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Integrating phylogeny, molecular clocks, and the fossil record in the evolution of coralline algae (Corallinales and Sporolithales, Rhodophyta)

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Abstract.—When assessing the timing of branching events in a phylogeny, the most important tools currently recognized are a reliable molecular phylogeny and a continuous, relatively complete fossil record. Coralline algae (Rhodophyta, Corallinales, and Sporolithales) constitute an ideal group for this endeavor because of their excellent fossil record and their consistent phylogenetic reconstructions. We present the evolutionary history of the corallines following a novel, combined approach using their fossil record, molecular phylogeny (based on the 18S rDNA gene sequences of 39 coralline species), and molecular clocks. The order of appearance of the major monophyletic taxa of corallines in the fossil record perfectly matches the sequence of branching events in the phylogeny. We were able to demonstrate the robustness of the node ages in the phylogeny based on molecular clocks by performing an analysis of confidence intervals and maximum temporal ranges of three monophyletic groups of corallines (the families Sporolithaceae and Hapalidiaceae, as well as the subfamily Lithophylloideae). The results demonstrate that their first occurrences are close to their observed appearances, a clear indicator of a very complete stratigraphic record. These chronological data are used to confidently constrain the ages of the remaining branching events in the phylogeny using molecular clocks.

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Introduction

The suitability of using molecular clocks and the fossil record as practical tools for dating branching events in the phylogeny of a particular group of organisms has been widely and contentiously debated (see the recent compilation by Hedges and Kumar 2009a). It is true that conflicting chronological results (sometimes quite significant) may be obtained depending on which method is used (Knoll 1992; Benton and Ayala 2003; Peterson and Butterfield 2005; Blair and Hedges 2005; Peterson et al. 2005; Hedges and Kumar 2009b). It is now clear that molecules may evolve at considerably different rates, thereby producing inconsistent temporal results (Ayala 1986; Rodríguez-Trelles et al. 2001, 2002, 2004; Welch and Bromham 2005; Ho and Larson 2006). Further, a poor fossil record (incomplete and/or of low quality) and difficulties in the satisfactory taxonomic identification of fossil taxa have been used as arguments to discredit fossils as accurate tools for dating evolutionary events (Patterson 1981; Hedges et al. 1996; Blair and Hedges 2005). Although there is no question that the fossil record is incomplete (Paul 1998), it has nonetheless been shown to offer an adequate history of life on Earth (Benton et al. 2000).

Increasingly, it is evident that using molecular clocks and the fossil record in combination strengthens the robustness of the dating of splitting events in a phylogenetic tree (Benton et al. 2009). The ideal situation is offered by a group of organisms with a continuous, very complete fossil record and with a robust, reliable phylogeny. The first prerequisite is fundamental because molecular clocks are necessarily calibrated by using data of first occurrences in the fossil record (Benton and Donoghue 2007; Hug and Roger 2007; Avise 2009). Coralline algae (Rhodophyta, Corallinales, and Sporolithales) are probably one of the closest groups to reaching such an ideal case.

Corallines have a very continuous fossil record (Wray 1977; Steneck 1983; Bosence

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1991; Aguirre et al. 2000). They first appear in the Early Cretaceous and progressively diversify up to a peak in the Early Miocene, with diversification stabilizing during the Neogene (Aguirre et al. 2000). Additionally, consistent molecular phylogenies based on anatomical-morphological traits, as well as on different molecular markers (18S rDNA, 18S rRNA, SSU-rDNA, *psb*A and nSSU), have been proposed (Bailey and Chapman 1996, 1998; Bailey 1999; Harvey et al. 2002, 2003; Vidal et al. 2003; Bailey et al. 2004; Broom et al. 2008; Farr et al. 2009).

The objectives of this paper are (1) to reconstruct the molecular phylogeny of the corallines, (2) to analyze the quality of the coralline fossil record by calculating the confidence intervals of the first occurrence of selected taxa, (3) to compare the ages of the first appearance of monophyletic taxa in the fossil record with the ages obtained by using molecular clocks, and (4) to propose an evolutionary timetree of the different taxa used in the phylogeny. We hypothesize first that the excellent and continuous fossil record of the corallines accurately reflects the evolutionary history of the group shown in the phylogenetic reconstructions, and second that a few pinpointed markers in the fossil record permit an accurate setting of molecular clocks that work for the whole evolutionary history of the lineage, allowing dating of the branching events.

The Corallines

A Neontological Perspective.—The corallines (orders Corallinales and Sporolithales [see below], class Florideophyceae, phylum Rhodophyta) are cosmopolitan autotrophic organisms that constitute the third most diversified group of rhodophytes in present-day seas (Brodie and Zuccarello 2007). They form a monophyletic clade (Gabrielson et al. 1985; Gabrielson and Garbary 1986; Garbary and Gabrielson 1990; van den Hoek et al. 1995; Brodie and Zuccarello 2007; Le Gall and Saunders 2007; Maggs et al. 2007) characterized by (1) a pseudoparenchymatous thallus formed by densely packed cell filaments, (2) cell walls impregnated with calcite, (3) pit connections, comprising a double membrane, linking cells along a filament or cells of adjacent filaments, (4) spores having cruciate or zonate division, and (5) spores either in isolated calcified cavities grouped into sori or assembled into a large cavity called a conceptacle (Silva and Johansen 1986).

Corallines include two morphological groups: the non-geniculate (non-articulated or encrusting) and the geniculate (articulated) forms. The thalli of the non-geniculate corallines are completely calcified and consequently have a high preservation potential. In contrast, geniculate corallines consist of calcified segments (intergenicula) articulated by filaments of non-calcified cells (genicula). The non-calcified cells of the genicula decay quickly after death and the calcified segments then fall apart and disperse in the sediment. This structural collapse makes recognition as well as correct taxonomic identification of geniculate corralines rather difficult.

