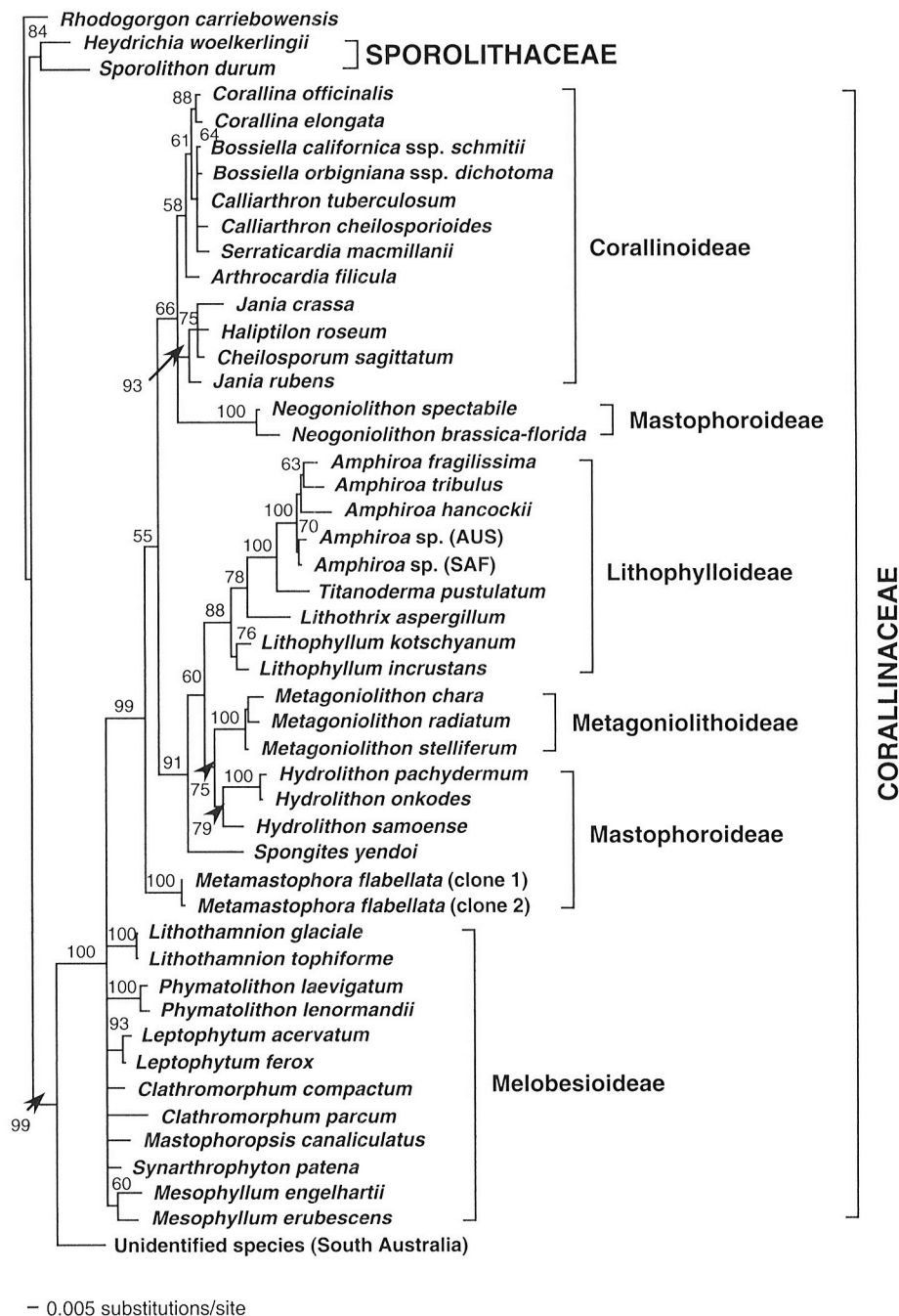


**Fig. 1.** Majority rule (50%) consensus of 66 equally parsimonious trees inferred from nuclear 18S rDNA gene sequences for 47 species of coralline red algae.

