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Sterols in red and green algae: quantification, phylogeny, and relevance for the interpretation of geologic steranes

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ABSTRACT

Steroids, a class of triterpenoid lipids with high preservation potential, are widely distributed in sedimentary rocks. All eukaryotes have a physiological requirement for these molecules, making steroids important biomarkers for aiding our understanding of eukaryote molecular evolution and geologic history. C_{26}–C_{30} sterols are the molecules most commonly incorporated or synthesized by eukaryotes, and correspond to C_{26}–C_{30} steranes ubiquitously and abundantly preserved in petroleums and sedimentary bitumens. Because these sterols occur in evolutionarily diverse taxa, it can be difficult to associate any particular compound with a single group of organisms. Nevertheless, geochemists have still been able to draw parallels between the empirical patterns in geolo gic sterane abundances and the age of petroleum source rocks. Paleobiologists have also used sterane data, in particular the patterns in C_{29} and C_{28} steranes, to support fossil evidence of an early radiation of green algae in latest Proterozoic and Paleozoic and the succession of the major modern phytoplankton groups in the Mesozoic. Although C_{29} sterols are found in many eukaryotes, organisms that produce them in proportional abundances comparable to those preserved in Proterozoic and Paleozoic rocks are limited. Based on a large, phylogenetically based survey of sterol profiles from the kingdom Plantae, we conclude that modern ulvophyte and early diverging prasinophyte green algae produce high abundances of C_{29} relative to C_{27} and C_{28} sterols most consistent with the sterane profiles observed in Paleozoic rocks. Our analysis also suggests that ancestral stem groups among the Plantae, including the glaucocystophytes and early divergent red algae are also plausible candidates.

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INTRODUCTION

Steranes, the geological derivatives of sterol lipids, have been used as molecular fossils to document a variety of eukaryotic groups (Moldowan *et al.*, 1990; Brocks *et al.*, 1999; Peters *et al.*, 2005). While sterols in modern biomass contain taxonomically specific patterns of unsaturations and functional groups, the fossilization of sterols presents a challenge to their use as biomarkers, because during their diagenetic transformation into steranes the molecules lose some of these features. Although sterols owe much of their molecular diversity to nonpreservable features like unsaturation and additional hydroxylation, the basic tetracyclic ring system and side-chain alkylation patterns are retained during diagenesis. The most abundant classes of C_{26}–C_{30} sterols are distinguished by different alkyl substitutions in the A-ring and side-chain; of these, the most common are the C_{27}, C_{28}, and C_{29} sterols and they are preserved as C_{27}, C_{28}, and C_{29} steranes.

Sterols are ubiquitous among eukaryotes, and it can be difficult to assign any particular sterol to a single group of organisms, because identical hydrocarbon skeletons may be synthesized by phylogenetically diverse taxa. Despite this limitation, it has been suggested that because green algae produce abundant C_{29} sterols (Volkman *et al.*, 1994), they are the most likely source of abundant C_{29} steranes found in latest Proterozoic and Paleozoic crude oils and bitumens (Grantham & Wakefield, 1988; Schwark & Empt, 2006; Knoll *et al.*, 2007).

In the past, organic geochemists have attributed C_{29} steranes to land plants, because plants are known to produce a strong predominance of C_{29} sterols (Volkman, 1986). The presence of abundant C_{29} molecules in rocks older than the earliest known land plants led, in turn, to the suggestion that green algae, the ancestors of land plants, might be the source of the oldest C_{29} steranes (Volkman, 2003; Schwark & Empt, 2006). Multiple observations have been marshaled in support of this interpretation:

the presence of C_{29} sterols in some green algae (Volkman *et al.*, 1994); the discovery of probable Proterozoic fossil green algae (Butterfield *et al.*, 1994); the finding that the majority of present-day C_{29} sterols in the ocean are of marine origin in some typical coastal marine environments (Pearson *et al.*, 2000); and a dearth in Paleozoic rocks of body fossils and biomarkers attributable to the chlorophyll a+c phytoplankton (diatoms, dinoflagellates, coccolithophorids) that dominate primary production in post-Paleozoic shelf environments (e.g. Knoll *et al.*, 2007).

Steranes, in trace concentrations, have been reported from bitumens as old as *c.* 2.8 billion years (Brocks *et al.*, 1999), but they do not become substantial components of the biomarker record until the Ediacaran period (635–542 Ma). C_{29} steranes are abundant in the earliest sterane-dominated bitumens, and they remain dominant relative to C_{28} steranes throughout the Paleozoic (Grantham & Wakefield, 1988; Schwark & Empt, 2006; Knoll *et al.*, 2007). C_{28}/C_{29} ratios, however, increase markedly during the Mesozoic and Cenozoic eras, in broad synchrony with the radiations of chlorophyll a+c phytoplankton (Falkowski *et al.*, 2004). This marked change in dominant sterol ratios away from the high proportional abundance of C_{29} steranes in pre-Mesozoic bitumens has been interpreted as evidence that primary production in Paleozoic oceans was dominated by green algae (Falkowski *et al.*, 2004; Schwark & Empt, 2006; Knoll *et al.*, 2007).