Harvey and Woelkerling (2007) considered the coralline algae as a single order (Corallinales) with three families divided into seven subfamilies (Table 1). This taxonomic scheme is based on phylogenetic analyses using a combination of morphological, anatomical, biochemical, ultrastructural, and molecular data (Bailey and Chapman 1996, 1998; Bailey 1999; Harvey et al. 2002, 2003; Vidal et al. 2003; Bailey et al. 2004; Kim et al. 2007; Broom et al. 2008; Farr et al. 2009).

In a recent paper, Le Gall et al. (2010) proposed changing the familial taxonomic status of Sporolithaceae to an ordinal level, Sporolithales Le Gall, Payri, Bittner & Saunders, comprising a single family, Sporolithaceae, based on a molecular phylogenetic study. Their results show that members of the genera *Sporolithon* and *Heydrichia* form a monophyletic group more closely allied to the genera *Rhodogorgon* and *Renouxia* of the order Rhodogorgonales than to the rest of the members of the former order, Corallinales. Le Gall et al. (2010) proposed separating the new order Sporolithales on the basis of the means of spore division (cruciate in the Sporolithales versus zonate in the Corallinales) and kind of structure of spore production (isolated cavities in the Sporolithales versus conceptacles in the Corallinales).

TABLE 1. Families, subfamilies, and genera of present-day coralline algae (after Harvey and Woelkerling 2007). Genera in bold are geniculate corallines.

Family and subfamily	Genus
Corallinaceae	
Metagoniolithoideae	***Metagoniolithon***
Corallinoideae	***Alatocladia, Arthrocardia, Bossiella Calliarthron, Cheilosporum, Chiharaea, Corallina, Haliptilon, Jania, Marginosporum, Masakiella, Serraticardia, Yamadaea***
Mastophoroideae	*Hydrolithon, Lesueuria, Lithoporella, Mastophora, Metamastophora, Neogoniolithon, Pneophyllum, Spongites*
Lithophylloideae	***Amphiroa**, Ezo, Lithophyllum/Titanoderma, **Lithothrix**, Paulsilvella, Tenarea*
Hapalidiaceae	
Austrolithoideae	*Austrolithon, Boreolithon*
Choreonematoideae	*Choreonema*
Melobesioideae	*Clathromorphum, Exilicrusta, Kvaleya, Lithothamnion, Mastophoropsis, Melobesia, Mesophyllum, Phymatolithon, Synarthrophyton*
Sporolithaceae	*Heydrichia, Sporolithon*

These two characters were used by Verheij (1993) to define the family Sporolithaceae. According to the results of Le Gall et al. (2010) coralline algae include two orders, Sporolithales and Corallinales, the latter comprising two families and seven subfamilies. Fossil representatives of Rhodogorgonales are unknown. The suitability of the separation of the order Sporolithales is a taxonomic problem that is beyond the scope of this paper, which focused on the evolutionary history of the coralline algae at the family and subfamily levels. This taxonomic proposition, however, does not affect our conclusions, because Sporolithales is a monophyletic group in the analysis of Le Gall et al. (2010), which coincides with our results, and, therefore, in terms of phylogenetic relationships, there is no difference whether the group is considered as a family or as an order with a single family.

A Paleontological Perspective.—Corallines have a very good fossil record due to calcification of the cell walls. Nevertheless, as commented above, non-geniculate corallines have a higher preservation potential than geniculate ones.

Several evolutionary reconstructions have been proposed by paleophycologists (Ishijima 1936; Johnson 1956; Maslov 1956; Endo 1961; Adey and Macintyre 1973; Poignant 1974, 1979). Except for the hypotheses of Ishijima (1936) and Endo (1961), these reconstructions show a polyphyletic origin for the corallines. Each evolutionary line in these phylogenies contains several genera identified following doubtful taxonomic criteria, but the taxonomy of the fossil corallines has changed substantially in the last two decades (Braga et al. 1993; Braga and Aguirre 1995; Aguirre et al. 1996; Bassi 1998; Basso et al. 1998; Aguirre and Braga 1998, 2005a; Rasser and Piller 1999; Vannucci et al. 2000; Braga 2003; Iryu et al. 2009). Braga et al. (1993) were the first authors to realize that a large number of the taxonomic criteria used in the identification of Recent corallines can also be applied for the classification of their fossil counterparts. Only a few genera cannot be correctly recognized as fossils because they are identified by anatomical features that do not fossilize (Braga 2003). However, under exceptional conditions, anatomical traits of taxonomic relevance, such as the distribution of the spermatangia in male conceptacles, can be preserved in fossil specimens (Braga 2006), and even fragments of DNA can remain in Pleistocene and sub-Recent corallines (Hughey et al. 2008).

Materials and Methods

Phylogenetic Analysis.—We have determined the phylogeny of 40 species of rhodophytes; 39 of these are coralline algae and the remaining species belongs to the outgroup. This analysis reproduces the molecular phylogeny based on the 18S rDNA gene sequence proposed by Harvey et al. (2003). Bailey et al. (2004) and Broom et al. (2008) have proposed more recent phylogenies including a longer list of coralline species. Although these

databases are larger than the one used by Harvey et al. (2003), the obtained polyphyly of the subfamily Mastophoroideae (see discussion below) led to the authors to conclude that more work is needed to solve the phylogeny of the group. Consequently, we have used the database of Harvey et al. (2003).

Gene sequences were extracted from the GenBank (accession number codes indicated in Table 1 of Harvey et al. [2003]). Sequence alignment was performed in CLUSTALX (Thompson et al. 1997) using the default setting for gap opening and extension penalties. Then, the nucleotide blocks with the most phylogenetic information were identified using GBLOCKS (Castresana 2000; Talavera and Castresana 2007). This step guarantees that the nucleotide positions with less evolutionary information for the phylogeny are discarded, without changing the rest of the alignment positions. The final alignment consists of 1653 nucleotides (average 1602) for the 40 species, comprising 1212 conserved positions, 441 variable positions, and 103 singletons. The compositional sequence analysis was done using the program MEGA4 (Tamura et al. 2007). This procedure of alignment is different from the one used by Harvey et al. (2003); they used CLUSTAL W to automatically align molecular sequences followed by a manual alignment with regard to the secondary structure for sequence using SeqPup; then, they eliminated those sequences that could not be unambiguously aligned (Harvey et al. 2003: p. 898).