other nongeniculate mastophoroids (*sensu* Woelkerling 1988), within the Corallinoideae. Cabioch (1972, 1988) did not consider the presence or absence of genicula to be a phylogenetically reliable character at the rank of subfamily. For this reason, she included within the Corallinoideae all taxa (geniculate and nongeniculate) in which uniporate tetrasporangial conceptacles and cell fusions were present but secondary pit connections were lacking. Although our data support Cabioch's proposal that *Neogoniolithon* is more closely related to the Corallinoideae than to other nongeniculate taxa, our results do not imply that all Mastophoroideae *sensu lato* also belong to this lineage. For example, Cabioch (1972, 1988) considered *Hydrolithon* and *Neogoniolithon* as closely related

members of a separate evolutionary lineage within the Corallinoideae. However, our results imply that these genera are only distantly related and do not share a recent common ancestor (Figs 1, 2).

*Neogoniolithon* is distinguished from other mastophoroid taxa by two features. First, only in *Neogoniolithon* are spermatangia present on the floor and roof of the male conceptacle (Penrose 1991, 1992). Likewise, in most Corallinoideae, spermatangial initials are formed on the floor and side-walls of male conceptacles (Johansen 1976, 1981). In other mastophoroids (*Hydrolithon*, *Lesueuria*, *Lithoporella*, *Mastophora*, *Metamastophora* and *Spongites*) spermatangia are restricted to the conceptacle floor. Second, carposporangial conceptacles



**Fig. 2.** Maximum likelihood tree based on nuclear 18S rRNA gene sequences depicting phylogenetic relationships inferred between 47 species of coralline red algae.

are characterized by a thin, flattened and discontinuous fusion cell giving rise to gonimoblast filaments. Only in *Neogoniolithon* are gonimoblast filaments produced from the dorsal surface of the coenocytic fusion cell; this feature has been reported for *N. fosliei* (Heydrich) Setchell & L.R. Mason, *N. accretum* W.H. Adey & Vassar and *N. pacificum* (Foslie) Setchell & L.R. Mason (Masaki 1968; Penrose 1992). In other mastophoroids, gonimoblast filaments are initiated from the margin of the fusion cell (see Penrose 1992 and references therein). The origin of gonimoblast filaments may also suggest a possible link between *Neogoniolithon* and some Corallino-

ideae. In the tribe Corallineae (Corallinoideae), as in *Neogoniolithon*, gonimoblast filaments arise from the dorsal or marginal surfaces of the fusion cell (Ganesan 1967, 1968; Johansen 1969, 1976, 1981; Johansen & Silva 1978; Murata & Masaki 1978). In the tribe Janiaceae (including *Cheilosporum*, *Haliptilon* and *Jania*) gonimoblast filaments are restricted to the margins (Johansen 1977; Johansen & Silva 1978).

Penrose (1992) reported that tetrasporangial conceptacles in *Neogoniolithon* are formed by filaments peripheral to the fertile area. In mature tetrasporangial conceptacles, roof filaments are oriented parallel to the thallus surface and may pro-

trude laterally into the pore canal; a central columella is absent. This mode of tetrasporangial conceptacle development [referred to as 'SUR' by Johansen (1976) and Type 1 by Johansen (1981)] is also found in the Corallineae (Ganesan 1968; Johansen 1976, 1977, 1981).

In summary, the sister taxon relationship inferred between *Neogoniolithon* and the Corallinoideae (Figs 1, 2) is further supported by three reproductive features held in common: (1) spermatangia are not restricted to the chamber floor, but also are found on the roofs and side-walls of male conceptacles; (2) gonimoblast filaments arise from the dorsal surface of the fusion cell (except in the Janieae); and (3) the development of the tetrasporangial conceptacle cavity and roof is fundamentally similar in both taxa, corresponding to the Type 1 mode described in Johansen (1981).

### The phylogenetic position of *Hydrolithon*

In our study, nongeniculate *Hydrolithon* spp. were resolved as sister to species of the geniculate genus *Metagoniolithon* with significant support (BP = 81%, 75%).