C_{29} sterols are made by a wide taxonomic range of phytoplankton, including diatoms, dinoflagellates, chrysophytes, haptophytes, and eustigmatophytes, as well as the green algae (Volkman, 1986; 2003), but the organisms that generated Ediacaran and Paleozoic bitumens and petroleum are additionally characterized, at least collectively, by a low C_{28}/C_{29} sterane ratio. To assess the hypothesis that green algae were more important in Paleozoic marine ecosystems than they are in modern oceans, the proportional abundances of sterols in this group must be evaluated in both phylogenetic and ecologic context.

Sterol analyses must also be extended to the closest phylogenetic relatives of green algae. This includes the Rhodophyta, which also have a Proterozoic fossil record (Butterfield, 2000; Xiao *et al.*, 2004; Elie *et al.*, 2007), and the Glaucocystophyta, a relatively obscure group of unicellular phototrophs that has no fossil record but would be expected, on phylogenetic grounds, to exist if both the green and the red algal lineages were present. Details of rhodophyte phylogeny remain in flux, but the most basal and most derived forms are known (Olivera & Bhattacharya, 2000; Rodriguez-Ezpeleta *et al.*, 2005). Together, the Rhodophyta, the Glaucocystophyta, and the Viridiplantae (green algae + land plants) form the monophyletic kingdom, Plantae. This kingdom has been more recently called the Archeoplastida because it appears to be the *only* kingdom of photosynthetic eukaryotes to contain primary plastids (with the possible exception of the recently discovered photosynthetic rhizarian *Paulinella chromatophora*; Yoon *et al.*, 2006b). Branching order remains incompletely resolved among the major groups of the Plantae; however, monophyly of the three constituent clades and of the group as a whole is strongly supported (Rodriguez-Ezpeleta *et al.*, 2005).

In order to investigate and constrain the possible sources of C_{29} steranes in marine sedimentary rocks, we quantitatively evaluated the sterol profiles of all known major groups of the Plantae. Comparisons of sterol profiles were made at various resolutions, from the broad ecologic classifications of freshwater versus marine, to taxonomic comparisons at the phylum, class, and family levels. Relative phylogenetic position – early divergent versus derived taxa – was also considered in relation to sterol profiles. The results of these analyses provide a framework for interpreting the C_{28}/C_{29} sterane ratio in the sedimentary record.

METHODS

Biomass

Algae were grown in batch cultures in large flasks (2 or 4 L Erlenmeyer flasks or 2 L Fernbach flasks) under air lift conditions with 24 h of light (see Table S1, in Supporting Information, for list of strains). All cultures were grown under the same temperature and light regime. Freshwater algae were grown on the minimal media, Bold's basal media. Marine cultures were grown on f/1 marine media with sterile filtered sea water. Cultures were harvested at the end of log phase growth, which was approximately 30 days, except for desert isolates, which grew more slowly. *Acrochaetium* (a macroscopic alga), *Coleochaete succata*, *Compsopogon* sp.?, *Chlamydomonas reinhardtii*, and *Chlorella vulgaris* were supplied by Carolina Biological Supply. *Cyanophora paradoxa* (LB 555), *Glaucocystis nostocinearum* (64), and *Klebsormidium flaccum* (LB 1958) were supplied by the University of Texas Culture Collection. *Scenedesmus bajacalifornica*, *Cylindrocystis brebissonii* (LG2 VF30), *Bracteococcus* sp.? (BC2-1), and *Chlorosarcinopsis gelatinosa* (SEV2-VF1) were generously supplied by L. Lewis and Z. Cardon from the Biotic Crusts project. *Chlorococcidiopsis* sp.? (CCMEE 171), *Chlorosarcinopsis* sp.? (CCMEE 174), and *Stichococcus* sp.? (170) were supplied by the Culture Collection of Microbes in Extreme Environments. *Pycnococcus* sp.? (CCMP1998), *Dixonella grisea* (CCMP1916), and *Cyanophora paradoxa* (CCMP329) were supplied by the Provasoli–Guillard Culture Collection of Marine Phytoplankton (CCMP). *Halosphaera* (Kodner, 2007) was obtained in phycomate form from field samples, collected in winter 2006 from northern Puget Sound, Washington; cells were isolated by micropipette and washed with Millipore water (Millipore, Billerica, MA, USA) prior to lipid extraction.

Sterol extractions

Total lipids were extracted using the Bligh–Dyer method (Bligh & Dyer, 1959). Sterols were separated from the neutral lipid fractions by thin layer chromatography plates or by silica gel column chromatography. Sterols, after conversion to the trimethylsilyl or acatate derivatives, were analyzed by gas

chromatography-mass spectrometry on a HP 6890 gas chromatograph equipped with a Varian CP-Sil-5 column (Varian, Inc., Palo Alto, CA, USA) (60 m, 0.32 mm ID, 0.25 μm film thickness) fused silica capillary column or an Agilent HP-5MS column (Agilent Technologies, Santa Clara, CA, USA) (30 m, 0.25 mm ID, 0.25 μm film thickness) coupled to a HP 5973 mass-selective detector operated at 70 eV. Molecular identifications were made using comparisons with reference spectra from authentic standards or available in the literature.

Additional sterol data

In addition to sterol profiles determined in this study, comprehensive data for sterols of red and green algae were compiled from the literature. Data were included only if quantitative information was published. Green algae were sorted into classes according to accepted phylogenetic relationships (Huss *et al.*, 1999; Fawley *et al.*, 2000; Karol *et al.*, 2001; Senousy *et al.*, 2004; Kodner, 2007). The genus *Chlorella* presents a particular phylogenetic problem because it is known to be polyphyletic, with named *Chlorella* species segregating into both the Chlorophyceae and the Trebouxiophyceae. *Chlorella* species phylogenetically verified as chlorophytes were placed with Chlorophyceae. All others that had not been phylogenetically evaluated were placed in their traditional position within the Trebouxiophyceae.