The evolutionary model that best fits with the data, the Tamura and Nei plus invariant sites and a gamma distribution model (TrN + Γ + I), was obtained with a MODELTEST (Posada and Crandall 1998) implemented in MODELTESTSERVER (Posada 2006) using the Bayesian Information Criterion or the Hierarchical Likelihood Ratio Test (HLRT).

We have obtained a maximum-likelihood (ML) phylogenetic tree, because this is a robust method for inferring the most probable and accurate topology of the tree (Whelan et al. 2001), and a Bayesian tree to cross-validate the resulting topology and branch lengths. The ML phylogeny was inferred with the program PAUP, version 4.0 for MAC OS (Swofford 2002). Heuristic searches in PAUP* used tree bisection-reconnection branch swapping on ten random-addition sequence replications.

The statistical significance of the resulting tree was deduced with a nonparametric bootstrap analysis performing 100 replicas on PAUP* with the same ML parameters as above. The bootstrap values are frequently used as good indications of the monophyly of a particular group (Whelan et al. 2001), but it must be considered with caution as demonstrated by Tarrío et al. (2001).

For the Bayesian inference analyses, we used MrBayes, version 3.1 (Ronquist and Huelsenbeck 2003) and selected general time-reversible plus invariant sites and a gamma distribution model (GTR + Γ + I). Two independent runs of Metropolis-coupled Markov chain Monte Carlo analyses were performed, each with four chains. Chains were run for 5,000,000 generations, sampling parameters and a tree every 100 generations. We discarded the first 12,500 trees (''burning'') to obtain a consensus tree. Stationarity was checked by analyzing the average standard deviation of split frequencies and by the program Tracer, version 1.4 (Rambaut and Drummond 2007).

Molecular Clocks.—First, using the likelihood ratio test (LRT), we checked to see whether the ML phylogenetic tree was an ultrametric, clock-like tree by comparing the ML values of the tree forcing a molecular clock and not forcing one. The results confirmed that the tree was not ultrametric (LRT = 500.798; d.f. = 41; χ^2 = 56.94). Then, we estimated the rate of molecular change with a penalized maximum-likelihood approach using the program r8s, version 1.70 (Sanderson 2004). We used a Penalized Likelihood method with a smoothing factor equal to 1000 and a penalty function of ancestor-descendant, using an additive scale for rate penalty. Optimization was obtained with the truncated-Newton method with bound constraints (Sanderson 2002). The program r8s can be applied to calculate the absolute rate of molecular change, thus enabling us to estimate the time of branch divergences in the phylogenetic tree based on molecular clocks. Furthermore, this program

TABLE 2. Observed and estimated ages of the three selected taxa. Dates of first occurrences observed in the stratigraphic record (time range of the stage or substage in left column) of the three monophyletic groups used for constraining the age of phylogeny splitting events of the coralline algae compared with dates obtained with the analysis of confidence intervals and the maximum temporal range (middle and right columns). There is a significant correspondence between the observed dates and the ages calculated, indicating a very complete fossil record. This, in turn, guarantees that the ages obtained for the nodes of the phylogenetic tree using molecular clocks (as indicated in Fig. 2 and Appendix) are most likely close to the real times.

Monophyletic clade	Observed stratigraphic time	Age of confidence interval	Age of maximum temporal range
Sporolithaceae	136.4–130 Ma (Hauterivian)	139.1 Ma (Valanginian)	137.7 Ma (Valanginian)
Hapalidiaceae	115–112 Ma (late Aptian)	117.3 Ma (late Aptian)	116.3 Ma (late Aptian)
Lithophylloideae	65.5–61.7 Ma (Danian)	70.5 Ma (Maastrichtian)	68.8 Ma (Maastrichtian)

can be used to assess changes in the molecular variation rates following different evolutionary models.

At least one chronological datum obtained from the stratigraphic record is required to date the branching events in the phylogeny with molecular clocks. The r8s program allows several chronological data to be introduced, thus obtaining better time constraints. Additionally, the program is able to run with time intervals, which is more realistic when the data are extracted from the stratigraphic record, giving more accurate calibrations (Hug and Roger 2007).

In the study case, the age ranges used for the molecular timing refer to chronological intervals of the first occurrence at the stage or substage level of the families Sporolithaceae and Hapalidiaceae and the subfamily Lithophylloideae (Table 2). The identification of fossil components of these monophyletic groups is easy and straightforward, thus precluding taxonomic biases. The family Sporolithaceae includes non-geniculate corallines with spores borne in isolated cavities grouped into sori. Hapalidiaceans are non-geniculate corallines characterized by tetra-bisporangial reproductive structures (conceptacles) with multiple pores in the roofs and cells of contiguous filaments connected by fusions. Finally, representatives of the subfamily Lithophylloideae show a single pore canal in the roof of the tetra-bisporangial conceptacles and cells of adjacent filaments are not fused. This latter feature separates the lithophylloids from the remaining subfamilies of the family Corallinaceae (Mastophoroideae, Corallinoideae, and Metagoniolithoideae), in which cells are connected by cell fusions. The family Corallinaceae has both geniculate and non-geniculate coralline species (Cabioch 1971, 1972, 1988; Bailey and Chapman 1996, 1998; Bailey 1999; Harvey et al. 2003; Vidal et al. 2003; Bailey et al. 2004; Broom et al. 2008).

The ages of the first occurrence of these three groups in the stratigraphic record allow the dating of the nodes of the phylogenetic tree to be accurately constrained on the basis of the molecular clocks (Appendix). The absolute ages of the selected time intervals are from the Geologic Time Scale of Gradstein et al. (2004).