*Hydrolithon* is primarily distinguished from other genera placed in the Mastophoroideae on the basis of features associated with tetrasporangial conceptacles. In *Hydrolithon*, tetrasporangial conceptacles are formed by filaments interspersed between the developing sporangia, as well as those surrounding the fertile area. Enlargement of the conceptacle cavity involves the disintegration of the filaments amongst the sporangia (Penrose 1992; Penrose & Woelkerling 1992; Penrose & Chamberlain 1993). Thus, cells of filaments comprising the roof are typically oriented perpendicularly to the conceptacle roof surface and do not protrude laterally into the pore canal (Penrose & Woelkerling 1992; Keats & Chamberlain 1994). Depending on the species, tetrasporangia may be borne peripherally or scattered across the chamber floor; a central columella composed of sterile filaments (paraphyses) may be present or absent (Penrose & Woelkerling 1992; Penrose & Chamberlain 1993). Developing tetraspores of *Hydrolithon* form a germination disc with a four-celled central element, and this feature is also believed to be characteristic of the genus (Chihara 1974; Penrose 1992; Penrose & Chamberlain 1993).

Male conceptacles contain spermatangial branches that are confined to the chamber floor (Penrose 1992; Penrose & Chamberlain 1993). Gonimoblast filaments arise marginally from the fusion cell (Penrose 1992; Penrose & Chamberlain 1993).

In *Metagoniolithon*, the tetrasporangial cavity is formed by the disintegration of cells derived from subepithallial initials, and tetrasporangia form a ring restricted to the periphery of the conceptacle cavity (Ganesan 1971; Ducker 1979). The pore (ostiole) of tetrasporangial conceptacles is likewise formed by the disintegration of cavity and cortical cells that are centrally located in the conceptacle roof. The early stages of female and male conceptacle development also involve disintegration of cavity cells, but the conceptacle pores are formed by the overgrowth of surrounding cortical tissue (Ducker 1979). Following fertilization, a continuous fusion cell is formed, from which gonimoblast filaments are initiated marginally (Ganesan 1971; Ducker 1979). Terminal carpogonia are located peripherally inside the female conceptacle;

spermatangial initials are borne across the chamber floor of male conceptacles (Ganesan 1971; Ducker 1979).

Thus, *Hydrolithon* and *Metagoniolithon* share three reproductive features that may support a phylogenetic relationship between them. These include: (1) fusion cells that give rise to marginal (peripheral) gonimoblast filaments; (2) spermatangial initials that are restricted to the floor of male conceptacles; and (3) Type 2 mode of tetrasporangial conceptacle development involving the disintegration of cavity cells among reproductive cells (Johansen 1981).

### *Hydrolithon pachydermum* comb. nov.

*Porolithon pachydermum* (Foslie) Foslie is a distinctive, widespread and very common alga in the Caribbean that often forms extensive pavements over reef substrates (Littler *et al.* 1995; Littler & Littler 2000). It was collected from Puerto Rico and examined in this study (Table 1).

Although some authorities continue to recognize *Porolithon* (e.g. Littler & Littler 2000), others do not. Woelkerling (1985) and Penrose & Woelkerling (1988) studied generic type specimens as well as recent collections of *Hydrolithon*, *Porolithon* and *Spongites*. Vegetative features studied did not provide a reliable basis for distinguishing among the three genera. Therefore, Penrose & Woelkerling (1988) subsumed *Hydrolithon* and *Porolithon* within *Spongites* but noted that the last genus was probably not natural. Later, Penrose & Woelkerling (1992) found that *Hydrolithon* and *Spongites* could be delimited on the basis of reproductive features. They found that *Hydrolithon* and *Spongites* possess patterns of tetrasporangial conceptacle development that are fundamentally different. (These are described in detail in other portions of the text.) Penrose & Woelkerling (1992) also concluded that *Porolithon* represented a heterotypic synonym of *Hydrolithon*; later, *Fosliella* Howe was also subsumed in *Hydrolithon* (Penrose & Chamberlain 1993).