Statistics

The sterol dataset was assembled using sterol profiles for 182 algae in the form of percentage of total sterol fraction for each of three molecular classes (C_{27}, C_{28}, and C_{29}; see Table S1 for a list of taxa with references). Statistical differences among sterols of different algal groups were determined using the statistical program JMP, using two-factor ANOVAs for various factors, with a least squares mean model. Data were transformed to the arcsine square root of the percentage, and analyses were run on both raw percentage values and on transformed data. Results are reported from the percentage data, as often there was little difference in statistical support between raw and transformed data. Statistical support for each factor in the ANOVA as well as interactions among factors was noted. Tukey tests were applied as a *post hoc* analysis of the statistical summary. Summary statistics and the distribution of each molecular class within each taxonomic grouping were determined as the mean and sample standard deviation on the original percentage values.

RESULTS AND DISCUSSION

Phylogenetic analysis of sterol distributions

Statistical analyses of the total dataset demonstrate that sterols are differentially distributed among the Plantae. A single factor analysis using carbon number (C_{27}, C_{28}, and C_{29}) suggests that the distribution of these sterol classes among the algal taxa differs significantly from random, with a *P*-value of < 0.001. A subsequent series of two-factor ANOVAs, using a least squares mean model and the distribution of C_{27}–C_{29} sterols as factors, was used to compare the complete datasets from Rhodophyta and Chlorophyta. This phylum-level classification into red and green algae was performed in order to look for statistically significant differences among the sterols of these groups. The glaucocystophytes were omitted from these analyses because data were only available for two species, and these species have dramatically different profiles. The relationship of phylum (red or green algae) to sterol distribution is highly significant ($P < 0.0001$). This suggests that the trends in sterol class distribution have deep phylogenetic origins and are not confined to higher-resolution patterns of similarity such as groupings within a particular genus or family. The ANOVA calculations establish statistical support for our subsequent analyses of sterol distributions at various taxonomic levels.

Figure 1 shows the relative percentage of C_{27}, C_{28}, and C_{29} sterols for each taxon listed in Table S1. Mass spectral data for newly described taxa are listed in Table S2 (Supporting Information). The patterns for red and green algae are evident in Fig. 1. The visual clustering, as well as the statistical calculations noted above, supports previous generalizations for these groups. Red algae predominantly make C_{27} sterols but may produce C_{28} in large quantities and C_{29} in small quantities. The green algae are more variable. They show a strong clustering in the region of C_{29}; however, there is a broad distribution of taxa across the C_{28}–C_{29} axis and a few genera have high proportional abundances of C_{27} sterols. Only two glaucocystophytes were available for analysis, and the data reported here constitute the first reports of sterol composition in this group. Our results show that at least one species of glaucocystophyte (*Glaucocystis nostochinearum*) has the ability to make a high percentage of C_{29} sterols.

A detailed survey of fractional C_{29} sterol composition was conducted within the green algae (Fig. 2). The Prasinophyceae are the only class of green algae not characterized by a dominance of C_{29} compounds. This is surprising, considering that these organisms may be the most important green algal phytoplankton in modern oceans. Previous sterol analyses of taxa within the Prasinophyceae show that they may be characterized by either dominant C_{28} or C_{29} molecules, and in general contain a single major product (Volkman *et al.*, 1994). The Prasinophyceae contain some individual species characterized by C_{29} sterol dominance, while others within the group have high proportional abundances of C_{27} or C_{28} molecules.

Further investigation into the sterol distribution within prasinophytes reveals that there is phylogenetic significance in these patterns of sterol abundance. The Pyramimonadales are the earliest branching clade within the class (Fawley *et al.*, 2000). Taxa that produce dominant C_{29} sterols are all found within this earliest divergent lineage of prasinophytes. In contrast,