Paleontological Data.—The accuracy of the chronological data of first appearances of the three selected monophyletic taxa has been demonstrated by calculating confidence intervals and the maximum temporal ranges. Both metrics calculate the time range in which a particular taxon can be found earlier than the first occurrence observed in the stratigraphic record (or later than the last appearance in the case of extinctions), thus giving an indication of the completeness of the fossil record (Marshall 1990, 1998).

The formula to calculate the confidence intervals is

$$r_c = \alpha R_o,$$

where r_c is the confidence interval, the time period in which it is possible to find a specific taxon earlier than observed in the stratigraphic record; R_o is the observed stratigraphic range; and α is a constant that depends on the confidence level (C) and the number of stratigraphic levels in which a particular taxon occurs (H):

$$\alpha = \left[(1-C)^{-1/(H-1)}\right] - 1$$

This analysis was proposed for local sections (Strauss and Sadler 1989), although it

has also been successfully used for global data (Marshall 1990; Benton 2004). In this paper, H is defined as the number of localities where a particular taxon has been cited with a temporal resolution at the stage or substage level (''Stage-Level Data'' from Aguirre et al. [2000]). In the case of the family Sporolithaceae, the data have been improved by adding data compiled recently by Braga and Bassi (2007). The observed stratigraphic range (R_0) is calculated from the base of the stage or substage in which each taxon first occurred to the present day (the three monophyletic groups are recent taxa). Absolute ages refer to the global geochronological timescale (Gradstein et al. 2004). All calculations were made using the 95% confidence level ($C = 0.95$).

The maximum temporal range was calculated by using the formula (Strauss and Sadler 1989):

$$R_t = R_o(H+1)/(H-1),$$

where R_t is the estimated theoretical time interval; R_o and H are as previously indicated.

Results

Molecular Phylogeny.—The Bayesian and the maximum-likelihood (ML) phylogenetic trees obtained show identical topology. Therefore, for the sake of simplicity, we will refer to the ML tree throughout the text (Fig. 1).

Three monophyletic groups can be identified in the ML tree (Fig. 1). The basal group is the family Sporolithaceae and the other two groups, the families Hapalidiaceae and Corallinaceae, form a clade phylogenetically related to the basal sporolithaceans (Fig. 1). The family Hapalidiaceae is a single monophyletic clade that comprises non-geniculate members of the subfamilies Melobesioideae and Choreonematoideae. The family Corallinaceae can be divided into two clades, one of which groups the geniculate forms of the subfamily Corallinadoideae; the other is a taxonomically and anatomically heterogeneous clade including both geniculate and non-geniculate species of the subfamilies Mastophoroideae, Lithophylloideae, and Metagoniolithoideae (Fig. 1).

Molecular Clocks and Paleontological Data on First Occurrences.—The ages of the branching events estimated using molecular clocks have been calibrated taking into consideration the first occurrences of sporolithaceans, hapalidiaceans, and lithophylloids in the stratigraphic record (Table 2). The accuracy of the chronostratigraphic data on the first appearance of the selected monophyletic taxa has been tested by analyzing confidence intervals (r_c) and maximum temporal ranges (R_t). The ages calculated using both metrics are very similar to the ones observed in the stratigraphic record (Table 2). Therefore, the probability of finding representatives of the family Sporolithaceae, first recorded in the Hauterivian, can be expanded to the Valanginian. The family Hapalidiaceae, first occurring in the late Aptian, can be recorded earlier but still within the Aptian. Finally, the probability of recording members of the subfamily Lithophylloideae in the fossil record can be extended to the Maastrichtian (Table 2).

These results indicate that the fossil record of the three monophyletic groups is very complete and, therefore, their first occurrences can confidently be used to calibrate the ages of the nodes in the phylogeny with molecular clocks (Appendix). The resulting timetree is shown in Figure 2.

Discussion

Molecular Phylogeny.—The ML tree obtained reproduces those obtained by previous authors (Bailey 1999; Harvey et al. 2003; Vidal et al. 2003; Bailey et al. 2004; Broom et al. 2008), although with some differences. In the phylogenetic hypothesis proposed by Harvey et al. (2003), the family Sporolithaceae occurs as a paraphyletic group. Our results show this group as a monophyletic clade (Fig. 1), coinciding with the phylogeny proposed by other authors (Vidal et al. 2003; Bailey et al. 2004; Farr et al. 2009; Le Gall et al. 2010). Members of the sporolithaceans show unique anatomical features and reproductive structures, making them easily identifiable and confirming the molecular phylogeny. Representatives of the family Hapalidiaceae, which is confirmed in our results (Fig. 1) and in other analyses (Harvey et al. 2003; Broom et