Our molecular data clearly indicate that *P. pachydermum* is closely related to *H. onkodes* and *H. samoense*. These three species form a clade whose monophyly is moderately supported (BP = 62%, 79%) (Figs 1, 2). Sequence comparisons indicate that the 18S rRNA sequences for *P. pachydermum* and *H. onkodes* differ by only six nucleotides. Assuming an 18S rRNA molecular clock for coralline algae equivalent to 1% sequence divergence per million years (J.C. Bailey, unpublished observations), we conclude that *P. pachydermum* and *H. onkodes* may have diverged from their common ancestor approximately 340,000 years ago. Based on our observations, we have chosen to transfer *P. pachydermum* (Foslie) Foslie to *Hydrolithon* as *H. pachydermum* (Foslie) Bailey, Gabel & Freshwater *comb. nov.*

### The phylogenetic positions of *Metamastophora* and *Spongites*

In our trees, *M. flabellata*, the type species of *Metamastophora*, is robustly positioned (BP = 96%, 99%) as sister to all other coralline taxa bearing uniporate conceptacles (Figs 1, 2). *Metamastophora* is unique among the mastophoroids in two respects. First, the thallus is branched and arborescent and attached to the substrate by a stipe and holdfast (Woelkerling 1980a, 1988). Second, both cell fusions and secondary pit connections have been documented for this species (Woelk-

erling 1980a). Woelkerling (1980b) assigned *Metamastophora* to the Lithophylloideae (*sensu* Johansen 1976) because secondary pit connections were occasionally observed in the species. Later, Woelkerling (1988) placed the genus in the Mastophoroideae after concluding that the predominant form of intercellular connection observed (i.e. cell fusions) should serve as the final arbiter for the subfamilial classification of *Metamastophora*. Our study indicates that *Metamastophora* does not belong in the Lithophylloideae as it is currently circumscribed (Bailey 1999); whether or not *M. flabellata* will remain classified in the Mastophoroideae *sensu stricto* is uncertain (see below).

In *Metamastophora*, filaments that surround and grow over the fertile area form tetrasporangial and gametangial conceptacles (Woelkerling 1988). In tetrasporangial conceptacles, a columella is present and tetrasporangia are formed only around the periphery of the conceptacle chamber (Woelkerling 1980a, b, 1988). Following presumed karyogamy, a large continuous fusion cell develops that gives rise to peripherally located gonimoblast filaments. Mature carposporangia form a ring around the periphery of the conceptacle chamber (Woelkerling 1980a). Spermatangia are restricted to the chamber floor of the male conceptacle.

In our phylogenetic trees (Figs 1, 2), *Spongites* is placed sister to a clade including *Hydrolithon*, *Metagoniolithon* and members of the Lithophylloideae (BP = 83%, 91%). In *Spongites*, tetrasporangial conceptacle roofs are formed by filaments surrounding the fertile area. As a result, roof filaments are oriented parallel to the roof surface and may protrude into the pore canal (Penrose 1991; Penrose & Woelkerling 1991; Penrose & Chamberlain 1993). In tetrasporangial conceptacles a columella may be present or absent (Afonso-Carrillo 1988). Spermatangia are restricted to the floor in male conceptacles, and carpogonial conceptacles are characterized by a thin, flattened fusion cell that gives rise to marginal gonimoblast filaments (Afonso-Carrillo 1988; Penrose 1991).

As described above, the ontogeny of asexual and sexual conceptacles in *Metamastophora* and *Spongites* is similar, although these species are morphologically distinct. According to the system of Johansen (1981), both genera are characterized by the Type 1 pattern of tetrasporangial conceptacle development. However, our results imply that these taxa do not belong to the same clade (Figs 1, 2).