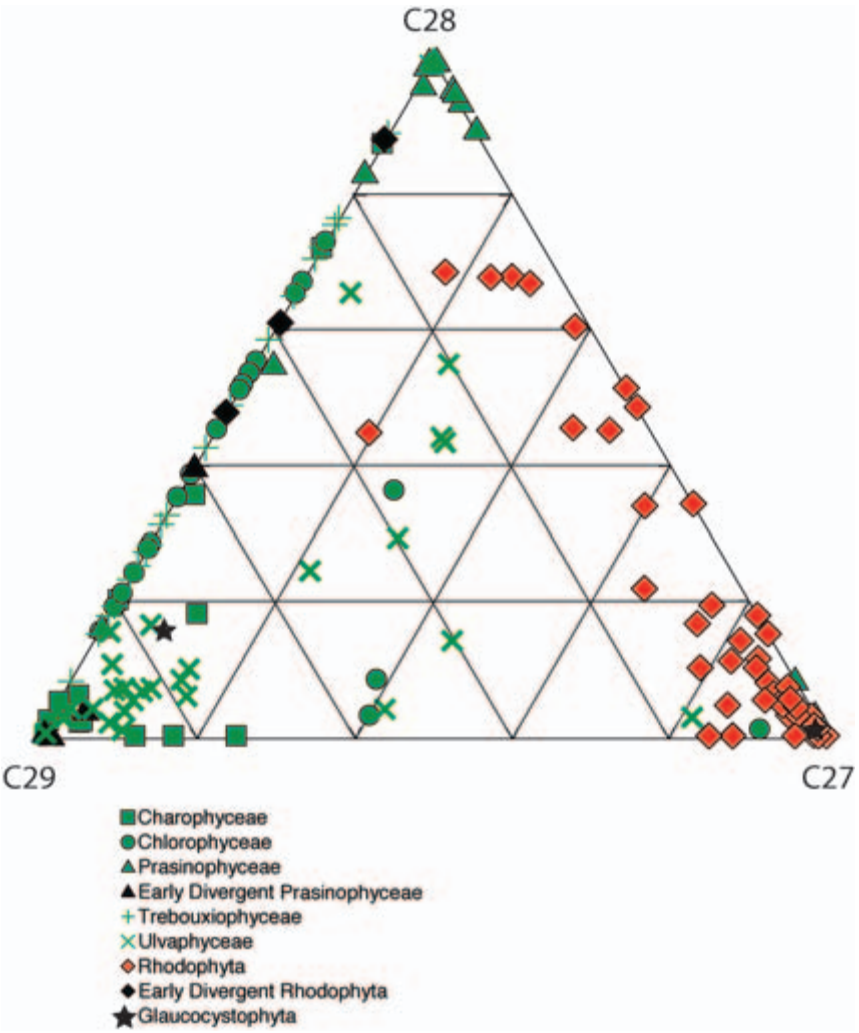

Fig. 1 Ternary diagram showing the relative percentages of C_{27}, C_{28}, and C_{29} sterols among modern members of the Plantae, including green algae, red algae, and glaucocystophytes. Early divergent species are shown in black. Individual taxa and associated references are given in Table S1 in Supporting Information.

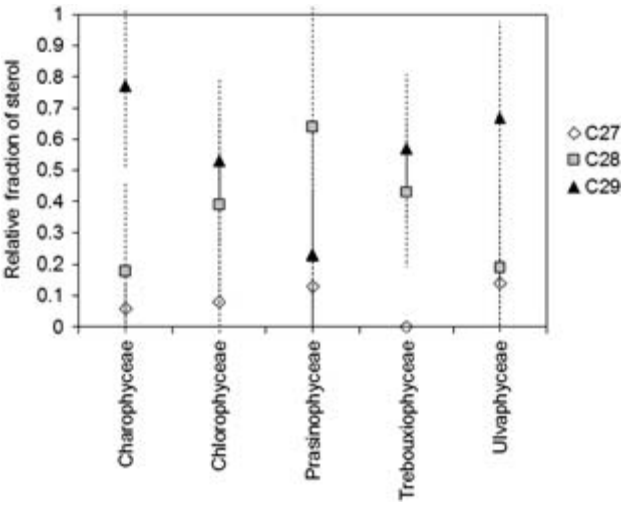

Fig. 2 The mean fractional contents of $C_{27–29}$ sterols across green algal classes, with population standard deviations.

almost all prasinophytes with dominant C_{28} sterols are from the genus *Tetraselmis*, a member of the Chlorodendrales, the most derived prasinophyte lineage. Only one species reported here that would be classified in the Pyramimonidales produced a high percentage of C_{28} (99%) sterols. It has been reported as an unidentified species of *Pyramimonas* (Patterson *et al.*, 1992). The otherwise strong trend toward C_{29} molecules in this clade may call into question this genus identification. Prasinophyceae and other very small microalgae can be difficult to identify morphologically, and this particular organism was not an isolate from a major culture collection. Given its dominant C_{28} sterol, we question its stated identification – an uncertainty also noted by the original authors. In the following analysis that distinguishes between early- and late-diverging prasinophytes, we have therefore omitted this species.

All other groups of green algae have mean percentages of C_{29} sterols that are high relative to their C_{27} and C_{28} sterols (Fig. 2), although the standard deviations for all classes except Charophyceae are broad enough to be overlapping. In addition to the Charophyceae, the Ulvophyceae are dominated by

species with high proportional C_{29} abundances. The common occurrence of C_{29} sterols in the Ulvaphyceae may be significant because our ulvaphyte dataset is composed entirely of marine taxa. The Charophyceae are less likely to be significant in the interpretation of marine steranes, because modern representatives of this group are all freshwater algae. Finally, the Chlorophyceae and Trebouxiophyceae distribute their C_{28} and C_{29} sterols in widely varying proportions. In modern ecosystems, both of these groups are dominated by freshwater taxa, and freshwater bias is reflected in the taxa available for analysis here. However, despite the dominance of freshwater species in these classes, they also can be important components of marine phytoplankton. In particular, it has been observed that trebouxiophycean algae related to the genus *Nannochloris* can be dominant in marine ecosystems (Henley *et al.*, 2004). C_{29} sterols are also produced by the other two main clades within the Plantae, the Glaucocystophyta and the Rhodophyta. The limited information available about the glaucocystophytes shows that C_{29} sterols dominate in one of the two taxa analyzed here. As mentioned above, the red algae in general do not show dominance of C_{29} sterols (Fig. 1), but data also suggest differing patterns between early- and later-divergent phylogenetic groups within the red algae. These results prompted further examination of the distribution of C_{29} sterols among all of the earliest-diverging taxa within the Plantae.