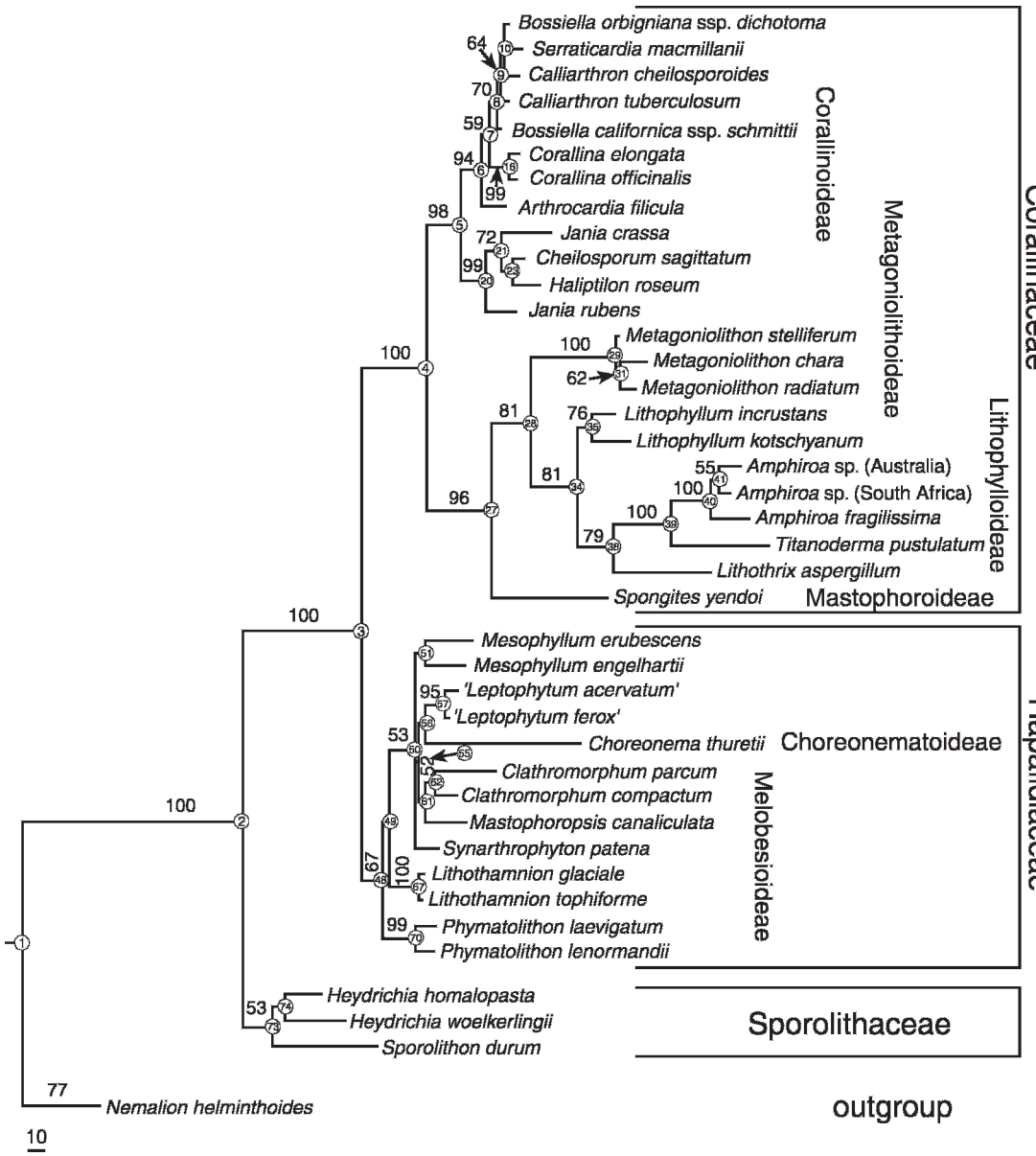

FIGURE 1. ML phylogenetic tree. The resulting tree allows the differentiation of three monophyletic groups (in order of appearance: families Sporolithaceae, Hapalidiaceae, and Corallinaceae). The names of the subfamilies have been added within each family. Circled numbers represent the nodes ordered as in the Appendix. Numbers above the branches indicate bootstrap values (only values above 50% have been included). Scale bar indicates number of nucleotide substitutions.

al. 2008; Farr et al. 2009) as monophyletic, also show exclusive anatomical and reproductive features.

Harvey et al. (2003) differentiated two subfamilies within the hapalidiaceans; the Choreonematoideae and the Melobesioideae. The subfamily Choreonematoideae, as described by Woelkerling (1987), includes only the parasitic species *Choreonema thuretii* (Bornet in Thuret & Bornet) Schmitz. Broadwater et al. (2002), on the basis of an anatomical, morphologic, and ultrastructural study, concluded that this species is closely related to other melobesioids but shows extensive anatomical and ultrastructural modifications due to its parasitic life habit. Furthermore, the

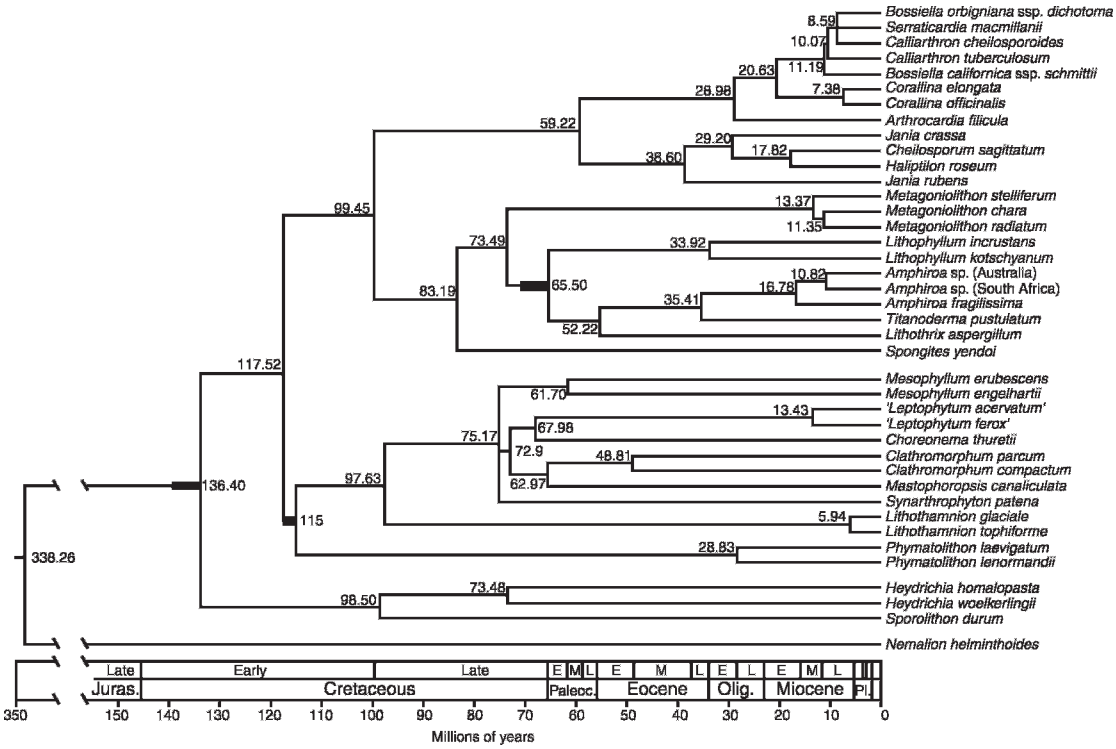

FIGURE 2. A timetree of corallines. Numbers at the nodes are the divergence times (in millions of years) of the branching events based on molecular clocks. The black bars indicate the confidence intervals (as shown in Table 2) of the first appearance of those clades in the geological record.

differentiation of the subfamily Choreonematoideae following Harvey et al. (2003) implies that the subfamily Melobesioideae is paraphyletic. The discussion of the taxonomic validity of the subfamily Choreonematoideae is beyond the scope of this paper, but our results show that it is not supported by molecular phylogeny.