In this study and others, traditionally unorthodox relationships have been inferred among some mastophoroid taxa (*Hydrolithon* and *Spongites*), *Metagoniolithon* and members of the Lithophylloideae based on 18S rRNA sequence analyses (Bailey & Chapman 1998; Bailey 1999). This clade is strongly supported in our analyses (BP = 83%, 91%), but are there any other data that might also unite these taxa? There are. In fact, the development of tetrasporangial conceptacles in *Hydrolithon*, *Metagoniolithon* and members of the Lithophylloideae (*sensu* Bailey 1999) is fundamentally similar. Johansen (1981) designated this mode of development as 'Type 2' and the process is characterized by three features of primary importance. First, the conceptacle chamber, pore canal and pore are formed by the programmed disintegration of cavity cells located among the sporangial initials. Second, in these taxa apical pore plugs are absent. Third, the conceptacle roof comprises cells oriented more or less perpendicularly to the roof surface that are derived from filaments within the fertile area.

The 'odd taxon out' in this clade is *Spongites*, in which Type 1 tetrasporangial development occurs. *Spongites* is positioned sister to *Hydrolithon*, *Metagoniolithon* and members of the Lithophylloideae, in which Type 2 development is found. Type 1 development, in which the conceptacle chamber, pore and roof are formed by overarched filaments surrounding the fertile area, is found within the Corallinoideae, *Metamastophora*, *Neogoniolithon* and *Spongites*. The topology of our trees suggests that Type 2 tetrasporangial conceptacle development is a derived feature within the Corallinaceae (Figs 1, 2).

Finally, it should be noted that the clade subtended by *Metamastophora*, which excludes the Melobesioideae but includes all other Corallinaceae, is also strongly supported (BP = 96%, 99%) (Figs 1, 2). At least one reproductive feature seems to support the monophyly of this assemblage. In most members of the subfamilies Corallinoideae and Lithophylloideae and in *Hydrolithon*, *Metamastophora* and *Spongites*, a single, continuous fusion cell forms following fertilization that covers nearly the entire fertile area in carposporangial conceptacles (Johansen 1981). Because this feature is characteristic of other mastophoroid genera *sensu lato*, we hypothesize that they too will eventually be placed (somewhere) within this large clade.

#### Comments on the Melobesioideae

Character support, as measured by bootstrap values, for the monophyly of the Melobesioideae has declined as more (and more distantly related) taxa have been added to the 18S rRNA data set (Bailey & Chapman 1996, 1998; Bailey 1999). Although bootstrap values are not directly comparable across studies containing different taxa, this observation nevertheless deserves attention. In this study the subfamily was not resolved as monophyletic (Figs 1, 2). It is therefore pertinent to review those features that unite the subfamily for comparison to those described for other taxa above.

Tetrasporangial development in the Melobesioideae involves the breakdown of cavity cells overlying the fertile area. However, tetrasporangial roofs are multiporate (i.e. numerous ostioles are formed, one over each tetrasporangium) and the pores are formed around a gelatinous plug which occludes the pore until the tetrasporangia are mature (Johansen 1981; Woelkerling 1988). Johansen (1981) termed this the Type 3 mode of tetrasporangial conceptacle formation. Gametangial conceptacles are formed by the overgrowth of filaments adjacent to the fertile area. According to Lebednik (1978), spermatangia are formed on the floor and roof in all genera of the Melobesioideae. Furthermore, in the Melobesioideae, fertilization is followed by the formation of discontinuous, irregularly shaped fusion cells or several very small fusion cells that may or may not cover the entire fertile area in carposporangial conceptacles; in some species a fusion cell of any sort has yet to be observed (e.g. Adey & Sperapani 1971; Lebednik 1977; Townsend 1979; Johansen 1981; Woelkerling & Foster 1989; Chamberlain 1991; Woelkerling & Harvey 1992; Wilks & Woelkerling 1995; Keats & Maneveldt 1997; Cabioch & Mendoza 1998; Mendoza & Cabioch 1998). In carposporangial conceptacles, gonimoblast filaments arise from the periphery of the fusion cell in most species examined, although in a minority of species they may be borne at the periphery and

on the dorsal surface of the fusion cell (Adey & Sperapani 1971; Townsend 1979; Chamberlain 1991; Woelkerling & Harvey 1992; Chamberlain & Keats 1994, 1995; Düwel & Wegeberg 1996; Keats & Chamberlain 1997; Keats & Manneveltdt 1997; Cabioch & Mendoza 1998; Mendoza & Cabioch 1998). Together this unique combination of features strongly implies that the subfamily Melobesioideae is monophyletic and clearly delimited from other coralline subfamilies. It is therefore difficult to reconcile this conclusion with the results of our phylogenetic analyses (Figs 1, 2), which are at odds. Gene sequence data from more melobesioid species seem to be required to test the hypothesis of monophyly adequately for this taxon.