Based on the current phylogenetic consensus, the earliest diverging lineage within the Rhodophyta is well defined as the Cyanidiales, represented here by three taxa (Olivera & Bhattacharya, 2000; Yoon *et al.*, 2006a). The two species of Glaucocystophyceae in our dataset are both considered early emerging for the purpose of these analyses (Olivera & Bhattacharya, 2000; Palmer *et al.*, 2004; Rodriguez-Ezpeleta *et al.*, 2005; Yoon *et al.*, 2006a). The base of the green algal tree is less well resolved than that of the red algae, and no one family has been identified as the earliest diverging lineage (Palmer *et al.*, 2004; Rodriguez-Ezpeleta *et al.*, 2005). There is, however, a major split between the Streptophyta (charophycean green algae plus the land plants) and the other four classes of green algae that make up the Chlorophyta (the Chlorophyceae, Trebouxiophyceae, Ulvaphyceae, and Prasinophyceae). The Prasinophyceae are the earliest divergent class, and as mentioned previously, the Pyramimonidales are the earliest branching clade within the class. The Pyramimonidales are represented by four taxa in our dataset. These four taxa, plus the three Cyanidiales and two Glaucocystophyceae, were included in our early divergent analysis.

Black data points on the ternary diagram (Fig. 1) highlight the sterol compositions of the earliest divergent organisms among all three phyla of the Plantae: Chlorophyta, Glaucocystophyceae, and Rhodophyta. The distribution of total sterols in these early divergent taxa is statistically different from the distribution of total sterols in the Plantae ($P < 0.007$), suggesting that early divergent members are not just a random subset of the whole database. When these nine species are compared to the total data for the Plantae, the mean proportion of C_{29} is higher than C_{27} ($P < 0.0001$), and the mean fraction of C_{29} sterols from the early divergent group also may be statistically higher than the fraction of C_{28} ($P = 0.018$). When the nine early divergent species are compared only to the more derived groups, the fraction of C_{29} in early divergent groups also is higher ($P = 0.012$). Interestingly, among only the more derived groups, the mean proportions of each molecular class (C_{27}, C_{28}, and C_{29}) are statistically identical to each other. Some of the data for the early divergent groups (specifically glaucocystophytes and Pyramimonidales) are bimodal and thus have high standard deviations. More data from early divergent groups are needed to evaluate further statistical support for the relationships explored here. Nonetheless, our analysis suggests that not only did the last common ancestor of the red, green, and glaucocystophyte algae make C_{29} sterols, but that these may have been the dominant sterols in stem group Plantae.

Ecology and sterol distribution

We further investigated the relationship between sterol distributions and phylogeny in the context of the ecology of constituent species. This distinction is important because the majority of biomass that contributed to the C_{28}/C_{29} sterane ratio in Proterozoic and Paleozoic rocks is considered to be marine-derived. We addressed this issue by identifying the organisms in our dataset as freshwater or marine and comparing the respective sterol contents. In particular, the number of green algae analyzed (Table S1) was both large and ecologically diverse enough to provide a detailed assessment of sterol phylogeny and ecology within the phylum. In general, the marine taxa are represented by the Ulvaphyceae and the Prasinophyceae, although we also include two marine members of the Chlorophyceae from the genus *Dunaliella*. Marine Trebouxiophyceae have also been reported, but none are represented in our dataset.

Figure 3 shows the mean values for percentages of $C_{27–29}$ sterols within marine taxa of green and red algae, compared to

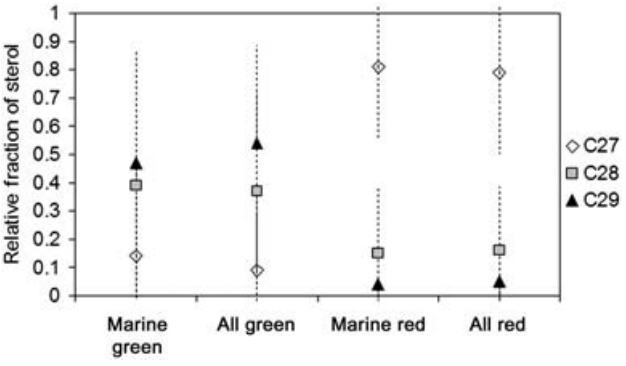

Fig. 3 The mean fractional contents of $C_{27–29}$ sterols across marine species of red and green algae, with population standard deviations. For comparison, the mean values from analysis of total (marine plus non-marine) data for each group also are displayed.

the mean for all species within each of these groups. We find that marine algal taxa are representative of their phyla as a whole. Again, the standard deviations are large, but the overall patterns are conserved. In all green algae, sterols are found with relative abundance $C_{27} < C_{28} < C_{29}$, while in all red algae, this pattern is reversed. Marine species are no different from the averages for all species. The slightly lower average abundance of C_{29} sterols in marine green algae is heavily influenced by the relative enrichment of C_{28} sterols at the expense of C_{29} sterols among some marine prasinophytes.

This same ecologic analysis is not currently possible using only the early divergent algal species because of lack of representation of both marine and freshwater species within each of the groups. The glaucocystophytes are primarily freshwater organisms, and the Cyanidiales are thermo- and acid-tolerant, but not marine. All known Pyramimonadales are marine.