The family Corallinaceae can be divided into two monophyletic groups. One group is the subfamily Corallinoideae and the other includes three subfamilies: Metagoniolithoideae, Lithophylloideae, and Mastophoroideae (Fig. 1). Kim et al. (2007) also concluded that the subfamily Corallinoideae is monophyletic. A bootstrap value below 50% for the subfamily Mastophoroideae is coincident with the values obtained in previous phylogenetic analyses (Bailey and Chapman 1996, 1998; Bailey 1999; Harvey et al. 2003; Bailey et al. 2004).

Bailey et al. (2004) included in their analysis seven species of mastophoroids based on anatomical and reproductive characters: *Neogoniolithon spectabile* (Foslie) Setchell and Masson, *N. brassica-florida* (Harvey) Setchell and Masson, *Hydrolithon pachydermum* (Foslie) Foslie, *H. onkodes* (Heydrich) Penrose and Woelkerling, *H. samoense* (Foslie) Keats and Chamberlain, *Spongites yendoi* (Foslie) Chamberlain, and two clones of *Metamastophora flabellata* (Sonder) Setchell. The molecular phylogeny unexpectedly showed that the subfamily Mastophoroideae was polyphyletic (Bailey et al. 2004), coinciding with the conclusion drawn by Broadwater et al. (2002) based on the ultrastructure of members included in this subfamily. Some mastophoroid species are linked to the geniculate corallines of the subfamily Corallinoideae, and other species are phylogenetically related to the subfamilies Lithophylloideae and Metagoniolithoideae. Broom et al. (2008), adding one species to the mastophoroid in Bailey et al. (2004), *Hydrolithon improcerum* (Foslie and Howe) Foslie, came to the same conclusion on the polyphyletic origin of the subfamily. Hughey et al. (2008) also obtained a polyphy-

letic relationship between the mastophoroids *Neogoniolithon brassica-florida* and *Spongites yendoi* based on a molecular phylogeny including recent and fossil DNA data of coralline species. All these results suggest that further work is needed to disentangle the puzzling taxonomic status of the subfamily Mastophoroideae (Bailey et al. 2004; Broom et al. 2008).

Age of the Nodes in the Phylogeny.—The ages of the nodes in the ML tree estimated from molecular clocks are shown in the Appendix and in Figure 2. These dates and the sequence of origination of the groups in the phylogeny fit with chronological data of first occurrence of taxa in the fossil record.

The species *Sporolithon rude* (Lemoine) Ghosh and Maithy from the early Hauterivian (Early Cretaceous) (Arias et al. 1995) and *Sporolithon phylloideum* (Bucur and Dragastan) Tomás, Aguirre, Braga and Martín-Closas from the late Hauterivian (Moussavian et al. 1993; Tomás et al. 2007) are the oldest records for the corallines. This confirms the phylogenetic reconstructions, because the family Sporolithaceae is the basal group within the corallines (Figs. 1, 2), and agrees with the classic ideas based only on anatomical, vegetative, and reproductive structures (e.g., Johnson 1956, 1961; Cabioch 1972; Townsend et al. 1995).

Hapalidiaceans evolved later according to the ML tree (Fig. 1). The oldest representative of this family so far is the species *Lithothamnion tenuicrustatum* Ishijima from the late Aptian of West Kalimantan (Borneo, Indonesia) (Ishijima 1978). This species shows multiporate tetra-bisporangial conceptacles and cell fusions characteristic of the subfamily. Therefore, the fossil record precisely matches the molecular results (Fig. 2).

The genus *Lithothamnion* shares anatomical features with representatives of the sporolithaceans, suggesting an evolutionary relationship between the two groups (Adey et al. 1982; Townsend et al. 1995; Farr et al. 2009). Additionally, the multiporate sporangial conceptacles of the hapalidiaceans probably derived from the fusion of isolated sporangial cavities of the sporolithaceans (Tomás et al. 2007). This polarity in the occurrence of characters is also consistent with the ML phylogeny of corallines.

Within the family Hapalidiaceae, the genus *Mesophyllum* can be identified as a melobesioid with a predominantly concentric arrangement (coaxial) of cell filaments at the ventral part of the thallus (Lemoine 1928; Aguirre and Braga 1998; Braga 2003). The node separating *Mesophyllum* from the similar genus *Synarthrophyton* is dated at 75.17 Myr (Maastrichtian; Late Cretaceous) according to the molecular clocks (Fig. 2). The species *Mesophyllum vignyense* (Lemoine) Lemoine is the oldest record of the genus, found in Maastrichtian and Paleocene sediments (Lemoine 1923). The age of the first stratigraphic occurrence of the genus is consistent with the age estimated by the molecular clocks.

The family Corallinaceae represents the most recent splitting of monophyletic taxa in the ML phylogeny of corallines. The age of the basal node of the family is estimated at 99.45 Myr (base of Cenomanian; lowermost Late Cretaceous) according to the molecular clocks (Fig. 2). This family, composed of geniculate and non-geniculate corallines with uniporate tetra-bisporangial conceptacles, includes four subfamilies (in the order of their appearance on the basis of the molecular clocks): Mastophoroideae, Lithophylloideae, Corallinoideae, and Metagoniolithoideae (Figs. 1, 2). The discussion on the chronostratigraphic origin of the family and subfamilies requires some comments.