### Taxonomic and evolutionary implications

Our analyses demonstrate that the Mastophoroideae is a polyphyletic taxon that cannot be maintained as it is presently circumscribed. Furthermore, our results cast considerable doubt on the phylogenetic affinities of other genera currently placed in the Mastophoroideae. Taxonomic changes are clearly warranted but at this juncture we have elected not to develop an interim scheme that is likely to be replaced in the near future. Too many questions remain unanswered, too few taxa have been examined and too many subjective alternatives are available for consideration. For example, nomenclatural priority applies at the rank of family and below. Setchell (1943) erected the Mastophoroideae and the type of the subfamily is *Mastophora* (J. DeCaisne 1842). *Mastophora* was not examined in this study; it is therefore unknown which, if any, of the four mastophoroid lineages (*sensu lato*) should be retained within the Mastophoroideae *sensu stricto*. Nor have the type species of *Neogoniolithon* (*N. fosliei*) or *Spongites* (*S. fruticosus* Kützinger) been examined. From a cladistics perspective it is possible to argue that *Neogoniolithon* should be classified within the Corallinoideae (Figs 1, 2). However, we are unwilling to do so because bootstrap support at the critical node is not robust (BP = 53%, 66%). As a third example, there are numerous possible subjective classifications that could be proposed for the strongly supported (BP = 83%, 91%) clade that includes *Spongites*, *Hydrolithon*, *Metagoniolithon* and members of the Lithophylloideae (Figs 1, 2). As noted above, however, the ontogeny of tetrasporangial conceptacles in *Spongites* differs from that found in other genera placed in the clade. The taxonomic significance that should be afforded by this observation is presently unclear.

In this paper we have elaborated on reproductive similarities among the four mastophoroid lineages discovered here and their sister taxa as inferred from the molecular data. Additional studies are required to determine whether these similarities are actually homologous (synapomorphic) or are the result of convergent evolution, i.e. these characters need to be examined using cladistic methods in a study encompassing data for the entire order. Such a project is beyond the scope of the present investigation. For these reasons, we do not alter the classification of the Mastophoroideae *sensu lato* here, but will address these and other issues in a separate treatise.

### The 'unidentified species'

The unidentified species examined in this investigation was collected in South Australia (Table 1; Figs 1, 2) attached to a

stone also bearing material of *H. samoënsis*. The specific identity of this organism is presently unknown and its vegetative and reproductive anatomy is under study. Preliminary investigations, including observations of flared epithallial cells, indicate that the species probably belongs in the genus *Sporolithon* (A. Harvey, personal communication). The species may or may not be new to science; our results only imply that the organism is not conspecific with *S. durum* (Foslie) R.A. Townsend & Woelkerling. The 18S rRNA sequence obtained for the organism has been critically examined. BLASTn searches using the entire sequence or only the 5' or 3' ends of the sequence indicate that the gene was obtained from a coralline alga (results not shown). Evidence for contamination or a chimeric sequence was not found. Thus, our combined evidence suggests that it probably represents a species belonging to the Sporolithaceae.

### ACKNOWLEDGEMENTS

The authors are grateful to A. and R. Harvey, P. Mitrovski and J. Pawlik for collecting species examined in this study. M.J. Humphrey and A. Harvey provided technical assistance. This research was supported by NSF MCB-0084197 awarded to J.C.B. and NSF DEB-9806743 awarded to R.A. Andersen (Bigelow Laboratory for Ocean Sciences) and J.C.B.

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Accepted 20 May 2003