Other, related data suggest that there is no link between environmental distributions and sterol composition within these, or other, algal groups. A comparison of sterols from *Dunaliella acidophila* (Chlorophyta) and *Cyanidium caldarium* (Rhodophyta) – both non-marine acidophiles – shows that they share no similarities in sterol composition despite the crucial role of membranes and their lipids in acid tolerance (Pollio *et al.*, 1988; Seckbach *et al.*, 1993). In another example, growth of *Dunaliella salina* on media with varying salinity shows that the relative dominance of a particular sterol is invariant and conserved (Peeler *et al.*, 1989). Different groups of marine green algae that appear ecologically and functionally identical have different sterol profiles, again suggesting the lack of correlation between ecology and sterol profiles. For example, the prasinophyte genera *Tetraselmis* and *Pyramimonas*, which have very different sterol profiles but are often recorded from the same localities, have dominant C_{28} and C_{29} sterols, respectively (Medlin *et al.*, 2006). Similarly, charophytes and ulvaphytes have different environmental and evolutionary histories but similar sterol profiles. Phylogeny appears to play the dominant role in determining the sterol preferences of green algae.

C_{28}/C_{29} sterane ratios in the geologic record

Our sterol survey can be used to sharpen interpretations of the organic geochemical record. A prominent feature of Phanerozoic patterns of demethyl steranes is the secular change in the C_{28}/C_{29} ratio of bitumens. This ratio is low in Ediacaran and Paleozoic rocks but increases markedly through the Mesozoic and Cenozoic (see, for example, Fig. 3 in Knoll *et al.*, 2007, drawn from a large global sampling of Phanerozoic petroleums). These bitumen and petroleum samples formed from the integrated biomass of multiple organisms that existed in the ecosystem when the organic matter was generated. It is also important to recognize that the samples in such a dataset also integrate the secular and environmental variability that would have occurred over the multimillion-year time intervals required for source rock development. The high values of C_{28}/C_{29} observed in Mesozoic and Cenozoic rocks coincide with the radiations of dinoflagellates, coccolithophorid and diatoms as recorded by conventional fossils (Falkowski *et al.*, 2004), providing a reasonable explanation for this biogeochemical trend. Explaining the consistently low C_{28}/C_{29} sterane ratio in late Neoproterozoic and Paleozoic organic matter, however, requires more than the absence of chlorophyll a + c phytoplankton: it requires organisms characterized by C_{29} sterol dominance.

Figure 4 shows the ratio of C_{28} to C_{29} sterols in all classes of green algae as well as in the early divergent taxa across the

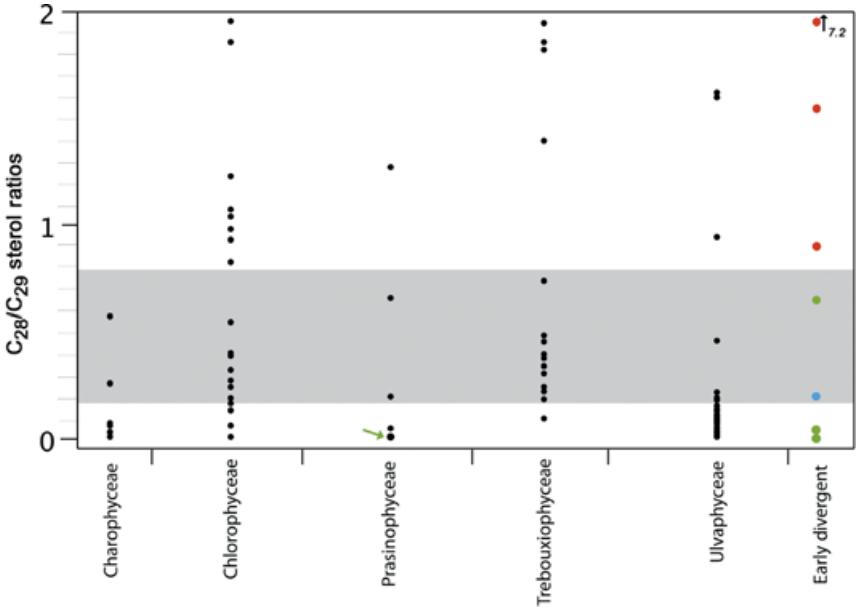

Fig. 4 C_{28}/C_{29} sterol ratios for all green algal species in the dataset (black). For comparison, all early divergent taxa are shown in a separate area on the right side and are color-coded according to phylum: green = green algae, red = red algae, blue = glaucocystophytes. One glaucocystophyte highlighted in Fig. 1 does not appear here, because the ratio is undefined; and two green algal species have C_{28}/C_{29} ratios = 0. The red point next to the arrow is off-scale (C_{28}/C_{29} ratio = 7.2). The green arrow in the column of prasinophytes points to *Halosphaera*, the only prasinophyte with a microfossil record. The gray box indicates the range of C_{28}/C_{29} sterane ratios found in bitumens and petroleums from Ediacaran and Paleozoic rocks.