Numerous recent taxonomic studies have demonstrated that many fossil mastophoroids have been erroneously assigned to the lithophylloid genus *Lithophyllum* (Braga et al. 1993); examples include *Spongites albanense* (Lemoine) Braga, Bosence and Steneck, originally described as *Lithophyllum albanensis* Lemoine, and *Hydrolithon corculumis* (Maslov) Braga, Bassi, Zakrevskaya and Petronova-Radionova, formerly *Lithophyllum corculumis* Maslov (Braga et al. 2005). Elliott (1959) described the species *Lithophyllum*? *shebae* from Cretaceous deposits of Iraq as an encrusting coralline alga with a uniporate tetra-bisporangial conceptacle. Although Elliott did not consider the type of intercellular connections, he reported, ''Individual cell-

walls usually difficult to distinguish clearly for measuring.... Apparently larger ones are probably due to two adjacent rows ill-defined at the junctions'' (Elliot 1959: p. 220). The presence of uniporate sporangial conceptacles indicates that Elliott's species certainly belongs to the family Corallinaceae. Moreover, the difficulty in distinguishing cell walls suggests that the cells are connected by fusions. These two characters make it plausible to consider Elliott's species as the oldest putative representative of the subfamily Mastophoroideae, in agreement with the timing obtained by using molecular clocks (Fig. 2). This datum, however, should be contrasted with a taxonomic reassessment of the type species.

The same taxonomic problem affecting mastophoroids applies to lithophylloids. At least 13 *Lithophyllum* species have been reported from Jurassic–Cretaceous sediments, but all of them were described without any indication about the sporangial conceptacles or the type of cell connections. Therefore, their generic attributions can no longer be sustained and must be reassessed. One exception, however, is *Lithophyllum premoluccense* var. *cretacicum* Maslov, from the Late Cretaceous deposits of Georgia (Maslov 1956). Recent revision of the type material by Braga et al. (2005) demonstrates that this is the first member of the subfamily Lithophylloideae recorded so far, because it shows no cell fusions. The age estimated with molecular clocks for the separation of the subfamily Lithophylloideae from the subfamily Metagoniolithoideae is 73.49 Ma (Fig. 2). This age estimate fits the observed fossil record of *L. premoluccense* var. *cretacicum* in Late Cretaceous sediments as well as the age obtained by calculating the confidence intervals and the maximum temporal range for the origin of the subfamily (Table 2).

The species *Pseudoamphiroa propria* (Lemoine) Moussavian from Maastrichtian–Thanetian deposits of the eastern Alps (Austria) is an encrusting coralline alga that shows no cell fusions (Moussavian 1989). It can therefore reasonably be assigned to the subfamily Lithophylloideae, also in agreement with the age obtained with molecular clocks (Fig. 2).

Distichoplax biserialis (Dietrich) Pia is an extinct coralline without cell fusions, similar to the present-day lithophylloid *Tenarea* (Denizot and Massieux 1965; Aguirre and Braga 2005b). Keij (1963, 1964) described the sporangial conceptacles of this species as large cavities protruding on the thallus surface, similar to the uniporate conceptacles of the lithophylloid species *Titanoderma cystoseirae* (Hauk) Woelkerling, Chamberlain and Silva. *D. biserialis* first appeared in the Late Cretaceous–Paleocene, an age range also coincident with the timing of the origin of the subfamily Lithophylloideae (Fig. 2).

The poor preservation potential of the geniculate corallines makes their feasible identification difficult; therefore, any fossil record of these corallines has to be considered cautiously (Bassi et al. 2000). The nature of *Amphiroa mattiroliana* Raineri, a putative geniculate coralline from the Cenomanian–Turonian deposits of Libya (Raineri 1920), is uncertain, because the lack of information on the type of intercellular connections precludes a precise subfamily attribution. According to the present-day taxonomy of corallines, the genus *Amphiroa* belongs to the subfamily Lithophylloideae, but the taxonomic criteria applied by Raineri (1920) are difficult to discern. The first reliable record of fossil corallinoids is from Paleocene deposits in Spain (Aguirre et al. 2007). The age of the node of the subfamily Corallinoideae is 59.22 Ma according to the molecular clocks (Fig. 2), strikingly coinciding with the Spanish findings.

Conclusions

1. A molecular phylogeny of 39 species of corallines allows the recognition of three monophyletic groups corresponding to the families Sporolithaceae, Hapalidiaceae, and Corallinaceae. Although the bootstrap values for the sporolithaceans and the hapalidiaceans are low, both the anatomical features and the reproductive structures of members of these two families are invariable, thus substantiating their monophyly. The monophyletic nature of the family Corallinaceae is supported by a

bootstrap value of 100%. Within this latter family, the bootstrap value of the subfamily Mastophoroideae is notably low (40%), suggesting that this clade could be polyphyletic, as reported by other authors (Broadwater et al. 2002; Bailey et al. 2004; Broom et al. 2008).

2. An outstanding coincidence between the phylogeny and the first occurrence of different taxa in the stratigraphic record has been shown. Both aspects are considered to be crucial for reliable dating of splitting events in a phylogeny based on molecular clocks, which is, in turn, essential to documenting and dating the sequence of appearances of evolutionary innovations characterizing the origin of different monophyletic groups. In the study case, the ages of the first stratigraphic records of the families Sporolithaceae and Hapalidiaceae, as well as the subfamily Lithophylloideae, have been used to estimate the age of the nodes in the phylogeny of the corallines. The accuracy of the chronological data of the three selected taxa has been demonstrated by our calculations of the confidence intervals and the maximum temporal range of their first occurrence. The results indicate that the first appearance of the three monophyletic groups in the fossil record is very close to the theoretical first occurrence calculated with the confidence intervals and the maximum temporal range. This, in turn, indicates that the corallines have an excellent (continuous and complete) fossil record.
3. Adjusting the molecular clocks to date branching events in a phylogeny allows the ages of the first occurrences of other taxa in the stratigraphic record to be predicted. Therefore, molecular phylogeny and the fossil record can be reconciled to accurately reconstruct the evolution of a group by relying on a few temporally pinpointed markers to set the molecular clocks and to calibrate molecular changes ticking at different rates within the clocks.