Plantae (including early divergent green algae). Values of the C_{28}/C_{29} ratio in Ediacaran and Paleozoic samples generally fall in the range of 0.2 to 0.8 (gray box in Fig. 4), with ratios at the lower end particularly common in older samples (e.g. Schwark & Empt, 2006; Knoll *et al.*, 2007). Consistent with this range, most ratios from all green algal classes fall within this box. Ninety-three percent of marine green taxa (94% of Ulvaphyceae and 89% of Prasinophyceae) have a C_{28}/C_{29} sterane ratio below 1. These data support previous suggestions that green algae are very likely responsible for Ediacaran and Paleozoic C_{29} steranes, although other stem group Plantae are also plausible candidates. The fossil record provides evidence for crown group red algae at 1200 Ma (Butterfield, 2000); therefore, it is expected that all the early divergent groups were present by that time.

All of these early divergent groups are microscopic, planktonic organisms. In the modern oceans, the most important marine taxa among the early divergent green algae appear to be the early divergent prasinophytes, a group that includes the genus *Halosphaera*, the proposed modern analog for many sphaeromorphic organic-walled fossils (marked with a green arrow in Fig. 4). It is also possible that glaucocystophytes and early divergent red algae have a larger presence in modern oceans than is currently appreciated. In a recent survey of the North Sea, clones were identified with sequences sister to the glaucocystophytes, as well as new lineages of microscopic Rhodophytes (Medlin *et al.*, 2006). As this portion of microeukaryotic diversity is uncovered using DNA-based molecular methods, the numbers of marine taxa from these earliest diverging lineages may grow. It is also possible that these groups had a larger marine presence in the past. Without preserved fossil evidence, however, the strongest support for the primary source of the Ediacaran and Paleozoic C_{29} steranes is the correlation between the putative ancient fossil record of early prasinophytes and the dominance of C_{29} sterols in *Halosphaera*.

Alternative explanations for high abundances of C_{29} steranes in Neoproterozoic and Paleozoic rocks also should be considered. One possibility is the green macroscopic seaweeds of the class Ulvaphyceae. This group produces prodigious amounts of C_{29} sterols, and 94% of ulvaphyte species surveyed (see Table S1) have very low C_{28}/C_{29} values. The fossil record of this group extends back at least 750 Ma to the fossil cladophoran alga, *Proterocladus*, from Spitsbergen (Butterfield *et al.*, 1994), perhaps the oldest fossil that can be identified as a green alga with confidence (Knoll *et al.*, 2007). Calcified ulvaphytes are abundant in Paleozoic carbonates. If these seaweeds contributed quantitatively to the Paleozoic C_{29} sterane pool, their biomass must have accumulated in coastal environments that are net sinks for carbon. In such a scenario, Mesozoic–Cenozoic radiations of brown algal macrobenthos and a concomitant drop in local green seaweed abundance might have contributed to the observed stratigraphic pattern of C_{28}/C_{29} abundance.

The geologic record of ancient organic matter overwhelmingly comes from continental shelves and platforms, yet the contribution of organic matter from marine macrophytes is rarely considered. In the context of the carbon cycle, seaweeds are significant contributors to total oceanic photosynthetic biomass. Estimates suggest that up to 28% of marine primary production occurs over the continental shelf, which constitutes less than 8% of the oceans (Longhurst *et al.*, 1995). Coastal ecosystems and their macrophytes and phytoplankton are the most productive environment per area in the ocean (Smith, 1981). Although the majority of carbon fixation happens in the open ocean, this production is thought to be a small biological sink for organic carbon, because it is recycled very efficiently by marine heterotrophs. Phytoplankton communities may export ≤ 10% of their fixed carbon to the deep ocean, and only a fraction of this ultimately is preserved in sediments. In contrast, it has been suggested that macroalgal biomass is efficiently transported out of its local environment. One model suggested that macroalgae are efficient exporters of organic carbon out of the coastal ecosystem, exporting up to 44% of their total biomass production (Duarte & Cebrian, 1996). This model did not address subsequent carbon burial, but it suggests that macroalgal biomass can be transported to deeper waters where it may have a higher chance for burial and preservation. Support for this idea comes from a survey concluding that shelf areas are not net organic carbon sinks, but supply biomass ultimately preserved elsewhere, such as continental slopes and canyons (De Haas *et al.*, 2002). In a study of the burial of photosynthetic organic carbon in Antarctica, macroalgal biomass was found to preserve in sediments to an equal or greater extent than diatoms, and it was suggested that seaweeds may leave a lasting signature on organic matter in the sedimentary record (Liebezeit & von Bodungen, 1987).

It is possible that ulvaphyte macroalgae were a quantitative source of C_{29} steranes on ancient continental shelves and platforms. Both phytoplankton and macroalgae began to become more abundant, complex, and diverse in the latest Proterozoic (Knoll *et al.*, 2006, 2007; Xiao *et al.*, 2006). This increasing complexity has been modeled as an increase in the surface area/volume ratio of macroalgae, which in turn has been correlated with increased productivity (Xiao *et al.*, 2006) and perhaps increased algal biomass burial. An increase in the relative importance of eukaryotic primary producers coincides with an increased abundance of sedimentary bitumens and petroleum that contribute to the molecular fossil record and in an overt increase in the absolute abundances of steranes in sedimentary bitumens. The prevalence and high concentrations of C_{27} and C_{29} steranes has been documented at many places around the globe (Summons & Walter, 1990; Peters *et al.*, 2005; Knoll *et al.*, 2007; and references therein). Xiao *et al.* (2006) suggest that the increase in complexity and production is seen in both phytoplankton and macroalgal populations through the Ediacaran; thus, both could be contributing to increased organic matter preservation during that

interval. In light of some questionable algal affinities for Ediacaran and Paleozoic organic-walled microfossils (Kodner, 2007; Yin *et al.*, 2007), it is possible that the macroalgae were an even greater source of C_{29} steranes than green phytoplankton. Nonetheless, the presence of crown group Chlorophyta in 750-Ma rocks may suggest that both early and later branching groups of prasinophytes were present in Ediacaran oceans. Prasinophytes and ulvaphytes could have contributed both C_{28} and C_{29} sterols to Ediacaran and Paleozoic sediments.