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Appendix

Estimated ages and substitution rates of all nodes of the phylogenetic tree of coralline algae. Ages are in millions of years ago (Ma). The rates of the molecular changes are similar, indicating that there is a very constant molecular substitution in the evolution of the group. This suggests the molecular clocks ticked at a very regular rate, which is useful for dating the splitting events in the timetree of the corallines. This accounts for the high correspondence in the age of the observed appearance of corallines in the stratigraphic record and the estimated ages using the molecular clocks.

Node (numbers of nodes as in Fig. 1)	Time range		Estimated age (Ma)	Rate of molecular change	
	Min. age (Ma)	Max. age (Ma)		Estimated	Local
1 [root]			338.26		
2	130.40	136.40	136.40	3.2749e-04	3.9261e-04
3			117.52	3.6653e-04	2.2435e-03
4			99.45	4.1216e-04	1.3057e-03
5 [Corallinoideae]			59.22	4.0409e-04	3.0075e-04
6			28.98	3.9777e-04	2.6001e-04
7			20.63	3.9691e-04	3.6236e-04
8			11.19	3.9464e-04	2.5632e-04
9			10.07	3.9583e-04	5.4156e-04
10			8.59	3.9900e-04	8.1833e-04
B. orbigniana			0.0	3.9778e-04	2.1118e-04
S. macmillanii			0.0	4.0142e-04	7.7433e-04
C. cheilosporoides			0.0	4.0052e-04	6.3354e-04
C. tuberculosum			0.0	3.9371e-04	1.2012e-04
B. californica			0.0	3.9219e-04	1.0813e-04
16			7.38	3.9853e-04	5.0231e-04
C. elongata			0.0	3.9905e-04	4.9168e-04
C. officinalis			0.0	3.9860e-04	4.0974e-04
A. filicula			0.0	3.9548e-04	2.9227e-04
20			38.60	4.0545e-04	4.3987e-04
21			29.20	4.1020e-04	5.7968e-04
J. cassa			0.0	4.1461e-04	6.2147e-04
23			17.82	4.0938e-04	3.7199e-04
C. saggittatum			0.0	4.0713e-04	2.3765e-04
H. roseum			0.0	4.1111e-04	5.4321e-04
J. rubens			0.0	4.0152e-04	2.6647e-04
27			83.19	4.5402e-04	1.4509e-03
28			73.49	4.8258e-04	1.4342e-03
29			13.37	4.8343e-04	5.0315e-04
M. stelliferum			0.0	4.8014e-04	9.0466e-05
31			11.35	4.8681e-04	8.9621e-04
M. chara			0.0	4.8936e-04	8.5286e-04
M. radiatum			0.0	4.8714e-04	5.3304e-04
34 [Lithophylloideae]	61.70	65.50	61.70	5.0450e-04	1.4364e-03
35			33.92	4.9239e-04	1.7421e-04
L. incrustans			0.0	4.8738e-04	2.4970e-04
L. kotschyanum			0.0	4.9071e-04	4.1021e-04
38			52.22	5.3196e-04	1.3403e-03

Appendix. Continued.

Node (numbers of nodes as in Fig. 1)	Time range		Estimated age (Ma)	Rate of molecular change	
	Min. age (Ma)	Max. age (Ma)		Estimated	Local
39			35.41	5.5105e-04	1.2234e-03
40			16.78	5.5535e-04	7.7938e-04
41			10.82	5.5492e-04	5.0758e-04
Amphiroa sp AUS.			0.0	5.5592e-04	7.2678e-04
Amphiroa sp SA			0.0	5.5363e-04	3.3543e-04
A. fragilísima			0.0	5.5782e-04	8.2919e-04
T. pustulatum			0.0	5.5963e-04	1.0080e-03
L. aspergillum			0.0	5.3596e-04	6.7190e-04
S. yendoi			0.0	4.5651e-04	5.0175e-04
48 [Hapalidiaceae]	112.00	115.00	112.00	3.3071e-04	1.3141e-03
49			97.63	3.0731e-04	2.1049e-04
50			75.17	3.0272e-04	3.7702e-04
51 [*Mesophyllum*]	61.70	65.50	61.70	2.9730e-04	3.1449e-04
M. erubescens			0.0	2.9595e-04	2.7454e-04
M. engelhartii			0.0	2.9301e-04	2.2551e-04
55			72.90	3.1452e-04	5.3293e-04
56			67.98	3.3080e-04	6.1501e-04
57			13.46	3.1879e-04	1.3315e-04
L. acervatum			0.0	3.1931e-04	3.5964e-04
L. ferox			0.0	3.1588e-04	8.9910e-05
C. thuretii			0.0	3.5780e-04	8.2765e-04
61			62.97	3.0956e-04	2.4376e-04
62			48.81	3.0874e-04	3.0874e-04
C. parcum			0.0	3.1435e-04	4.3376e-04
C. compactum			0.0	3.0244e-04	1.7350e-04
M. canaliculata			0.0	3.0606e-04	2.4979e-04
S. patena			0.0	2.9008e-04	1.2877e-04
67			5.94	2.8987e-04	1.1876e-04
L. glaciale			0.0	2.8997e-04	3.0568e-04
L. tophiforme			0.0	2.8871e-04	1.0189e-04
70			28.83	3.1331e-04	1.5276e-04
P. laevigatum			0.0	3.1164e-04	2.5176e-04
P. lenormandii			0.0	3.1049e-04	2.0980e-04
73 [Sporolithaceae]			98.50	3.2044e-04	2.7136e-04
74			73.48	3.0907e-04	1.9344e-04
H. homalopasta			0.0	3.0084e-04	1.8936e-04
H. woelkerlingii			0.0	3.0878e-04	3.0462e-04
S. durum			0.0	3.2651e-04	3.9307e-04
N. helminthoides			0.0	1.5096e-04	8.5847e-05