Other organisms present in Ediacaran and Paleozoic oceans also could have contributed C_{29} steranes. Many heterotrophic protists produce C_{28} sterols (Raedersdorff & Rohmer, 1985, 1987; Kodner, 2007), and there are reports that some protozoa produce C_{29} molecules as well (Smith & Korn, 1968; Raedersdorff & Rohmer, 1985, 1987; Volkman, 2003). Yet, heterotrophic protozoa remain understudied, and the relative importance of heterotropic biomass to total marine organic matter also is poorly known. A recent study of pico- and nanoplankton suggests that heterotrophic biomass is larger than phototrophic biomass at certain times of the year, usually following an algal bloom (Medlin *et al.*, 2006). More generally, the relative proportions of heterotrophic protozoans and eukaryotic algae in the oceans are unknown, and the relative biomass contribution of heterotrophic protozoans to organic sinks is also unknown. Still, we can be sure that their sterols would have contributed to the geologic distributions.

Metazoan grazing of phytoplankton would likely also play a role in modifying resulting sterane patterns. It is known that metazoans can incorporate and modify sterols from their food sources (Goad, 1981). Generally, C_{28} and C_{29} sterols are altered to C_{27} or C_{26} molecules. If the former process occurs on a large scale, then it has the potential to influence the ratios of C_{27}, C_{28}, and C_{29} sterols exported to sediments and subsequently the ratio of C_{27}, C_{28}, and C_{29} steranes that are preserved in both the present and the past. This relationship has yet to be defined quantitatively. Secondly, metazoan grazing would have had a manifestly secular aspect, taking into account radiations, mass extinctions, and their impacts on macroecology (Butterfield, 2007).

CONCLUSIONS

The Plantae as a kingdom, and sterols as functionally important lipids, are both central to any attempt to understand the early evolution of eukaryotes. With the possible exception of the recently discovered *Paulinella chromatophora*, the Plantae are the only photosynthetic eukaryotes that contain a primary plastid, so the common ancestor of this kingdom provides the point of origin for photosynthesis in eukaryotes as a whole. A physiological requirement for sterols, in turn, is one of a very few traits that unites the eukaryotic domain, and the resulting steranes are well preserved as molecular fossils. Our analyses of sterol production in the Plantae suggest that C_{29} sterols are plesiomorphic for the group. The C_{29} sterols occur in higher proportional abundance in the earlier diverging lineages of each phylum of the Plantae than they do in more derived groups. C_{29} sterols were found to be the most abundant sterols in all but the most divergent group of Prasinophyceae; and they also were dominant in Charophycean algae (much as they are in their descendents, the land plants); in the marine green macroalgae, the Ulvaphyceae; and in the presumed earliest-divergent prasinophyte lineage studied here, *Halosphaera*.

The C_{28}/C_{29} ratio of marine green algae suggests that within the green algae, the Ulvaphyceae or the early divergent Prasinophyceae could be responsible for the abundance of C_{29} steranes in Ediacaran and Paleozoic sedimentary rocks. It is also possible that stem group Plantae, marine glaucocystophytes, or early divergent rhodophytes related to the Cyanidiales could have contributed C_{29} sterols to the Neoproterozoic and Paleozoic organic carbon pool. Chlorophyte green algae are not thought to be major primary producers in modern oceans, but a greater marine presence of this family before the rise of chlorophyll a+c algae to ecologic dominance could have provided an additional source of C_{29} sterol-rich organic matter. The relative importance of these early divergent lineages will likely become clearer with further surveys of marine pico- and nanoplankton. The possible contributory role of C_{29} sterol-producing heterotrophs will also become clearer with comprehensive sterol surveys of protists within a phylogenetic framework. Metazoa could be an important source of C_{27} sterols – including those produced by grazing on C_{28} and C_{29} precursor compounds – and the degree to which this perturbs the primary producer sterol record of sedimentary organic matter remains unresolved.

Steranes offer a perspective on primary production in the Neoproterozoic and Paleozoic that is consistent with inferences from morphological fossils and molecular clocks. The Plantae appear to have played a greater role in continental shelf ecosystems of the late Proterozoic and Paleozoic than they have since the Mesozoic radiations of coccolithphorids, dinoflagellates and diatoms among the plankton, and laminarialean brown algae and crown group coralline red algae among the macrobethos. Further research will both illuminate finer details of this transition and permit investigation of its relevance to Mesozoic–Cenozoic evolutionary transitions in marine animals (Bambach, 1993).

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SUPPORTING MATERIALS

Additional Supporting Materials may be found in the online version of this article:

Table S1. Taxa included in sterol database and references.

Table S2. Mass spectra for sterol profiles cultures used in this study. Major mass fragments (m/z) given, with relative abundance of fragments in parentheses. Relative abundance of molecule in sterol profile of each species also provided.

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